Toward a Mechanistic Understanding of Hepatic Insulin Action and Resistance

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ABSTRACT

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The development of insulin resistance (IR) in the liver is one of the key pathophysiologic events in the development of type 2 diabetes mellitus, but most patients do not become uniformly resistant to the hepatic actions of insulin. Although insulin loses its ability to blunt glucose production, it largely retains its capacity to drive lipogenesis. This “selective IR” results in the characteristic hyperglycemia and dyslipidemia of type 2 diabetes. In this thesis, we take two approaches to better understand the mechanisms underlying selective IR. First, the compensatory chronic hyperinsulinemia (CHI) of insulin resistance downregulates levels of the insulin receptor (InsR). We have therefore modeled CHI in primary hepatocytes to demonstrate that the reduction in InsR number results in insufficient signaling capacity to halt glucose production while still leaving enough residual signaling capacity to promote lipogenesis. That is, the two processes are inherently differentially sensitive to insulin. Second, we hypothesize that FoxO1, a key insulin-inhibited transcription factor, coordinately regulates both hepatic glucose and lipid homeostasis. We have developed a transgenic mouse model heterozygous for a knocked-in allele of DNA binding-deficient FoxO1 and have proceeded to dissect the mechanisms by which FoxO1 differentially regulates glucose and lipid handling. We found that while the former requires FoxO1 to bind to its consensus sequences in target-gene promoters, the latter proceeds via a co-regulatory action of FoxO1. Taken together, these findings reveal novel connections between the glucose and lipid “arms” of the insulin-signaling pathway and how they may go awry in the run-up to diabetes.

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A.1. Professional Acknowledgements

A.1.1. Accili Laboratory

The chance to work in the Accili lab was one of the main reasons I was drawn to Columbia in the first place, and I have not been disappointed. The caliber of mind in the lab is unusually high, even in a field dominated by high-caliber minds. At first I felt quite daunted by this, but this challenge has forced me to grow exponentially as a scientist. I suppose it is part and parcel of the PhD process, but if only I knew when I began what I do now, thanks in large part to the questions and insights posed by my colleagues, I would have finished these projects a year ago already.

First and foremost, I would like to thank Mimmo Accili, my doctoral mentor extraordinaire, for guiding me through this entire process. It has been an unparalleled honor to be mentored by him, to be able to be counted among the ranks of the distinguished alumni of his group. It is no surprise to me now why he is universally considered one of the great pioneers of our field; whether liver, pancreas, adipose tissue, vascular endothelium, hypothalamus, or practically any other cell-type imaginable, Dr. Accili has made seminal contributions to our understanding of insulin action and diabetes. His intimate knowledge of each of these areas, each with a literature unto itself, is formidable to say the least, and therefore especially reassuring for me as a protégé. Moreover, I have always been drawn to mentors with a penchant for patience and good humor in order to offset my generally nervous and often catastrophic view of the series of struggles we call the scientific method. Dr. Accili embodies this sense of sagacious serenity perhaps best of any of my advisors yet. Whenever I was convinced the sky was falling, he would do exactly what I needed: smile and remind me, based on his accumulated wisdom, that this too would pass. Indeed, he has always been indefatigably patient with my somewhat neurotic approach to doctoral research, always helping me to remember what is most important and what I should and should not expend emotional energy worrying about. I have especially also appreciated the historical perspective he brings to our work. Considering that the collective scientific memory extends back about 20 years or so before we begin repeating experiments that have already been done and forgotten, it is important to be

able to call upon an advisor who has a much better memory than most and who also has an uncanny ability to meld cut-and-dry experimental results with the clinico-historical context from which they emerged. Dr. Accili has always inspired my own approach through his ability to bring the narrative arc of diabetes research to life.

I also owe a great debt to the other members of the laboratory. Aside from Dr. Accili, I clearly owe the most to Tommy Kolar and Ana Flete-Castro, our two tremendous technicians. Tommy performed almost all of my mouse genotyping and has the uncanny ability to fix any and all problems in the lab, technical or otherwise. It seems at this point that he can even anticipate problems and address them before they arise. Ana performed all of the ear tagging and tail cutting, as well as much of the metabolic testing of the mice. I suppose it seems a bit trite, but I would not stop short of dubbing her our resident "mouse whisperer." Nobody I have ever seen can handle mice as much without raising their blood glucose levels. As the third of our great triumvirate of essential personnel, I would like to thank Terra Chinn for gracefully fielding my many and varied stupid administrative questions over the years.

Among other colleagues, I first would like to thank Kyoichiro Tsuchiya, now at Tokyo Medical and Dental University. Ichiro served as an able steward of the DBD mouse colony between the depature of Alex Banks and my arrival. Without exaggeration, he also taught me almost everything I now know about how to work with mice, especially importantly including primary hepatocyte isolation. It has been a great privilege for me to inherit his collection of dissecting tools; as I pass them on to Fanny Langlet, I hope that I can continue to convey his legacy of gentle but determined scholarship in the lab. Next, I thank Alex Banks and Tadahiro Kitamura for designing and generating the *Foxo1*^{dbd} transgenic mouse line and Michihiro Matsumoto for bequeathing me his preliminary cell-line data on the DBD allele.

I thank Hongxia Ren for being a close friend and confidante throughout the process; her advice has been transformative in my approach to my individual projects and to my attitude toward PhD life in general. I also appreciate her allowing me to collaborate with her on her *Gpr17*$^{-/-}$ project, from which I hope we both have learned much. I thank Li Qiang for schooling me on everything I know about the WAT browning process and for giving me the HIB1B project I parlayed into my thesis proposal and qualifying exam. I also would like to acknowledge the other members of the laboratory and its close affiliates for

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Although they have graduated to be PIs themselves, I would also like to thank Rebecca Haeusler and Utpal Pajvani. I have always been particularly desirous of and receptive to their advice, both because they are leading experts on insulin signaling in liver and because trying to reach their own personal standards of quality in their work has been a goal of mine since reading their papers even before I joined the lab. I would doubly like to thank Dr. Haeusler for allowing me the honor of collaborating with her on her L-FoxO1,3,4 project, a story that I think is tremendously important and that has been extremely influential in my thinking. Finally, I thank both Dr. Hauesler and Sam Lee for allowing me to use the L-FoxO1,3,4 mice for my own purposes as well.

Finally, I must give my deepest thanks to the most numerous members of the Accili laboratory, the colony of around 4000 mice I have shepherded through the previous 3½ years. It goes without saying that they were as essential as I myself was to these projects. It seems glib to “thank” them given that their sacrifices are unwitting and in many ways unfair. It has been strenuous for me to become chronically desensitized to the mass slaughter of innocent animals, but I try always to take to heart a poster that used to hang in the animal facility at the Children’s Hospital of Philadelphia that read, “Animal research helps animals too.” Perhaps this is merely a means of rationalization, but as an animal lover myself who recently lost one of my life’s great loves, my Cairn terrier Oliver, I need to believe that the veterinary advances that helped him live through Lyme disease, two maulings, and chronic vertigo signify the benefits of animal research. And yet, what these mice have given up will always stay with me.

A.1.2. Other Laboratories

Good science cannot occur in a vacuum, and so I would like to thank the many others both at Columbia and elsewhere who have contributed to my projects and scientific development. Closer to home, I would

like to thank Elizabeth Millings of the Leibel lab for teaching me how to do the fatty acid oxidation assay and for being a calming force against the vicissitudes of learning the DNL assay. I would also like to thank the members of the Ferrante lab, especially Yuxi Lin, for never being annoyed at lending us PBS or vacuum filters when we inevitable run short. Finally among Columbians, I would like to thank Donna Conlon and Byoung Moon in the Ginsberg lab for teaching me how to use the Autospotter and for allowing me my many Friday afternoon spotting sessions.

Outside of Columbia, I first would like to thank Bin Fang from the Lazar lab at the University of Pennsylvania for performing all of the sequence alignment and statistical analysis for the RNA-seq study. I thank Läuge Schaffer of Novo Nordisk in Denmark for sending me a large supply of the S961 InsR antagonist. I thank Maria Angeles Zuriaga Herrero (laboratory of Kenneth Walsh, Boston University) for supplying me with Gsk3β-S9A adenovirus and Akiyoshi Fukamizu (laboratory of Keiko Hirota, University of Tsukuba) for providing me with HA-HNF4α2 plasmid. I finally would like to thank Rafael Mayoral Monibas of the Olefsky laboratory at UC San Diego for including me as a co-author on his study of the adipose tissue-specific SirT1 knockout even though my attempt to contribute to the project did not end all that successfully.

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A.1.4. Previous Mentors

When I was 16, I attended the New Jersey Governor's School in the Sciences, a month-long "nerd camp" for rising seniors interested in scientific research. While there, I was assigned to work on the Team in Molecular Biology, and our goal was to clone a gene encoding a fimbrial protein of *Bordetella avium* as a step toward developing a vaccine against this barnyard pest. Although our cloning worked, we were unable to successfully purify the protein. We were all feeling quite down after having spent a whole month on this pursuit, but our team leader, Prof. Louise Temple of Drew University, offered us a reassurance that has since become a personal maxim for me: "Science is not for the faint-of-heart." I have quoted her in each of the theses I have produced since then, as I have tried to remember this aphorism and stick by it as science has all too often had its dispiriting way with me. Evidently it has served me well.

For me, mentorship has always been more about the philosophical than the practical. What I remember most from each of my mentors has been advice they have given me rather than pointers on how to do a particular type of experiment. I would like to take this opportunity to thank them, on the record, for their many contributions to my development as a scientist. First, going back to my days at Burlington Township High School, I thank Steve Montgomery, Alex Marian, and Alberto Milanes, my teachers for AP Biology, Chemistry, and Calculus, respectively, whose inspired if unorthodox teaching styles imbued me with a scientific hubris that was ripe for dashing (although that would be an important lesson as well). At the University of Pennsylvania, I would like to thank my first PI, Dr. Bryan Wolf, for taking a chance on challenging a 17-year-old to start thinking about designing his own research project and then following him through completing it for four years. I also would like to thank Dr. Brant Burkhardt, then a postdoctoral fellow in the Wolf lab, who as my closest mentor taught me the bulk of what I still know about the design and execution of experiments and scientific writing. Next, at the University of Cambridge, I thank my master's advisor, Dr. Robert Semple, for providing me with an eye-opening, albeit at times frustrating, foray into translational research. I also would like to thank Drs. Stephen O'Rahilly and Kenneth Siddle of Cambridge for additional advising.

A.1.5. Thesis Committee

I would like to offer my deepest gratitude to the members of my committee: Drs. Anthony Ferrante, Ronald Liem, Judith Korner, and Steven Spitalnik. I had always heard horror stories of adversarial committees that sounded more like the stuff of a congressional hearing than of an educational experience. My committee members, from my qualifying exam through my progress meetings, have proven invaluable in setting the trajectory of my research projects. Their insightful questions and comments have inspired both me and Dr. Accili to think ever harder about the questions we are asking and how we interpret the answers. I am most grateful for their continued devotion of time and energy to my thesis defense, and look forward to engaging in a thoughtful and fascinating dialogue on 23 Sept.

A.2. Personal Acknowledgements

The PhD years can be a lonely, alienating time, and riddled with pratfalls into self-doubt. I used to apologize for the data I generated and would even secretly hope that it would be left by the wayside because I did not believe in myself as a competent scientist. Now, however, I will defend my thesis with confidence and pride. Part of this, of course, is the inevitable improvement in skill that one experiences over time, but a large part of it is due to the mental and emotional support provided by those I love. I would therefore like to extend my thanks to them.

First, to my friends. Most of all, I would like to thank my closest friend, Dr. Isabel Huang-Doran. We both began our graduate work – my M.Phil., her Ph.D. – with Rob Semple at Cambridge in the same week and soon found in one another kindred spirits. Working in the lab with her – commiserating over bad data, singing selections from Andrew Lloyd Webber in tissue culture, and other such misadventures – helped me to get over my initially paralyzing homesickness and feeling of isolation in a new country and come to make the most of the experience. I would also like to thank my other close friends who have provided me with considerable moral support and the occasional welcomed escape from being in and thinking about lab: Rachael Gordon of Pittsburgh, Sunaina Seelam of San Francisco, Juliana Calhoun of Los Angeles, Marina Minic of London, Mark Manguerra of New York, Ronit Sternberg of New York,

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Next, my family. First, I would like to thank my sister Taylor. The fact that we are extremely alike sometimes leads to a bicker, but the vast majority of the time means that I can turn to her for a welcome distraction from the perils of lab life. Since she moved to New York I have gotten much joy from our weekly dinners and weekend adventures to the beach or to Burlington, not to mention our wonderful trip to Paris. Our elaborate collection of absurdist inside jokes has always forced me to crack a smile even when I have been determined not to. Now that she will be moving over to Columbia, I look forward to having another Lion in the family.

I would like to thank my grandmother, Francine Schonfeld, for providing a much-welcomed reassuring voice during many, many periods of midnight consternation over the years. Whenever I have been worried or scared or confused, she has told me that everything would "come out great," that I would see because "you know, I'm always right!" And right she has been indeed. For my entire life she has been a source of warmth and wisdom that I have been unduly lucky to receive. I would also like to thank my other grandparents, Rene and the late Joseph Cook, for always making me smile and for making me know how proud they have been of my accomplishments.

How do I presume to "thank" my parents? How can I even begin? My parents have simultaneously been my life coach, psychologist, cheerleader, protector, provocateur, and friend. Often I have been angry when they have refused to allow me to wallow in self-pity brought about by difficulties and self-doubt in the lab. But that sort of can-and-will-do attitude has spurred me on to more and greater achievements than indiscriminate sympathy ever could have. It is not surprising in hindsight that their faith in me has been so unwavering, as they have always given everything possible of themselves such that their children could succeed. Whenever we need them, they are there; they share with us in everything. They have spent years working in jobs that could be charitably said to be unrewarding and saving whatever money they could to give Taylor and me a plainly enviable life. When we were in grade school, my mother always volunteered to be the class mom. Every soccer game I ever played, my father volunteered to run the sidelines. They created a world for us in which we knew we could succeed in life

based on a combination of the values they instilled in us and how they emphasized the importance of our own unique qualities as people. Everything I have always done, I have done to make them proud and to give back to them at least something of what they have given to me. It is one of the great moments of pride in my life that they will be present at my PhD thesis defense. I live a truly charmed life because of them. I love them immeasurably.

Finally, I would like to thank my fiancé and true love, Forewing Lum. Through most of my time in the lab, I have had a Valentine's Day card Forewing gave me pinned to the corkboard at my desk. In it, he lists a number of activities that we enjoy doing together, from the quotidian ("brushing our teeth together") to the sublime ("falling more in love each day"). Our values, our aspirations, our senses of humor, our fears hang together as a world unto ourselves, a culture all our own. Having built such a life with him over these past years, I have been able to venture forth each day through the oft-vexing vicissitudes of predoctoral life knowing that he, my safe haven, will be there at home for me that evening. I am not afraid or embarrassed to tell him that I have had a bad day, that things are not going as well as I hoped, because I know that he fundamentally believes that I am a winner. He is my unflappable partisan, and yet also an ever clear voice of reason.

Through my years with him I have gained so much more than the 20 lbs. that one can see on the outside. I have always been inspired by the way that he is neither unduly impressed nor intimidated by anybody; he is eminently self-possessed and always holds the courage of his convictions – the very quality that Joan Didion equated with self-respect. Because of him, therefore, I have become a notably more confident and assertive person (although my characteristic neurosis will always be there as well). This has stood me in good stead during these years of my scientific naissance, learning to believe in myself and my work. My productivity in the laboratory has been directly correlated with the consistent strengthening of our relationship through time. Now, being able to see all of my work come to fruition, I am utterly thrilled to be able to share these days with him and to bask in the glow of his pride as well. Although the coming year will be trying, I do not worry about the endurance of our bond. I love him forever-much in both breadth and depth.

DEDICATION

I dedicate this work to the memory of my grandfather, "Pa," **Seymour Schonfeld**.

Pa was born in Brooklyn in 1925 and grew up during the Great Depression. He served in the army during World War II and came home to marry his beloved Francine and have three children – the third my mother, Amy. He trained as a mechanical engineer at City College and rose from designing conveyor systems at Williamsburg Steel in the 1950s to becoming a vice president overseeing the then-fledgling computer operations of Chase Manhattan Bank during the 1970s and 1980s. Never content to stop learning, he spent his retirement teaching himself to play the piano, building a fleet of model airplanes and a neighborhood of doll houses for his granddaughters, perfecting his *New York Times* crossword pace, and parlaying his engineering drawing skills into sketching and painting the world around him. Most of all, however, he "kvelled" in the love and pride he shared with his wife, children, six grandchildren, and pet birds.

Pa passed away in 2013 at the age of 88. Remarkably, however, although he never strayed above the lean BMI range, he had lived with type 2 diabetes since his initial diagnosis in the late 1960s. Pa was truly a model patient, keeping copious records of his FSBG readings and using them, as his scientific training no doubt inspired him, to better understand his metabolism and how best to control it. Because of his conscientious and analytic approach, he was well controlled on oral medications for many years. Only in the last decade or so of his life did he require basal insulin injections, which he administered religiously and without complaint.

Given this history, it is no coincidence that I have dedicated the entirety of my time in biomedical research thus far – and very likely in the future – to studying various aspects of insulin action and production. During my time as a PhD student, I even presented Pa's story as a model of how much diabetes research has improved quality and length of life for so many to the CUMC Board of Advisors. Near the end of his life, however, his insulin dosage requirements dropped and neither I nor apparently his doctors considered the possibility that it might be a sign of a problem. We will never know if it was or

not, but I will never forgive myself for not thinking to question it at the time. I will take this as a lesson in the importance of bridging the knowledge we obtain through our work in laboratory to the lives of our patients and loved ones.

Pa was living proof of the old adage that still waters run deep. He never sought to draw attention but it was clear that behind his smiling eyes lay the soul of somebody who not only noticed but even reveled in the small wonders of life. He derived happiness from living in a world in which even the individual flowers and birds he sketched were imbued with a quiet dignity and intricate beauty that made everything feel as though it were meant to hang together by some benevolent grand design. He derived happiness from assembling his long and rich life into carefully curated and annotated photo albums and collections that tell of a journey well taken. But most of all, he derived happiness from giving everything he could for the ones he loved. For his country, he lied about his age to be able to begin his army service early. For his neighbors, he was eloquent and charming; everyone he knew around town from the pharmacist to the manager at Wendy's remarked that their days felt brighter for having enjoyed his company. For his colleagues, he led always by example of patience and grace, spurring them to keep up with their former boss even a quarter century into his retirement.

As for his family, however, there is no real way to express succinctly all that he gave, and of how much I miss all of that now. Yes, he gave of his considerable wisdom, he gave hugs and kisses, he gave countless memorable Thanksgiving dinners and hunts for the afikomen. Much as I miss those things, however, the hole in my heart that is left by his passing would not be refilled merely were those to return. Rather, I miss *him*, my real-life example of what a true human being is. I miss his warm smile and the way that it made me feel as though any worries I had were not so enormous that they could not be solved by approaching them as he would. I miss the way that he proved that the chaos of life in the modern world could be wrangled into order by being proactive yet patient. I miss his gentle sense of humor and how he laughed in his characteristic chuckle with and never at people. I miss the way he wanted to learn about everything. I miss the way he seemed to deeply understand everything I told him, even when some of it was not explicitly spoken. I miss the way that the world felt more humane and more purposeful because he was there to contribute his spirit to it.

I want all of this to be entered into the record forever in this dissertation because this work would not have been possible without Pa there to keep my head held high. And, just in case it was not already clear enough, I want to restate how incredibly much I will always love Pa.

Chapter 1

GENERAL INTRODUCTION

1.1. Hormonal Regulation of Energy Homeostasis

The First Law of Thermodynamics implies that energy must be supplied in at least equal measure to what the body must expend in carrying out the myriad processes of living [1]. The monosaccharide glucose is the metabolic fuel favored by most cell types in the body, in particular neurons and erythrocytes. Thus maintaining a relatively consistent, moderate blood glucose concentration (termed *euglycemia*) is a matter of grave physiologic importance. Evolution has devised an intricate network of hormonal signals that coordinate nutrient catabolism and anabolism in a variety of tissues in order to maintain euglycemia regardless of the time of day or feeding state [2].

Although much of the regulatory control of carbon metabolism remains controversial, a basic framework underpinning bodily energy flux has emerged through years of study. Following ingestion of food, nutrients are absorbed across the intestinal epithelium to enter the circulation. Upon passing through the islets of Langerhans, the endocrine compartment of the pancreas, these nutrients – especially glucose – trigger the secretion of the peptide hormone insulin from islet-resident pancreatic β cells [3 pp. 753-756]. Other mechanisms also exist to link nutrient ingestion with insulin secretion, including the secretion of “incretin” hormones from the gut epithelium as well as neural pathways [4].

Insulin in the bloodstream circulates to its various target organs whose cells express the insulin receptor (InsR). Insulin binds to this receptor to activate its downstream signaling pathways, as will be discussed in greater detail. Insulin is the body’s key anabolic signal, triggering the uptake and utilization of glucose from the bloodstream—especially by skeletal muscle and adipose tissue—as well as its storage as glycogen in liver and other sites. Insulin also coordinates the metabolism of lipids, including their biosynthesis by liver and adipose tissue as well as their inter-organ shuttling. In keeping with this general anabolic drive, insulin also promotes protein synthesis in a variety of tissues [3 pp. 744-756].

In the hours following food intake, insulin acts to partition dietary carbohydrates and lipids among various tissues in order to maintain euglycemia. As the energetic demands of cellular activity remain even while no new nutrients are absorbed, glucose is siphoned from the circulation, resulting in a lowering of blood glucose termed hypoglycemia. Even slight drops in blood sugar trigger the cessation of insulin secretion, thus curtailing the postprandial anabolic drive in order to reallocate energy reserves for

catabolic use. There are several overlapping mechanisms the body uses to signal this fed-to-fasting transition, including secretion of the peptide hormone glucagon from endocrine pancreatic α cells and glucocorticoids from the adrenal cortex as well as action of the sympathetic autonomic nervous system [3 pp. 744-756, 5 pp. 1503-1508].

These “counterregulatory” responses serve to buoy blood glucose levels back toward normal by halting glucose uptake by muscle and fat, stimulating β-oxidation of fatty acids by multiple cell types, and by stimulating hepatic glucose production (HGP). This latter mechanism involves both the breakdown of stored glycogen (glycogenolysis) and the regeneration of glucose from its glycolytic breakdown products and from certain amino acids (gluconeogenesis). The counterregulatory response also relieves insulin’s inhibition of the export of free fatty acids (FFA) and glycerol from adipose tissue to liver (the process of lipolysis) for use as β-oxdiative and gluconeogenic precursors, respectively. Following the next feeding period, the hormonal balance shifts back from counterregulatory factors to insulin [3 pp. 744-756, 5 pp. 1503-1508]. Both insulin and glucose itself work to suppress fasting-induced HGP in addition to insulin’s aforementioned anabolic actions [2, 6].

Under cases of prolonged fasting and starvation, the ability of the liver to produce glucose wanes due to diminished supply of gluconeogenic precursors. In this case, fatty acids liberated by lipolysis can be partially oxidized in the liver to ketone bodies, such as acetoacetate and β-hydroxybutyrate, which can then be further oxidized by the brain. This state, known as ketoacidosis, although meant to help fuel vital processes, comes at a great cost, as the disruption of acid-base balance if unchecked can result in altered mental status, coma, and death [3 pp. 744-756, 5 p. 1407].

This work focuses on metabolic regulation in the liver, arguably the most important orchestrator of peripheral energy homeostasis. Indeed, as mentioned, the liver acts as the body’s major rheostat for fine-tuning levels of glucose and lipids in the blood, and thus throughout the body. As will be discussed, the liver is also a relatively late but indubitably substantial contributor to the development of perhaps the most pressing public-health crisis of our time, diabetes mellitus.

1.2. Diabetes Mellitus Results from Functional Insulin Deficiency

1.2.1. Overview of Diabetes Mellitus

The hormonal control of metabolism as described above is an elegant and intricate balance carefully stricken between the actions of insulin and counterregulatory factors [5 pp. 1503-1509]. Perturbations in this delicate balance result in metabolic disease, the most epidemiologically pressing of which is diabetes mellitus [7]. Diabetes mellitus, however, is actually a constellation of metabolic disorders united by a common etiology: the failure of insulin to counterweigh the counterregulatory hormones [7, 8]. This imbalance is reflected in the etymology of *diabetes mellitus*, "honeyed discharge," refers to the spilling of glucose into the urine (glycosuria) [7, 9]. Glycosuria is the result of prolonged hyperglycemia, which in turn is due to the inability of insulin to fulfill all of its aforementioned goals in promoting glucose storage and utilization [9]. Meanwhile, counterregulatory hormones continue to drive their opposing processes, mobilizing glucose and decreasing its peripheral catabolism [7]. Based on epidemiologic data, the medical community has established three bright-line diagnostic criteria for diabetes [5 p. 1330, 10]: (i) a fasting blood glucose reading of 126 mg/dL or greater, (ii) a random blood glucose reading of above 200 mg/dL, and (iii) a HbA_{1c} value of 6.5% or greater. This last metric is based on glycosylation of hemoglobin that is proportional to ambient blood glucose levels; it is therefore an index of chronic glycemic control [11].

The "insulin failure" of diabetes, in practice, arises due to two main and related causes – either an absolute deficiency of insulin or a relative deficiency of insulin [8] . The most common cause of the first is autoimmune destruction of pancreatic β cells, referred to as type 1 diabetes mellitus (T1DM) [8]; less common causes of insulin deficiency include mutations in genes necessary for proper β-cell function (maturity onset diabetes of the young, or MODY) [12], pancreatitis [13], pancreatectomy [14], and the development of neutralizing antibodies to circulating insulin [15]. Treatment of these disorders requires first and foremost replacement of insulin to physiologic levels [16].

By far the most prevalent form of the disease, however, is type 2 diabetes (T2DM), which arises from *relative* insulin deficiency [17]. Relative deficiency relates not only to production of insulin but also to its action [17-19]. That is, the target cells of insulin become less responsive to its actions, a state referred to as insulin resistance (IR) [17, 20]. This problem can also be formulated as a particular dose of insulin

not triggering a particular response to its normal maximal level; reaching that usual maximum requires a higher-than-normal dose of insulin [17]. Pancreatic β cells are able to compensate for IR, sometimes for many years, by undergoing hyperplasia to produce enough insulin as necessary to maintain euglycemia [5 pp. 1351-1352]. Over time, however, β cells fail to keep up production of insulin sufficient to overcome IR. This two-hit model – IR followed by β-cell failure – encapsulates the pathogenesis of T2DM [17] .

Treatment of T2DM is in many ways more complex than treatment of T1DM [5 pp. 1364-1365]. Replacing "lost" insulin bypasses the pathologic lesion of T1DM, but in T2DM insulin therapy alone does not solve the underlying problem of IR [21]. In fact, type 2 diabetic patients often do not require insulin therapy unless their disease is quite advanced [22, 23]. Instead, they can be treated with a variety of drugs that act as insulin "sensitizers" [24]. By decreasing the severity of IR, patients' own β cells – which, unlike in T1DM, are still at least partially active – may be able to pick up the slack [21]. In reality, the ideal therapy for T2DM would not address IR but rather the root cause of IR itself. The literature on the causes of IR is extensive, but epidemiologically speaking, it appears in Western societies often to boil down to years of living an unhealthy lifestyle, usually manifested as diet-induced obesity (DIO) [25-27]. Concordantly, prescribing a "lifestyle intervention" focusing on diet and exercise in and of itself is a proven means of combatting T2DM, and acts in a powerful synergy with pharmacotherapy [5 pp. 1360-1364, 28].

Problematically, however, many diabetic patients do not adhere to their prescribed therapies, and thus the disease's many complications are able to rear their ugly heads. Untreated diabetes appears to wreak havoc in particular on blood vessels, and thereby secondarily damage the organs served by the affected vasculature [5 pp. 1417-1432]. The complications of diabetes have therefore traditionally been grouped into two main categories: microvascular and macrovascular [29]. The microvascular complications of diabetes arise largely due to hyperglycemia, while the macrovascular complications of diabetes are believed to relate more to derangements in lipid metabolism [30-34]. Because of these complications, it has been estimated that diabetes shortens life expectancy by up to 10 years [35].

1.2.2. Consequences of Hyperglycemia – Microvascular Complications

Persistent hyperglycemia results over time in damage to small-caliber blood vessels and by extension to the tissues they serve; this is referred to as the microvascular complications of diabetes [29]. Although the relationship between glycemia and the incidence of particular complications is not strictly linear, large public-health studies demonstrate a strong and continuous correlation between the two in both T1DM and T2DM [16, 30, 31]. In particular, the correlation between glycemia and diabetic complications becomes much tighter above HbA1c levels of 7%, and thus this value represents a common target for glycemic control [10].

Three sites in particular, each richly vascularized with small-caliber vessels, represent major foci of hyperglycemic complications: the retina, the renal glomerulus, and the peripheral nerve [5 pp. 1417-1420]. Over time, uncontrolled diabetes at these sites results in diabetic retinopathy, nephropathy, and neuropathy, respectively [5 pp. 1417-1420]. Diabetic retinopathy manifests as macular edema and has become the leading cause of adult-onset blindness in the United States [5 p. 1432]. Diabetic nephropathy has become the most common cause of end-stage renal disease (ESRD) [36], typically fatal within 4 years of diagnosis [5 p. 1417]. Over 60% of diabetic patients suffer from neuropathies [5 p. 1417], which includes a variety of presentations including neuropathic pain, autonomic dysfunction (*e.g.*, urinary incontinence, erectile dysfunction), and impaired motor coordination [37].

1.2.3. Consequences of Dyslipidemia – Macrovascular Complications

Diabetes had historically been thought of primarily as a disorder of carbohydrate metabolism, the microvascular complications of the disease serving as stigmata of chronic hyperglycemia. However, T2DM is also a disorder of lipid metabolism, and as a result rarely occurs in isolation. It is more typically associated with obesity, hypertension, and atherosclerosis, a pathologic assemblage referred to as “metabolic syndrome” and rooted in insulin resistance [38, 39]. T2DM, as with the IR state of metabolic syndrome generally, brings about characteristic lipid-metabolic abnormalities termed *dyslipidemia*. The typical abnormal blood lipid profile of T2DM includes high concentrations of TG and small, dense LDL and low concentrations of HDL [40]. Dyslipidemia, in turn, is thought to be responsible for the accelerated

development of atherosclerosis in major blood vessels including coronary and carotid arteries, hence "macrovascular complications" [41].

Macrovascular complications are responsible for the largest portion of diabetic morbidity and mortality [42]. Indeed, the leading cause of death among people with diabetes, affecting as many as 80% of diabetic patients, is cardiovascular disease (CVD), including heart attack and stroke [43]. In fact, diabetic patients are up to four times more likely to develop CVD than non-diabetics [44]. Although hyperglycemia can contribute to and exacerbate CVD [45], CVD arises in large part as a primary result of insulin resistance rather than solely secondary to derangements in glucose metabolism as do the microvascular complications [46]. This is not necessarily surprising, however, as insulin is a dominant regulator of lipid metabolism in a variety of tissues.

1.2.4. Hepatic Steatosis

Despite the importance of serum lipid profile and events at the arterial wall in the pathogenesis of CVD, the main focus of this thesis is on insulin action in the liver. The importance of this is not to be underestimated, however, as IR in the liver alone is sufficient to foment dyslipidemia and atherosclerosis [47]. Moreover, the liver itself is vulnerable to lipid abnormalities. Obesity and IR are strongly correlated with the buildup of triglyceride in the liver, a condition termed *hepatic steatosis* [48-50]. Although "simple" hepatic steatosis may be clinically benign, it can progress to increasing degrees of liver dysfunction in non-alcoholic steatohepatitis (NASH) and even to cirrhosis [50]. This spectrum of pathologies is collectively referred to as non-alcoholic fatty liver disease (NAFLD) [48]. The seriousness of NAFLD as a public-health problem is highlighted by the fact that NAFLD has become the leading cause of liver failure requiring transplant in the United States.

Hepatic steatosis appears to result largely from two main sources: (i) increased influx of fat from adipocyte lipolysis (especially in obesity) and (ii) increased *de novo* lipogenesis (DNL) in the liver itself [48, 51]. Increased TG stores in the liver may in turn give rise to increased secretion in the form of VLDL, contributing to dyslipidemia and atherosclerosis, and potentially to worsening IR. It is important to note,

however, that there is not a strictly linear relationship between liver TG content and VLDL secretion [47, 48, 52, 53].

Despite the strong epidemiologic correlation between IR and NAFLD, the direction of causality (if any) remains difficult to define. Triglycerides in and of themselves do not appear to be injurious to hepatocytes [54], although TG metabolites such as ceramides [55] or diacylglycerols (DAG) [56, 57] may interfere with insulin signaling. Consequently, TG may even be beneficial to hepatocytes as a "safe" storage form for lipid species that may otherwise adversely affect cellular function [58, 59]. In other words, histologic hepatic steatosis may represent a compensatory epiphenomenon roughly proportional in extent to the concentrations of the invisible "true" bad actors [60, 61]. Consistent with this interpretation, transgenic mice with liver-specific overexpression of enzymes catalyzing the esterification of FFA and DAG to TG are protected from IR despite florid hepatic steatosis [62, 63]. Even in the more clinically relevant model of high-fat diet feeding, hepatic steatosis did not impair the direct actions of insulin on isolated rat livers, implicating extrahepatic factors in the development of steatosis-correlated hepatic IR [64]. In addition to these models of steatosis without IR, it should also be noted that some of the most severe cases of IR – the insulin receptoropathies, as will be discussed in Section 1.6 – are associated with normal or even low liver TG levels [65].

Taken together, this evidence suggests that, if generalized IR does precede steatosis, it may be secondary to unchecked WAT lipolysis that would furnish ostensibly harmful FFA to the liver and further hinder hepatic insulin action [51, 60, 66]. This model is consistent with the widely held idea that the development of IR in WAT generally antedates hepatic IR by many years in the natural history of T2DM [67-71]. This may be especially important in obesity due to the enormously expanded pool of releasable fat and potentially detrimental alterations in fat composition [51, 66, 72].

Finally, although increased FFA flux appears to be an integral contributor to hepatic steatosis, the liver itself is not passive in the process. Rates of hepatic DNL have been found to be as much as fivefold higher than normal in NAFLD even during fasting and may be increasingly important even relative to FFA re-esterification as the disease progresses [48, 73, 74]. Patients with type 2 diabetes (*i.e.*, with hepatic IR) also exhibit higher rates of DNL [75]. Thus, whether or not hepatic IR is causally related to the initiation of

NAFLD, it may well be relevant to the disease's progression both as cause and effect. These issues will be revisited in Chapter 5.

1.3. The Proximal Insulin Signaling Pathway

In order to be able to understand the pathogenic mechanisms underlying insulin resistance, it is necessary first to consider the actions of insulin under normal circumstances (illustrated in Fig. 1.1A). Insulin acts by binding to the insulin receptor (InsR) on the surface of target cells. InsR is a heterodimeric receptor tyrosine kinase consisting of two extracellular α subunits and two integral membrane β subunits [76]. The α and β subunits are the product of a single precursor polypeptide that undergoes posttranslational cleavage followed by the association of the cleaved subunits by disulfide bonds [76].

The binding of insulin to its receptor triggers a conformational change that is translated through the receptor's transmembrane domains, ultimately resulting in the autophosphorylation and activation of the receptor's intracellular tyrosine kinase domains [77]. The autophosphorylation of tyrosine residues on InsR itself recruits several phosphotyrosine-binding proteins including the insulin receptor substrate (IRS) proteins, most importantly IRS1 and IRS2 [78]. InsR is able to phosphorylate several tyrosine residues within IRS1/2; these phosphotyrosines are then able to serve as docking sites for several SH2 domain-containing proteins [79, 80]. Thus, IRS1/2, which are themselves devoid of enzymatic activity, serve as scaffolds upon which the insulin-signaling complex can be arranged in response to the activation of InsR [78, 81, 82].

Arguably the most metabolically important Irs-binding protein is phosphoinositol-3-kinase (PI3K) [83]. PI3K is a modular enzyme composed of a regulatory subunit for which three genes exist (*PIK3R1*, *PIK3R2*, *PIK3R3*, encoding p85α, p85β, and p55γ, respectively) and a catalytic subunit, also encoded by three different genes (for p110α, β, and δ) [83]. The PI3K regulatory subunit contains an SH2 domain that binds to phosphotyrosine residues within IRS1/2 that, in turn, releases its inhibition of the associated catalytic subunit [83, 84]. The PI3K catalytic subunit is then able to phosphorylate the inner-leaflet plasma membrane glycolipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3) [84]. The generation of PIP_3 within the vicinity of the InsR/Irs complex allows for the

recruitment of protein kinase B, also known as AKT, one of the principal mediators of the metabolic effects of insulin signaling, via its pleckstrin homology (PH) domain [85]. Once localized to the plasma membrane by binding to PIP_3, AKT can be activated by 3-phosphoinositide-dependent kinase-1 (PDK1) via phosphorylation of threonine 308 within AKT's activation loop [86]. Several studies have indicated that the mammalian target of rapamycin complex-2 (mTORC2) is responsible for a second phosphorylation of AKT at Ser 473 [87-89]. Nevertheless, Ser 473 phosphorylation is also responsive to insulin, and the mechanism linking insulin signaling to Ser 473 phosphorylation by mTORC2 remains unclear [88].

Although the upstream components of the insulin-signaling pathway are able to regulate other pathways, such as the MAPK signaling pathway through ERK1/2 [83], the preponderance of existing scientific evidence suggests that AKT is the most distal step of the pathway that unites insulin's major effects on glucose and lipid metabolism [90]. In other words, it appears that these pathways diverge downstream of AKT. Having established this basic paradigm, more detailed descriptions of the mechanism of insulin's actions on glucose and lipid metabolism in the liver follow below.

1.4. Insulin Regulation of Hepatic Glucose Metabolism

1.4.1. Overview of Hepatic Glucose Metabolism

Two major peripheral target organs of insulin, skeletal muscle and adipose tissue, generally require insulin signaling to stimulate glucose uptake by inducing translocation of the GLUT4 glucose transporter to the cell surface [91]. Liver, however, expresses a different major glucose transporter, GLUT2, whose subcellular localization is not directly regulated by insulin and allows for the equilibration of glucose concentrations within the blood and the hepatocyte [92]. In order to retain glucose within the cell for further metabolism, hepatocytes express glucokinase (*GCK*), a hexokinase unique to liver and pancreatic β cells, which phosphorylates glucose to form glucose-6-phosphate (G6P) [93]. G6P is not subject to transport by GLUT2 and thus can be efficiently shunted to the various carbohydrate-metabolic pathways [93].

The two most important roads down which G6P proceeds are (i) the synthesis of glycogen, the major storage form of glucose, and (ii) catabolism via glycolysis and, ultimately, either lipid biosynthesis or

oxidative phosphorylation to generate energy in the form of adenosine triphosphate (ATP) [3 pp. 744-752]. In the case of glycogen synthesis, G6P is converted to glucose-1-phosphate (G1P) that reacts with a molecule of uridine triphosphate (UTP) to generate UDP-glucose. UDP-glucose units are then coupled to the growing end of a glycogen chain with the subsequent release of UDP [3 pp. 481-487]. Glycogen is broken down in the process of glycogenolysis by the action of glycogen phosphorylase, which re-generates G1P from glucose monomers within the glycogen chain. G1P can then be enzymatically converted back into G6P, which in turn can be dephosphorylated by glucose-6-phosphatase (G6Pase, the catalytic subunit of which is encoded by the *G6PC* gene) to yield glucose that can be released into the circulation [3 pp. 474-481].

The process of glycolysis transforms one molecule of G6P ultimately into two molecules of pyruvate [3 pp. 428-431]. Along the way, glycolysis results in the net generation of two molecules of ATP and of two molecules of NADH that act to shuttle electrons to the mitochondrial electron-transport chain for oxidative generation of more ATP [3 p. 446]. The two molecules of pyruvate are each converted by the pyruvate dehydrogenase complex (PDC) into acetyl-CoA that can proceed through the citric acid cycle to yield the net generation of two more molecules of ATP and six of NADH [3 pp. 517-524]. Acetyl-CoA is also the principal building block utilized in *de novo* lipogenesis, as will be described later [3 pp. 650-651].

Gluconeogenesis represents the reverse of glycolysis – the regeneration of glucose from pyruvate. Although this process proceeds merely by reversing most of the steps of glycolysis using the same enzymes, there are three main enzymatic transformations that differ between these processes and therefore are ideal loci of hormonal regulation [3 pp. 500-501]. First, pyruvate is produced from phosphoenolpyruvate (PEP) in one highly exergonic step by the ATP-generating enzyme, liver-type pyruvate kinase (L-PK, encoded by the *PKLR* gene) [3 pp. 443-445]. The reverse reaction therefore requires the input of significant energy and is not merely reversible through L-PK [3 pp. 500-501]. Instead, pyruvate is tagged with a CO_2 adduct by pyruvate carboxylase to produce oxaloacetate, which is in turn converted back to PEP by PEP carboxykinase (PCK1, commonly known as PEPCK), classically considered the main rate-limiting step of gluconeogenesis [3 pp. 502-503, 94]. The next glycolytic reaction requiring a gluconeogenic workaround is the conversion of fructose-6-phosphate (F6P) to

fructose-1,6-bisphosphate (F1,6BP) by phosphofructokinase-1 (PFK-1) [3 pp. 433-434]. F1,6BP is converted back into F6P in the gluconeogenic pathway by the action of FBPase-1 [3 pp. 504-507]. The third non-reversible reaction required to complete gluconeogenesis is the same as the final step of glycogenolysis: the dephosphorylation of G6P to glucose by G6Pase, the reverse reaction of Gck [3 pp. 480-481].

As will be described, each of these pathways can be regulated via both posttranslational and transcriptional mechanisms. One of the most important mechanisms of exacting this control is through the modulation of the relative circulating levels of insulin and glucagon [7]. As mentioned earlier, during times of fasting, glucagon levels rise causing the liver to switch from storage and utilization of glucose to HGP in order to maintain euglycemia [3 pp. 752-756]. Thus, in this scenario, glycogen synthesis (glycogenesis) is decreased in favor of glycogen breakdown (glycogenolysis) while glycolysis also gives way to its reverse pathway, gluconeogenesis [3 pp. 752-756]. The processes of glycogenolysis and gluconeogenesis that are stimulated by counterregulatory factors are subject to negative regulation by insulin, also both by posttranslational and transcriptional mechanisms [3 pp. 752-756].

The rate of HGP is highly sensitive to changes in the relative concentrations of insulin and glucagon [2]. The shift to higher concentrations of insulin following a meal reduces HGP within a matter of minutes, suggesting a predominance of posttranslational regulation of the proteins involved; over the course of a physiologic fast of several hours, about 75% of HGP results from glycogenolysis in response to counterregulatory signaling [95-97]. It therefore makes sense that the vast majority of the HGP-suppressing effect of insulin during physiologic fasting is due to its net inhibition of glycogenolysis in favor of glycogen synthesis [98-102].

The importance of gluconeogenesis is not to be overlooked, however. The relative contribution of gluconeogenesis to HGP appears to be greater in humans suffering from obesity and type 2 diabetes, as the percentage of HGP attributable to gluconeogenesis, as well as total HGP, are positively correlated with fasting plasma glucose [103]. Overall, the contribution of gluconeogenesis to HGP may be 50% higher in diabetic patients [103-105]. In fact, using an NMR-tracer method to follow carbon flux revealed

that glycogenolysis may even be decreased in diabetic patients and that increased gluconeogenesis is the culprit behind the fasting hyperglycemia of diabetes [106].

1.4.2. Posttranslational Regulation of Glycogenolysis by Insulin

Multiple mechanisms contribute to insulin's ability to shut down HGP. Insulin stimulates the activity of protein phosphatases such as PP1 that undo the activating phosphorylation events carried out by cAMP-dependent protein kinase (PKA) in response to glucagon [107, 108]. Targets of this form of regulation include glycogen phosphorylase [109] and glycogen synthase (GS) [110]. Insulin signaling also promotes Akt-dependent phosphorylation of glycogen synthase kinase-3α and -3β (GSK3α/β). Phosphorylated GSK3α/β are then unable to carry out their glucagon-stimulated inhibitory phosphorylation of GS, thus allowing glycogen synthesis to proceed [111]. Although insulin's inhibition of GSK3α/β has long been considered a major link between InsR and glycogen synthesis, the *in vivo* significance of this pathway in liver has recently been called into question [112].

1.4.3. Posttranslational Regulation of Gluconeogenesis by Insulin

Only following a prolonged fast of 24-48 h are hepatic glycogen stores depleted in humans, thus rendering the liver dependent upon gluconeogenesis to maintain euglycemia [113]. The ability of insulin to directly suppress gluconeogenesis, however, has been controversial [2]. Indeed, glycogenolysis appears to be far more sensitive to inhibition by insulin than does gluconeogenesis [98, 99]. A series of studies in dogs undergoing portal-vein insulin infusion has demonstrated that only at very high concentrations of insulin is there a net inhibition of net gluconeogenic flux [94, 114]. Even when insulin does appear to suppress net gluconeogenesis, the effect is transient except at the highest of insulin concentrations and may be due more to an increase in glycolysis than to a suppression of gluconeogenesis *per se* [94, 114]. In any event, inhibition of gluconeogenesis by insulin may be somewhat beside the point, as even in the fed state gluconeogenesis proceeds to produce G6P from pyruvate [94, 100, 101, 114]. The key difference lies in the fact that this G6P is shunted into insulin-stimulated glycogen synthesis rather than dephosphorylated and released into the circulation [97, 100, 115, 116].

The major effects of insulin on the acute regulation of gluconeogenesis *in vivo* may therefore be indirect [117, 118]. Insulin strongly suppresses the secretion of glucagon from pancreatic α cells, thus dampening the pro-gluconeogenic signal in fasting [119, 120]. Insulin also potently inhibits adipose-tissue lipolysis, thereby cutting off the supply of glycerol, an HGP substrate, and free fatty acids, which have been shown to potentiate HGP in a variety of ways [121-126]. Finally, insulin also decreases the release of gluconeogenic substrates such as lactate and alanine from skeletal muscle and other organs [127, 128].

Despite the foregoing, insulin has been shown to posttranslationally regulate several steps of gluconeogenesis [2]. As in the case of glycogenolysis, insulin triggers the activation of phosphatases such as PP1 that undo the pro-gluconeogenic actions of glucagon-stimulated PKA activity. Perhaps the most important example of this is in the regulation of fructose-6-phosphate (F6P)/fructose-1,6-bisphosphate (F1,6BP) balance by phosphfructokinase-1 (PFK1) and F1,6BPase (FBP1) [129, 130]. PKA stimulates the activity of fructose-2,6-bisphosphatase (FBP2), converting fructose-2,6-bisphosphate (F2,6BP) to F6P. FBP2, however, is a bifunctional enzyme that also possesses a phosphofructokinase activity that is stimulated by insulin-dependent dephosphorylation of FBP-2. Upregulation of FBP2's phosphofructokinase activity by insulin converts F6P back to F2,6BP, which acts as an allosteric activator of PFK1, thus driving the equilibrium between the PFK1/FBP1 reactions toward the former and driving net glycolysis [129, 130]. The increase in intracellular F2,6BP concentration has been posited to be the principal means by which insulin does exert its acute, if minor, repressive effect on gluconeogenesis [94, 114, 131]. Nevertheless, insulin also triggers the reversal of PKA-mediated inhibition of L-PK as well, both by directly activating phosphatase activity [132, 133] as well as by increasing intracellular levels of F1,6BP, an allosteric activator of the enzyme [129]. Finally, insulin can inhibit the activities of pyruvate dehydrogenase kinase-2 and -4 (PDK2/4), which serve as negative regulators of the PDC [134]. Thus, in effect, insulin can facilitate the conversion of pyruvate to acetyl-CoA, effectively preventing its becoming a potential substrate for gluconeogenesis [2].

1.4.4. Transcriptional Regulation of HGP by Insulin Through FoxO1

Although insulin and glucagon are able to cause rapid changes in HGP through purely posttranslational modifications of the enzymes involved, signaling by these hormones is also able to alter the long-term program of hepatic glucose metabolism by regulating gene expression [2]. Indeed, given the evolutionary imperative of preventing hypoglycemia, multiple overlapping transcriptional mechanisms have arisen to promote HGP in the long term [2]. Moreover, perhaps in order to prevent hypoglycemia, not all of these pathways are inhibited by insulin. To wit, glucose production in the fed state is significantly lower than during fasting but is not absent; input from other hormones (*e.g.*, adrenal corticosteroids) [135], the autonomic nervous system [136], and circadian factors [137] all modulate HGP independently of insulin signaling [138, 139].

The forkhead box O (FoxO) proteins, in particular FoxO1, FoxO3, and FoxO4, are a widely expressed class of transcription factors characterized by a common forkhead DNA-binding domain [140, 141]. We focus in particular on FoxO1 (also known as FKHR, encoded by *Foxo1* in mice and *FKHR* in human), the best-characterized and most abundant FoxO family member in hepatocytes [2, 142, 143]. Importantly, the FoxO proteins – especially FoxO1 – appear to represent the major insulin-regulated transcriptional coordinator of HGP [2, 144, 145]. Akt downstream of InsR phosphorylates FoxO1 at three sites – Thr 24, Ser 256, and Ser 319 – causing its exclusion from the nucleus and cytoplasmic sequestration through interaction with 14-3-3 proteins [143, 146-150]. This inhibitory effect of insulin is rapid, potent, and persistent. Glucagon signaling in the postabsorptive state activates FoxO1 [151], which in turn upregulates the expression of several fasting-inducible genes, most notably including *G6pc* [152], *IGF binding protein-1* (*Igfbp1*) [148, 153], *Pdk4* [154], and *Pck1,* although the last is controversial [142, 144, 145, 155]. FoxO1 acts on these genes through binding to conserved recognition motifs in their promoters termed *insulin response elements* (IRE). The phosphorylation and nuclear exclusion of FoxO1 in response to insulin then shows a strong temporal correlation with the decrease in *G6pc* and *Pck1* mRNA [94].

Whole-body knockout of FoxO1 is embryonically lethal due to the occurrence of arteriovenous malformations [156, 157]. Liver-specific deletion of FoxO1, dubbed the L-FoxO1 mouse model, results in

a significant enhancement in whole-body glucose tolerance due to decreased HGP [145]. Hyperinsulinemic-euglycemic clamp studies have shown L-FoxO1 livers to exhibit decreases in both gluconeogenesis and glycogenolysis [145]. Although blood glucose is normal in L-FoxO1 mice following a standard overnight fast, hypoglycemia becomes apparent following prolonged fasting [145]. Despite their reduced HGP, L-FoxO1 mice are not overall more sensitive to insulin; they do not show any difference in fasting or fed insulin levels or in insulin tolerance [145, 158]. Concomitant deletion of FoxO3 and FoxO4 in the liver (L-FoxO1,3,4) results in an enhancement of glucose tolerance beyond that of the L-FoxO1 mouse due to a further reduction in HGP [144, 159]. Conversely, transgenic expression of an insulin-insensitive mutant FoxO1 in liver results in glucose intolerance due to an inability of insulin to blunt HGP and tonically increases expression of *G6pc* [142, 160]. Similar findings were obtained with adenoviral overexpression of wild-type FoxO1 [161].

Multiple lines of evidence support the primacy of FoxO1 repression in insulin's overall regulation of HGP. In *C. elegans*, for example, deletion of the *Foxo1* orthologue *daf-16* rescues the developmentally arrested *dauer* phenotype of nematodes lacking the *Insr* orthologue, *daf-2* [145, 162, 163]. In a similar vein, deletion of *Foxo1* only in liver significantly extends the lifespan of whole-body *Insr* knockout mice [145]. Although most of these mice still die prematurely, they are spared the lethal neonatal hyperglycemia, ketoacidosis, and hepatic steatosis of $Insr^{-/-}$ mice [145, 164]. That the compound *l-Foxo1* ; $Insr^{-/-}$ mice phenocopy mice lacking *Insr* in all tissues except the liver further suggests the centrality of FoxO1 in mediating insulin's actions on hepatic metabolism [145, 165]. These striking findings of genetic epistasis between *Insr* and *Foxo1* are substantiated by further mechanistic studies on the InsR signaling pathway. Liver-specific deletion of *Irs1/2* [166] or *Akt1/2* [167] both result in hyperglycemia and glucose intolerance due to the inability of insulin to blunt HGP, associated with constitutively increased expression of *G6pc* and *Pck1* and decreased expression of *Gck*. In both of these cases all of these abnormalities can be completely reversed by concurrent liver-specific deletion of *Foxo1* [166, 167].

FoxO1 appears to represent a promising if elusive target in the treatment of diabetes; multiple studies have shown that inhibition of FoxO1 in insulin-resistant states has beneficial effects on glucose metabolism [155]. L-FoxO1 mice rendered insulin resistant by Western-type diet feeding showed a

significant reduction in fasting blood glucose, fasting insulin, and glucose intolerance relative to controls [168]. In the acute setting, anti-*Foxo1* antisense oligonucleotide (ASO) therapy of insulin resistant, diet-induced obese (DIO) mice resulted in decreased blood glucose and HGP, improving glucose tolerance and whole-body insulin sensitivity [169]. Similarly, infection of diabetic *db/db* mice with an adenovirus encoding the dominant-negative *Foxo1*-Δ256 also dropped blood glucose levels and reduced expression of *G6pc* and *Pck1* in liver [170].

1.4.5. Transcriptional Regulation of HGP by FoxO1-Associated Proteins

FoxO1 acts in tandem with other transcriptional effectors to regulate the HGP transcriptional program in mice. A particularly prominent FoxO1 co-regulator is PPARγ coactivator-1α (Pgc1α, encoded by the *Ppargc1a* gene), which is itself a FoxO1 target gene [145]. Pgc1α is induced in response to fasting, and adenoviral overexpression in liver activates the expression of *G6pc*, *Pck1*, and *Fbp2* and consequently upregulates glucose production [171, 172]. FoxO1 and Pgc1α synergistically activate the expression of *G6pc* and *Pck1* through a direct interaction [173] and Pgc1α, like FoxO1, can be inhibited by Akt-mediated phosphorylation [174]. Interestingly, FoxO1 is required for Pgc1α induction of *G6pc* and *Pck1* but not for the action of Pgc1α on mitochondrial oxidative gene targets [145].

FoxO1 also coordinates gluconeogenesis through functional interactions with CREB-regulated transcription coactivator-2 (CRTC2, formerly known as TORC2). CRTC2 is a co-activator of cAMP-response element binding protein (CREB), a major downstream effector of glucagon-induced gluconeogenic gene expression [175, 176]. CRTC2/CREB-induced gluconeogenic gene expression appears to predominate during very early fasting but then gives way to FoxO1 activity, through coordinated deacetylation of the two proteins [177]. Furthermore, CRTC2 itself can be degraded in response to insulin signaling, providing a FoxO1-independent means of tamping down glucagon-induced transcriptional activity [178]. Finally, FoxO1 has also been demonstrated to interact with hepatocyte nuclear factor-4α (HNF-4α) to regulate the expression of *G6pc* and *Gck* in fasting and feeding, respectively [179].

1.5. Insulin Regulation of Hepatic Lipid Metabolism

1.5.1. Overview of Hepatic Lipid Metabolism: Lipid Biosynthesis

The influx of nutrients into the liver in the fed state results in a net production of acetyl-CoA, most classically following the glycolytic breakdown of glucose to pyruvate, but also from other carbohydrates as well as certain amino acids [180]. Acetyl-CoA can be further metabolized via the citric acid cycle to yield ATP and NADH, although more is produced in the immediate postprandial state than is required for the generation of energy [180]. The liver therefore seeks to preserve the chemical energy stored in acetyl-CoA for use during fasting periods by polymerizing these carbons into fatty acids. This process is termed *de novo* lipogenesis (DNL) [180].

The liver strikes a balance between the anabolism and catabolism of fatty acids that responds to the metabolic conditions of the moment [3 pp. 744-747]. The liver takes into account signaling inputs from hormones (*e.g.*, insulin, glucagon) as well as changes in the relative concentrations of nutrients themselves [3 pp. 743-784]. The main regulator responsible for setting this balance is acetyl-CoA carboxylase-α (ACC1, encoded in liver by the *ACACA* gene), which catalyzes the first committed step of fatty acid biosynthesis [181, 182]. As its name indicates, ACC1 transforms acetyl-CoA into its carboxylated adduct, malonyl-CoA [181]. Malonyl-CoA, then, serves two functions: it both serves as the major substrate for polymerization to fatty acids and inhibits the import of fatty acids to the mitochondrion in order to prevent catabolism of the newly synthesized products [181-183].

The major enzyme responsible for transforming individual two-carbon units of malonyl-CoA into fatty acids is the enzyme fatty acid synthase (FAS, encoded by the *FASN* gene) [3 pp. 653-658]. This single enzyme remarkably performs the seven different catalytic activities necessary for fatty-acid biosynthesis, powering these reactions through the cleavage of malonyl-CoA's highly energetic thioester bond [3 pp. 653-658]. The end product of the FAS reactions is the sixteen-carbon saturated fatty acid, palmitate [3 p. 656]. Much as in the case of malonyl-CoA, palmitate serves both as substrate and allosteric regulator [3 pp. 660-662]. Palmitate can be transformed into longer-chain fatty acids through the action of fatty acid elongases, as well as into desaturated fatty acids by desaturases such as stearoyl-

CoA desaturase-1 (SCD1) [3 pp. 657-658]. Palmitate also allosterically inhibits ACC1 in an example of negative feedback [3 pp. 660-662].

Three individual fatty acyl-CoA esters can be esterified (or, in the case of free fatty acids derived from adipose-tissue lipolysis, hepatically re-esterified) with the three carbons of glycerol to form triacylglycerol, or triglyceride (TG), the major storage form of fatty acids [3 pp. 658-660]. Triglycerides can be stored in the liver or can be exported for uptake and use by other tissues. For this purpose, the liver packages triglycerides along with cholesterol esters and apolipoproteins into very low-density lipoproteins (VLDL) [184]. Insulin has been shown to inhibit the secretion of VLDL by stimulating the degradation of its major protein component, apoB [46, 185].

In the healthy liver, DNL accounts for only about 4% of TG secreted into the circulation basally, although this number rises to a high of 23% at 4 h following a meal [186, 187]. By comparison, esterification of FFA liberated from WAT accounts for 77% and 44% of secreted TG in the fasted and fed states, respectively [188-190]. Meal composition also plays a role in determining the relative contribution of DNL to circulating TG, with high-carbohydrate foods more potently driving lipogenesis than fat- or protein-rich foods [189, 191-197]. However, in the pathologic state of hepatic steatosis associated with insulin resistance, a far greater percentage of the hepatic TG and associated VLDL secretion is derived from DNL [73-75].

1.5.2. Transcriptional Control of Lipid Biosynthesis

The transcriptional control of lipid biosynthesis is largely, although not exclusively, driven by two “master” regulators, sterol regulatory element binding proteins (SREBP) and carbohydrate response element binding protein (ChREBP), as well as by FoxO proteins. Their respective contributions to the process are described below.

Sterol regulatory element binding protein-1c (SREBP-1c)

The program of biosynthesis of lipids in liver is remarkably well coordinated at the transcriptional level by the SREBPs, a trio of transcription factors that recognize and bind to sterol-response elements (SRE) in the promoters of target genes [198]. There are two genes encoding SREBPs, namely *Srebf1* and *Srebf2*; the latter encodes SREBP-2, which is directly responsible for the transcriptional activation of all of the major enzymes involved in cholesterol biosynthesis and transport [198]. *SREBF1* contains two different transcriptional start sites to yield two isoforms, SREBP-1a and SREBP-1c, differing in their first exon [198]. SREBP-1c is the most abundant SREBP-1 in liver [199]. Target genes of this transcriptional complex include all of the major lipogenic enzymes described above, including *Fasn, Acaca, Scd1* and the enzymes involved in the esterification of TGs from fatty acids. SREBP-1c also positively regulates its own expression [198]. Whole-body knockout of *Srebf1* leads to embryonic lethality in a considerable majority of animals [200]. In those that survive, it appears that upregulation of SREBP-2 partially compensates for the loss of SREBP-1 at the price of markedly upregulated cholesterol synthesis [200]. Whole-body knockout specifically of the exon unique to the -1c isoform results in blunted feeding-induced expression of *Fasn*, *Acaca*, *Scd1*, and other lipid-biosynthetic enzymes, resulting in decreased circulating and hepatic TG in response to re-feeding [201].

The derangements in feeding-induced lipid synthesis underscore the fact that regulation of SREBP-1c is one of the principal methods of insulin's control of liver fat metabolism [202]. Although it is a transcription factor, regulation of SREBP-1c by insulin occurs in large part outside of the nucleus [198]. At baseline, SREBP-1c is an integral membrane protein localized to the ER membrane in complex with SCAP, another integral membrane protein that shepherds SREBP-1c into vesicles bound for the Golgi apparatus [203, 204]. Preventing this translocation event are INSIG-1 and INSIG-2a [205]. Once arrived at the Golgi, the proteases S1p and S2p catalyze sequential cleavage events that liberate a cytosolic, *N*-terminal fragment of the SREBP-1c precursor protein [206]. This cleaved SREBP-1c is then free to translocate to the nucleus to perform its function as a transcription factor [207].

Insulin affects SREBP-1c at each of these stages [208]. First, insulin promotes the packaging of the precursor SREBP-1c/SCAP complex into Golgi-bound vesicles at the ER [203]. Second, insulin

decreases protein levels of INSIG2a, presumably relieving its inhibition of SREBP-1c/SCAP translocation [209]. Third, insulin triggers the phosphorylation and inhibition of LIPIN-1, a negative regulator of SREBP activity within the nucleus [210]. Finally, insulin positively regulates *Srebf1* gene expression, again at least in part due to positive feedback of SREBP-1c [209, 211-213].

The intermediaries linking insulin signaling to SREBP-1c expression and cleavage have been elucidated in recent years. Both the cleavage and expression of SREBP-1c require signaling from InsR through PI3K and Akt [213]. Akt's activation of SREBP-1c proceeds via mammalian target of rapamycin complex-1 (mTORC1) [214, 215]; Akt activates the mTORC1 kinase through inhibition of its inhibitors, TSC-1/2 [216]. Blocking hepatocellular mTORC1 via treatment with rapamycin or by deletion of TSC-1 results in an inability of insulin to promote SREBP-1c expression or cleavage, thereby suppressing insulin-induced DNL and lipogenic gene expression [213, 217, 218]. Insulin's regulation of SREBP-1c diverges downstream of mTORC1, however, as insulin-induced cleavage of SREBP-1c requires the activity of mTORC1 substrate S6 kinase (S6K) while expression of SREBP-1c in response to insulin does not [218]. A further potential mechanistic link between insulin and SREBP-1c is through GSK3β, which has been shown to phosphorylate SREBP-1c and thereby inhibit its activity [219]. The inhibition of GSK3β by Akt downstream of InsR, therefore, would relieve GSK3β's inhibition of SREBP-1c, further promoting its activity [185, 219, 220]. A gap in our understanding persists, however, between these distal signaling events and the actual processing and transcription of SREBP-1c.

Increasing our understanding of the regulation of SREBP-1c is of great clinical importance given its alleged role in the pathogenesis of NAFLD and potentially hepatic insulin resistance. Liver samples from humans with obesity [221] and NAFLD [222] have been shown to have higher expression of SREBP-1c and its targets. Several animal models of fatty liver disease, such as the leptin-deficient *ob/ob* mouse and the sucrose-fed hamster, likewise demonstrate elevated levels of SREBP-1c in liver [223-225]. Supporting the importance of increased SREBP-1c levels, mice engineered to express a constitutively active mutant SREBP-1c in the liver developed hepatic steatosis and insulin resistance [226, 227]. Conversely, deletion of SCAP (resulting in an inability to cleave SREBP-1c) prevented hepatic steatosis in both *ob/ob* and sucrose-fed hamster models of NAFLD by reducing DNL [225].

Carbohydrate response element binding protein (ChREBP)

In a manner similar to SREBP-1c, ChREBP is a transcription factor capable of coordinately inducing many genes in the lipogenic program [228]. While SREBP-1c is primarily controlled by insulin signaling, ChREBP activity, as its name implies, is induced following exposure to carbohydrates acting in a signaling capacity [228-230]. At least three metabolites of glucose, each requiring the initial action of glucokinase [231, 232], have been reported to induce ChREBP to stimulate glucose-induced gene expression [233-236]. As the expression of *Gck* is itself strongly influenced by insulin, even ChREBP activity is indirectly affected by insulin signaling [202].

ChREBP shares many common targets with SREBP-1c, including *Fasn*, *Acaca*, and *Scd1* [228]. This helps to explain why livers lacking nuclear SREBP-1c, for example due to deletion of InsR, still demonstrate some feeding-induced expression of these lipogenic genes [201, 202, 237]. Furthermore, it appears that synergism between insulin-stimulated SREBP-1c activity and glucose-stimulated ChREBP activity is required for maximal activation of several lipogenic genes [232, 238]. On the other hand, only ChREBP is able to induce the expression of liver-type pyruvate kinase (*Pklr*), responsible for catalyzing the final step of the glycolytic pathway [239, 240].

FoxO proteins (FoxO1, FoxO3, FoxO4) in Lipid Biosynthesis

The importance of FoxO proteins in the regulation of HGP is now firmly established [2]. Empirical evidence has also mounted to implicate FoxO proteins in the control of hepatic lipid metabolism as well but the interpretation of these data has been more nuanced [158, 161, 168, 241-245]. Nevertheless, it appears overall that inhibition of FoxO proteins, in particular of FoxO1, may represent another important *modus operandi* of insulin's control of fat anabolism in the liver.

Our laboratory's initial publication of the liver-specific FoxO1 knockout mouse (L-FoxO1), though highly supportive of a key role for FoxO1 in the control of hepatic glucose metabolism, did not demonstrate any notable abnormalities in lipid parameters [145]. L-FoxO1 mice generated by another group (albeit using a different liver-specific promoter to drive Cre expression) did demonstrate slight but significant elevation in liver TG in the fed state [158]. One potential reason for the absence of a lipid

phenotype as dramatic as the glucose phenotype in the L-FoxO1 mice may be the fact that these measurements were performed in chow-reared mice. Indeed, subsequent studies done in mice subjected to various metabolic challenges have elicited a more robust connection between FoxO1 and hepatic lipid metabolism. L-FoxO1 mice rendered insulin resistant by Western-type diet (WTD) feeding show exacerbated hepatic steatosis compared to controls in the fed state [168]. This phenotype is due at least in part to an impaired ability to generate 12α-hydroxylated bile acids, which serve as endogenous signaling molecules that limit TG deposition, in the absence of liver FoxO1 [168].

Gain-of-function models also have helped to uncover potential connections between FoxO1 and hepatic lipid handling. Compared to controls, livers from mice overexpressing a constitutively nuclear FoxO1 showed decreased feeding-induced DNL coupled with lower expression of lipogenic genes including *Gck*, *Pklr*, *Fas*, *Acaca*, and *Srebf1* [160]. In keeping with an effect of FoxO1 on lipogenic gene expression, FoxO1 has been shown to block the activating *O*-glycosylation of ChREBP, reducing its stability and ability to transactivate expression of lipogenic genes [246]. Interestingly, mice overexpressing a constitutively nuclear form of FoxO1 acutely rather than congenitally show increased hepatic TG, due apparently to increased insulin sensitivity potentially driving SREBP-1c [247].

Altered TG secretion may also underlie FoxO1 regulation of hepatic lipid homeostasis, although it is important to note that secreted (*i.e.*, circulating) TG levels are not necessarily reflective of hepatic TG storage [63, 248-251]. FoxO1 has been shown to induce expression of microsomal TG transfer protein (MTP), which in turn regulates lipid loading onto apoB for secretion as VLDL [242, 243]. Mice transgenically overexpressing a constitutively nuclear FoxO1 exhibited increased TG secretion proportional to increased MTP levels while acute knockdown of FoxO1 expression by siRNA led to the opposite [243], although other studies on the constitutively nuclear mutant have shown decreased TG secretion [160]. Some data have suggested that FoxO1 can directly regulate the expression of apoB [243, 252] and apoC-III [244] as well, in both cases leading to accumulation of TG in plasma. On the other hand, in the context of insulinopenic hyperglycemia, L-FoxO1 mice exhibit increased VLDL secretion leading to higher serum TG without an effect on liver TG content [241].

Most of these models have attempted to cast FoxO1's ostensible regulation of hepatic lipid metabolism as mechanistically separate from its canonical regulation of HGP. However, recent studies in our laboratory have argued in favor of a unified, parsimonious explanation for FoxOs' dual effects on glucose and lipid handling in the liver [253]. L-FoxO1,3,4 mice show significantly higher levels of liver TG attributable to higher rates of DNL [245, 253] and potentially to lower rates of fatty acid oxidation [245]. The significant increase in DNL in these mice is not, however, associated with increased expression of most classical lipogenic enzymes (*e.g.*, *Fasn*, *Acaca*) [253]. Rather, it appears that a robust and reproducible increase in *Gck* expression independent of feeding state coupled with decreased *G6pc* in the absence of FoxO may be responsible [253]. This constitutively decreased *G6pc*:*Gck* ratio results in significantly increased flux through glycolysis and DNL regardless of feeding state [253].

Thus, FoxOs may be the key to the transcriptional control of the fasting/feeding transition [253]. During fasting, when insulin signaling is low, active FoxO orchestrates an increase in *G6pc* expression while inhibiting the expression of *Gck*, thereby shunting carbons into HGP at the expense of DNL [253]. In the fed state, when insulin signaling inhibits FoxO activity, these effects are reversed, favoring DNL over HGP [253]. These mechanisms may also be at play in L-FoxO1 mice, which also show increased *Gck* expression with decreased *G6pc* [144, 145, 167], but may be, as in the case of glucose metabolism by FoxO, partially compensated for by intact FoxO3 and FoxO4 [144, 159, 160, 179, 254].

The establishment of *Gck* as a FoxO target gene is a seminal contribution given its utter centrality in insulin's control of carbon flux through the hepatocyte [255-258]. Although its vaunted insulin responsiveness had previously been thought to be due to regulation by SREBP-1c [211, 259], this view is no longer generally accepted [258] leaving an explanatory void now filled by FoxO. Indeed, mice with liver-specific deletion of *Akt1/2* show a near total loss of Gck, but concomitant knockout of *Foxo1* restores Gck levels nearly to normal (presumably knockout of *Foxo3* and *Foxo4* would result in a complete rescue) [167]. Thus, insulin signaling appears to orchestrate DNL at least partially in parallel through regulation of FoxO → *Gck* and SREBP-1c → *Fasn*/*Acaca*/etc. [253]. Naturally, the coordinated control of *G6pc* vs. *Gck* expression also need not be mutually exclusive with FoxO regulation of other lipid-regulatory pathways (*e.g.* bile acid metabolism, etc.).

1.5.3. Overview of Hepatic Lipid Metabolism: Fatty Acid Oxidation

During times of fasting the liver and other tissues produce glucose in order to maintain euglycemia. This is not the only mechanism for fueling the processes of life in the absence of dietary nutrients, however. In the post-absorptive state, as insulin levels fall, the inhibition of adipocyte lipolysis decreases, allowing for adrenergic signaling to stimulate the release of free fatty acids for transport back to the liver where they can be oxidized to provide energy [183].

The process of fatty acid oxidation (FAO) occurs in the mitochondrial matrix [3 pp. 636-637]. In order to enter the mitochondrion, however, the fatty acid must first be "activated" through coupling to acetyl-CoA, forming an acyl-CoA. The energy stored in the acyl-CoA thioester bond can then be expended in coupling the fatty acid to carnitine through the action of carnitine palmitoyltransferase-1 (CPT-1). The fatty acyl-carnitine adduct is then whisked through to the mitochondrial matrix where the carnitine moiety is removed by carnitine palmitoyltransferase-2 (CPT-2) and the fatty acid is recoupled to acetyl-CoA. The mitochondrial fatty acyl-CoA is then free to undergo oxidation. Fatty acids are oxidized through the removal of two-carbon acetyl-CoA units (*i.e.*, the exact reverse of the process of DNL) proceeding from the β (carboxyl) end of the fatty acid chain. A series of four enzymatic reactions are necessary to catabolize saturated fatty acid chains. Catabolism of unsaturated fatty acids requires additional enzymes [3 pp. 636-642].

Each of the acetyl-CoA units removed from a fatty acid chain can then be further metabolized by the citric acid cycle and oxidative phosphorylation, both of which also take place inside the mitochondrion, just as for acetyl-CoA derived from glycolysis [3 pp. 660-662, 182]. Fatty acids are therefore able to provide fuel for the liver as it engages in energetically costly processes of HGP, as the brain and erythrocytes are themselves unable to oxidize fatty acids and strongly "prefer" to metabolize glucose [3 pp. 649-662, 744-752]. Fatty acids themselves, however, are unable to be converted to glucose in animals due to the net loss of the lipid-derived carbons as CO_2 during the irreversible steps of the citric acid cycle [3 pp. 535-538]. Fatty acids do not only provide the fuel for gluconeogenesis, however; they also allosterically prevent glucose oxidation and promote gluconeogenesis, thus sparing glucose for the brain [125]. During times of prolonged fasting or starvation, acetyl-CoA derived from FAO can be used

directly to provide energy to the brain. That is, the liver is able to condense two molecules of acetyl-CoA to generate acetoacetate, which can be further metabolized to acetone and to β-hydroxybutyrate. These three “ketone bodies” can then be converted back to acetyl-CoA in other tissues and used in place of pyruvate-derived acetyl-CoA [3 pp. 649-650].

A key transcriptional coordinator of the FAO program is peroxisome proliferator-activated receptor-α (PPARα) [260, 261]. PPARα is a nuclear hormone receptor that, in conjunction with retinoid X receptor (RXR), responds to a variety of fatty acid species by inducing FAO genes [262] including lipid transporter *Cd36* [263], *Cpt1*, various acyl-CoA dehydrogenases (*e.g.*, *Acadm*), and acyl-CoA oxidase-1 (*Acox1*). In addition to its role in promoting HGP, PGC-1α also stimulates FAO at least in part by serving as a co-activator of PPARα [174, 264]. Interestingly, it appears that the hypolipidemic action of PPARα may also include transrepression of FoxO1, particularly with respect to its induction of apoC-III expression [252].

No liver-specific PPARα knockout model has yet been published. Whole-body PPARα-knockout mice show decreased fasting β-oxidation of long-chain fatty acids [265-267], although these mice are also protected from the development of obesity-induced insulin resistance [268]. Nevertheless, the relatively easy activation of PPARα with synthetic ligands such as the fibrate class of drugs has allowed it to be harnessed as a therapeutic avenue in the treatment of hyperlipidemia [269].

1.5.4. Regulation of Hepatic Fatty Acid Oxidation by Insulin

In an oft-quoted article from 1992, J. Denis McGarry posited that Oskar Minkowski, who is credited with linking diabetes mellitus to the pancreas, had focused too much on the sweet taste of his pancreatectomized dog’s urine and not sufficiently on the acetone scent of the dog’s breath in pondering the pathogenesis of diabetes [270]. Had Minkowski instead been more attuned to olfactory stimuli, McGarry argued, he and his successors would have come to think of diabetes as primarily a disorder of fatty acid metabolism [270]. Insulin deficiency, as in type 1 diabetes, results in unchecked lipolysis that leads to extremely high levels of FFA in the blood [183]. FFAs exacerbate hyperglycemia at multiple levels, including by hindering glycolysis and promoting gluconeogenesis in liver [121, 125, 126, 270];

reducing FFA levels has been shown to ameliorate hyperglycemia [271]. Furthermore, the skyrocketing FFA levels also drive very high rates of FAO and ketone body production, hence the aroma of nail polish remover that Minkowski was meant to have noticed emanating from his dog [270]. In patients with type 1 diabetes, this buildup of ketone bodies can result in radical alterations in blood pH, termed diabetic ketoacidosis (DKA), and results in short order in coma and death [5 p. 1407].

The exact mechanism of insulin's suppression of FAO however, has not garnered as much experimental attention in recent years as has its promotion of DNL. It is apparent from what has been heretofore discussed that insulin exerts indirect control over the process. Insulin inhibits adipose tissue lipolysis, thereby cutting off the supply of ketogenic precursors to the liver [270]. Insulin also stimulates the uptake of glucose directly by promoting Glut4 translocation in muscle and adipose tissue and indirectly by "trapping" glucose as G6P in hepatocytes through its induction of *Gck* and posttranslational regulation of various steps of glycolysis [3 pp. 749-755]. Insulin-stimulated glycolytic flux in liver and skeletal muscle generates acetyl-CoA that, through mass action, will be metabolized to malonyl-CoA by ACC1 for DNL [3 pp. 744-750]. Malonyl-CoA is a potent inhibitor of CPT-1, preventing the import of fatty acids into the mitochondrion for FAO [181]. Insulin is also able to regulate ACC1 activity by promoting the removal of inhibitory phosphates placed by PKA in response to glucagon or epinephrine. This, then, also stimulates the production of malonyl-CoA to inhibit FAO [3 pp. 660-662].

The above means of insulin control of FAO at the global level require contributions from extrahepatic tissues. Whether hepatocyte insulin signaling *per se* controls FAO more proximally than solely by altering malonyl-CoA production outside of the mitochondrion [272] remains unclear. Mice lacking InsR specifically in hepatocytes (LIRKO) show elevated plasma ketones in the fed state, associated with increased expression of *Cpt1* and *Hmgcs2*, the latter a ketogenic enzyme [202], suggesting a transcriptional element of regulation. One way in which insulin signaling may influence FAO-related gene expression is via Akt phosphorylation of PGC-1α at Ser 570, which has been shown to prevent its activation of FAO in a hepatoma cell line [174]. This issue will be further discussed in Chapter 5.

1.6. Molecular Mechanisms of Insulin Resistance

1.6.1. Insulin Receptoropathies as a Model of Pure Insulin Resistance

Insulin resistance is a *sine qua non* of type 2 diabetes mellitus; fasting hyperglycemia results from inadequate insulin signaling. While hyperglycemia is thought to account for the microvascular complications of diabetes, it is insufficient to explain the characteristic dyslipidemia and attendant atherosclerosis associated with the disease [5 pp. 1420-1421, 46]. Thus, dysregulated insulin action *per se* may drive such lipid abnormalities. Moreover, as the liver is one of the principal regulators of bodily glucose and lipid metabolism, it is thought to be central to the pathophysiology of insulin resistance.

Based on our understanding of the hepatic insulin-signaling pathway as described earlier, we can formulate predictions as to the consequences of impaired insulin action (illustrated in Fig. 1.1B). We can consider, for instance, the case of the liver InsR knockout (LIRKO) mouse, in which all insulin signaling is curtailed specifically in the liver [273]. In this case, the hepatocyte is indifferent to the presence of insulin. As a result, there is no activation of the insulin-signaling cascade (*e.g.*, phosphorylation of Irs or Akt). If we first consider the glucose arm of the pathway, the inability to activate Akt in response to insulin results in the inability to phosphorylate and thereby inactivate FoxO proteins. FoxOs therefore remain largely nuclear in localization and promote glucose production, perhaps by inducing the expression of gluconeogenic enzymes. Insulin also is unable to engage in its normal posttranslational regulation of enzymes involved in glucose flux. Thus, on the whole, the hepatocyte moves to release glucose through glycogenolysis and gluconeogenesis, contributing to the characteristic hyperglycemia of diabetes. This prediction is borne out by the glucose intolerance exhibited both by the LIRKO mouse prior to liver failure [273, 274] and, more importantly, in humans with insulin receptoropathies due to *Insr* mutations or acquired autoantibodies against InsR [65].

We also predict that dysfunction of InsR would abrogate the stimulatory effect of insulin on hepatic lipid biosynthesis. Specifically, insulin-stimulated cleavage of SREBP-1c downstream of Akt would be impaired, thus resulting in decreased expression of lipogenic enzymes. In addition, as in the case of the glucose arm, insulin would not be able to engage in posttranslational regulation of lipid partitioning,

such as its normal inhibition of VLDL secretion. Thus, on the whole, insulin-stimulated lipid biosynthesis would be expected to be low.

Again, these mechanistic predictions are validated both in mouse and human models of insulin receptoropathy. The LIRKO mouse liver shows significantly reduced SREBP-1c processing in response to re-feeding, thus resulting in defective induction of *Fasn* and *Scd1* expression [47], which in turn translates into decreased DNL [202]. Interestingly, liver TG content is normal at steady state in the LIRKO mouse, potentially due to homeostatic compensation by ChREBP [202]. Human insulin receptoropathy patients likewise exhibit low to normal liver fat content and normal rates of DNL, the latter value not frankly low perhaps due to minor residual signaling capacity of the defective receptors or hyperglycemia-associated ChREBP activity [65]. Diminished TG biosynthesis by InsR-deficient livers both in mouse and man also results in low to normal plasma TG levels, although this often translates into pro-atherogenic small, dense VLDL enriched in cholesterol relative to TG [47, 65, 275].

1.6.2. Garden-Variety Insulin Resistance Is Selective in Nature

Although the phenotype of patients with insulin receptoropathies tidily comports with our basic understanding of the hepatic insulin-signaling pathway, it partially contradicts the prevailing epidemiologic picture of IR. All type 2 diabetic patients by definition manifest fasting hyperglycemia due to impaired insulin action, but the vast majority of these patients do not exhibit the benign triglyceride profiles of insulin-receptoropathy patients [40, 41]. Instead, these individuals, who often have developed IR associated with diet-induced obesity, are prone to dyslipidemia including elevated circulating triglycerides and LDL cholesterol as well as excessive deposition of triglyceride in the liver (hepatic steatosis) [40, 41, 248]. As aforementioned, this radically altered lipid phenotype is thought to account for the increased risk of cardiovascular disease in IR and diabetic patients [40, 41]. Thus, understanding the connection between garden-variety IR and defective lipid homeostasis is of paramount clinical importance.

In an influential essay [276], Brown and Goldstein argued that the underlying abnormality in the garden-variety IR leading to most cases of type 2 diabetes is actually an uncoupling of insulin's normal actions on the glucose and lipid arms of the pathway. In this paradigm (illustrated in Fig. 1.1C), the

glucose arm (including downstream of the FoxOs) becomes resistant to insulin, both as predicted and empirically verified in diabetic patients. On the other hand, the excessive hepatic lipogenesis exhibited by most IR patients may actually represent some preservation of insulin's normal stimulation of this process. In other words, the lipogenic pathway including SREBP-1c remains *relatively* sensitive to insulin compared to the glucose arm. IR in these patients is "selective," unlike the "pure" IR of insulin receptoropathy.

Chronic hyperinsulinemia (CHI) may represent a primary etiologic linkage between the combined excessive glucose production and excessive lipogenesis of the insulin-resistant liver. That is, excessive glucose released by the liver triggers compensatory secretion of insulin, resulting in the well-documented hyperinsulinemia of all forms of IR [276, 277]. In the case of insulin receptoropathies, InsR itself is defective and so is minimally responsive to any concentration of insulin [65]. In common forms of IR, however, InsR itself is not necessarily qualitatively defective, and thus the heightened levels of insulin can continue to activate its signaling cascade in the hepatocyte [276]. Evidently this compensatory hyperinsulinemia is sufficient to drive the lipid arm of the pathway, thus resulting in excessive lipid deposition, but is not able to adequately affect the glucose arm, hence excessive glucose production [276]. The mechanism of this CHI-induced IR, which is likely to result from downregulation of InsR [278], will be discussed at greater length below.

1.6.3. Differential Sensitivity to Insulin May Drive Selective IR

The most important question, then, is the biochemical mechanism underlying this apparent uncoupling of the glucose and lipid arms. One possibility with considerable support in the literature is that the glucose and lipid arms are *inherently differentially sensitive* to insulin. Specifically, a greater degree of insulin signaling is ordinarily required to blunt glucose production (*i.e.*, less sensitive) than to stimulate lipogenesis (*i.e.*, more sensitive). In biochemical terms, the ED_{50} for insulin's action on lipogenesis is expected to be lower than the ED_{50} for insulin's action on HGP (Fig. 1.2).

Based on this idea, we propose that pure IR and selective IR do not necessarily represent a paradox. Rather, as illustrated in Fig. 1.2, we hypothesize that they represent different stages along a

single continuum of insulin action. In either case, IR can stem from a quantitative defect in InsR signaling that alters the effective concentration of insulin "seen" by the hepatocyte rather than arising solely from some postreceptor defect as some have posited. At one end of the IR spectrum, as indicated by the blue shading in Fig. 1.2, lies the effective absence of InsR that leads to pure IR. In this region, the effective insulin concentration lies below the ED_{50} of both the glucose and lipid arms. Thus, pure IR would represent a state with too few functional InsR available to transduce any metabolic signals efficiently, resulting in the expected defects in both pathways.

On the other hand, in garden-variety (selective) IR, some degree of signaling through InsR remains intact. As indicated by the pink shading in Fig 1.2, the effective insulin concentration experienced by the cell is less than the ED_{50} for the glucose arm but is greater than the ED_{50} for the lipid arm. Thus, in practical terms, the residual capacity for transducing the insulin signal would be sufficient to drive the more-sensitive lipid arm *relative to* its effect on the less-sensitive glucose arm. This model implies that, if one's degree of hepatic insulin action were hypothetically to deteriorate, one could in theory proceed from selective toward pure IR. In other words, were signaling through InsR to worsen, insulin would progressively lose its ability to drive lipogenesis. Any residual ability to properly regulate HGP that were retained in the partially IR state – albeit insufficient to keep blood glucose levels below the clinical cutoff for diabetes – would then be completely lost in pure IR, further exacerbating glucotoxicity.

This theoretical construct presents two major questions. First, what is the mechanism behind the loss of functional InsR? Second, how does incremental loss of InsR differentially affect downstream signaling pathways?

1.6.4. Downregulation of InsR Signaling by Hyperinsulinemia

The reason behind the effective absence of InsR is clear in the case of pure IR, but what causes this loss of functional InsR in selective IR? We suspect that the mechanism responsible for diminished InsR signaling in selective IR is the same as that driving the inappropriate deposition of lipid: hyperinsulinemia.

As described above, hyperinsulinemia results from the attempt of pancreatic β cells to compensate for excessive HGP. Chronic hyperinsulinemia *per se*, however, is a known cause of IR, even

in the absence of inchoate IR preceding the development of hyperinsulinemia [277]. For example, patients with insulinoma, a benign insulin-secreting tumor, can be considered a model of primary hyperinsulinemia. These patients often exhibit reduced insulin action (*e.g.*, by hyperinsulinemic-euglycemic clamp) in proportion to their hyperinsulinemia that is reversed following removal of the tumor [277, 279]. Moreover, mice engineered to express 8 or 32 copies of the human insulin gene (resulting in circulating insulin levels 2 or 4 times control) display marked glucose intolerance along with hypertriglyceridemia, characteristic of selective IR. Studies of first-degree relatives of patients with T2DM provide further evidence of a more "physiologic" primary hyperinsulinemia as an early event in the development of IR [270]. Even though these supposedly healthy people showed completely normal glucose tolerance test (GTT) profiles, their insulin levels both before and during the test were twice as high as controls [280]. This finding appears to dovetail with an apparent genetic predisposition toward primary hyperinsulinemia in these family members [281], suggesting that the GTT findings may actually have been inappropriately normal for the high insulin and thus reflective of early IR [270]. It merits reemphasizing that the physiologic importance of CHI to IR need not be limited to rare case of primary hyperinsulinemia, as CHI is a cardinal feature of all forms of IR prior to β-cell failure.

CHI is believed to induce IR at least in part by causing a functional downregulation of InsR, an example of negative feedback employed by numerous receptor-ligand pairs [277, 278, 282, 283]. The extent of IR downregulation in the face of CHI would be expected to dampen signaling through InsR in a dose-dependent manner [284, 285]. Thus, in a patient experiencing mounting IR, the steady rise in insulin levels over time would result in a progressive loss of hepatic InsR function such that the effective hepatocyte "insulinization" falls in parallel [286]. Were garden-variety IR to deplete InsR such that the effective insulin concentration were delimited to the pink-shaded region in Fig. 1.1, the clinical stigmata of selective IR would arise. Again, in the case of pure IR, the severe defect in InsR function renders the hepatocyte utterly indifferent even to the extreme hyperinsulinemia described in such patients.

The paradigm described above suggests that most IR patients should show a partial loss of functional InsR [287]. Specific binding of insulin to purified human liver plasma membranes isolated during liver biopsy was found to be significantly decreased—but not absent—in both non-diabetic and diabetic

obese patients compared to non-obese controls [288, 289]. Several studies provide evidence of InsR downregulation in a variety of other human cell types as well [290-293]. Such studies have not, however, directly assessed the simultaneous effect of partial InsR downregulation on the differential insulin signaling to lipid *vs.* glucose metabolism.

The biochemical mechanism behind CHI-induced InsR downregulation is complex and involves both alterations in receptor numbers and affinity for insulin. Hormone-bound InsR, as in the case of other receptor tyrosine kinases, is internalized and degraded in a variety of cell types including liver, thereby decreasing the total population of InsR. CHI may also modulate InsR number by decreasing levels of *Insr* mRNA [294], although others have not found such an effect [295]. Second, CHI decreases insulin's affinity for its receptor. Each InsR has two insulin binding sites, the second of which is only occupied at high concentrations of insulin. Binding of a second insulin to InsR occurs at a lower affinity than the first, decreasing the overall average affinity of insulin binding [296]. CHI also alters the splicing of *Insr* mRNA to favor the lower affinity long form (InsR-B) *versus* the higher affinity short form (InsR-A) [297], although the significance of this mechanism in liver, where InsR-B predominates under normal circumstances, is unclear [298].

1.6.5. Insulin Signaling Diversity Results from Isoform Combinations

The second major question posed by our model is how a functional loss of InsR translates into differential effects on residual insulin signaling to downstream pathways. In other words, what is the molecular mechanism behind the phenomenological dose-response curve difference between glucose and lipid metabolism?

Thus far, the only documented cause of the pure IR phenotype is insulin receptoropathy. This suggests that the various defects that result in selective IR lie at the postreceptor level. It is convenient to think of insulin signaling in a streamlined fashion (*e.g.*, InsR → Irs → PI3K → Akt), a model that is difficult to reconcile with selective IR. However, in reality, each of these upstream steps in the pathway is actually represented by a group of effectors. There are, in fact, four IRS isoforms, three catalytic and five regulatory PI3K subunit isoforms, and three Akt isoforms. Consequently, activation of the singular InsR

can actually engage many different, albeit related, downstream signaling pathways that can generate the signaling diversity needed to account for inherent differences in the insulin sensitivity of metabolic pathways [83].

In order for this model to be plausible, there must exist demonstrable differences in the ability of isoforms of each of these upstream signaling "nodes" to transduce the insulin signal to downstream metabolic pathways [83]. Transgenic mouse models manipulating these signaling intermediates show this indeed to be the case. Below we consider the cases of the Irs and Akt isoforms, although similar work has also been done with regard to the various PI3K subunit combinations.

Insulin-receptor substrates (IRS)

There are two major IRS isoforms in liver, IRS1/2. Deletion of both of these isoforms in the liver results in glucose and insulin intolerance due to increased HGP that can be rescued by concomitant deletion of FoxO1 [166]. As in the LIRKO model, although hepatic TG levels are normal on a chow diet, liver-specific Irs1/2-deficient mice show a significant decrease in induction of *Srebf1c* expression and its lipogenic gene program in response to re-feeding as well as reduced serum TG [166]. The liver phenotypes of the whole-body knockouts of IRS1 and IRS2 are confounded by detrimental effects on other tissues [299].

Loss of IRS1/2 together resembles a total loss of insulin signaling [166]. On the other hand, loss of individual Irs isoforms result in different phenotypes, consistent with the idea of isoform-specific signaling driving differential insulin responsiveness. Liver-specific deletion either of IRS1 or IRS2 resulted in decreased insulin sensitivity under hyperinsulinemic-euglycemic clamp conditions due to a complete loss of insulin inhibition of HGP [300] but a similar study found no difference in these parameters in IRS2 liver knockout mice [301]. Although there was no difference in liver TG content on chow diet, deletion of *Irs1* decreased the degree of steatosis in the livers of HFD-fed mice compared to control while liver deletion of *Irs2* had no effect [300]. This was mirrored by dampened feeding-responsive expression of lipogenic genes such as *Fasn* and *Gck* that are more dramatic in the IRS1- *versus* IRS2-deficient liver [300]. An RNAi-based approach to acute silencing of *Irs1* and/or *Irs2* expression in adult liver, however, suggested that IRS1 is more integral to insulin's control of glucose metabolism and IRS2 to the lipid arm,

although this conclusion was largely based on gene-expression patterns rather than on physiologic readouts [302].

Although reconciling such data may be difficult due to acute *versus* chronic effects of IRS knockdown, they emphasize the overall possibility that insulin signaling may differentially regulate far-downstream aspects of glucose and lipid metabolism through differential activation of IRS isoforms. This interpretation is bolstered by mechanistic data from primary rat hepatocytes demonstrating that CHI-induced insulin resistance specifically leads to decreased mRNA and protein levels of IRS2 but not of IRS1 [295]. Insulin-stimulated tyrosine phosphorylation of IRS2 and association of IRS2 with PI3K in response to insulin are abrogated in CHI-treated cells [295]. On the other hand, these parameters are preserved with respect to IRS1, in addition to insulin-induced expression of *Srebf1c* [295]. (Similar experiments performed in rat hepatoma cells have, however, found decreased protein levels of IRS1 in response to CHI [303].) While these experiments suggest that SREBP-1c is more under the control of IRS1 than IRS2, generally consistent with the transgenic mouse data, experiments in cultured MEFs suggest that IRS2 more efficiently affects the phosphorylation of FoxO proteins [304].

Akt

As in the case of IRS, there are two major isoforms of Akt operating in liver, Akt1/2. Liver-specific deletion of both major isoforms, in a fashion again similar to both the LIRKO and liver-specific IRS1/2-knockout mice, develop glucose intolerance due to the inability of insulin to suppress HGP. This phenotype is associated with increased expression of *G6pc* and *Pck1* and these abnormalities can be rescued by concomitant deletion of FoxO1 [167]. Although the effect of liver-specific Akt1/2 knockout on hepatic or plasma lipids has not been reported, insulin-stimulated expression of *Srebf1c* and *Gck* were significantly decreased relative to controls [167]. Again, prior experimental evidence suggests the possibility of differential regulation of metabolism by Akt1 *versus* Akt2. In cultured adipocytes, for example, CHI treatment suppressed insulin-stimulated glucose uptake by Glut4 but did not prevent the blunting of lipolysis downstream of FoxO1 because the latter process is controlled by both Akt1 and Akt2 while the former is regulated only by Akt2 [305].

In the context of liver, tissue-specific elimination of Akt2 results in a significant impairment of insulin inhibition of HGP (specifically of glycogen synthesis and catabolism) without effects on *G6pc* or *Pck1* expression [112]. Hepatic Akt2-knockout livers do not manifest obvious deficiencies in lipid metabolism in the chow-fed state, although short-term feeding with a high-carbohydrate diet unmasks a significant lowering of DNL in the Akt-deficient liver [158, 306]. Moreover, induction of metabolic stress through various genetic (*e.g.*, leptin deficiency) and dietary (*e.g.*, Surwit HFD feeding) manipulations reduced steatosis in the Akt2-deficient livers due to decreased lipogenic gene expression and coordinately lower rates of DNL [306]. Interesting, subsequent experiments demonstrated that the decreased rates of DNL in HFD-fed Akt2-null livers occurs alongside decreased expression of *Fasn* and *Gck* despite normal induction of *Srebf1c* [158]. Thus, on the whole, Akt2 plays a role in hepatic lipid metabolism under conditions of metabolic stress but is a prime regulator of hepatic glucose metabolism under normal physiologic conditions.

There has been no study to date focusing solely on liver-specific Akt1 deficiency. Whole-body *Akt1* knockout mice, unlike global *Akt2* knockouts, are susceptible to perinatal mortality and exhibit growth defects but, also unlike *Akt2*$^{-/-}$ animals, do not demonstrate notable perturbations in glucose metabolism [307-309]. This suggests that the bulk of insulin's effects on metabolism are mediated through Akt2 while Akt1 is more important in mediating mitogenic aspects of insulin and growth factor signaling [310-313]. It is important, however, to note these studies did not explore aspects of whole-body or hepatic lipid metabolism in depth in the *Akt1* knockout mice.

Signaling diversity may be generated through the Akt "node" not only through the unique activities of Akt1 *versus* Akt2 but also through differential activating phosphorylation of Akt1/2. Both Akt1 and Akt2 are phosphorylated in response to insulin signaling on two main residues, Thr 308 (in Akt1)/Thr 309 (in Akt2) and Ser 473 (in Akt1)/Ser 474 (in Akt2). Again, although both of these sites are phosphorylated in response to insulin signaling, the individual kinases responsible for phosphorylating these two residues differ. In the case of Thr 308/309, the phosphate transfer is catalyzed by PDK1 following PI3K activation within the classical upstream insulin-signaling paradigm [86, 314]. On the other hand, Ser 473/474 does not appear to be phosphorylated by PDK1, but rather by mTOR complex 2 (mTORC2) [87-89]. A number

of other kinases have also been implicated in the phosphorylation of Ser 473 [315]. Although some have speculated that phosphorylation of Thr 308 is dependent on an initial "gatekeeping" phosphorylation at Ser 473, the two phosphorylation events have been shown to be capable of independent regulation and are both required for full activation of Akt activity [88, 316-318].

Experimental evidence suggests that differential phosphorylation of Thr 308 *versus* Ser 473 results in selective regulation of downstream signaling pathways [316]. Liver-specific deletion of rictor (LiRiKO), the defining constituent of mTORC2, abrogates Ser 473 phosphorylation in response to insulin signaling while leaving Thr 308 phosphorylation intact [88]. This manipulation results in the loss of Akt's ability to phosphorylate and inhibit FoxO1 and GSK3β but not of its ability to trigger the activation of mTORC1 and its substrates, S6K1 and 4EBP1 [88]. Interestingly, however, despite the preservation of mTORC1 activation, LiRiKO mice exhibit defects in insulin regulation of both HGP and DNL [88]. Cells from whole-body rictor knockout mice have also demonstrated defects in phosphorylation of FoxO but not of TSC2 → mTORC1 → S6K1 [89]. On the other hand, liver-specific deletion of *Pdk1* (L-*Pdk1*KO) resulted in deficient phosphorylation of FoxO1 and S6K1 but not of GSK3β [319]. In both LiRiKO (*i.e.*, phospho-Ser 473-deficient) and L-*Pdk1*KO (*i.e.*, phospho-Thr 308-deficient) livers there was a significant decrease in *Gck* expression [88, 319]. Moreover, in both of these models, rescuing *Gck* expression via adenovirus rescues the expression of some or all of the classical ChREBP-inducible lipogenic genes but not *Srebf1c* [88, 319]. Interestingly, in the LiRiKO mouse, even the restoration of glucose flux and lipogenic gene expression is not sufficient to rescue DNL [88].

1.7. Hypotheses and Specific Aims

Although we know a great deal about how insulin acts and how resistance to those actions can contribute to metabolic disease, a great many questions remain outstanding. Perhaps principal among these stands the phenomenon of selective IR, as the unfavorable combination of escalated HGP and DNL give rise to the mutually reinforcing hyperglycemia and dyslipidemia of T2DM. A great part of our problem in elucidating the pathogenesis of selective IR is the fact that we still do not completely understand the intimate relationship between insulin's actions on hepatic glucose and lipid metabolism even in the healthy liver. However, I have identified two promising avenues of investigation that may lend us greater insight into these pressing questions.

First, as discussed at length above, I hypothesize that **chronic hyperinsulinemia drives selective IR due to inherent differences in the insulin responsiveness of the glucose and lipid arms of the pathway**. Thus, in Chapter 3, I describe a model of chronic hyperinsulinemia in isolated primary hepatocytes that does indeed recapitulate selective IR. I further substantiate InsR downregulation as a potential mechanism of CHI-induced selective IR by demonstrating that acute titration of InsR levels *per se* differentially affects insulin's ability to halt glucose production *versus* stimulating *de novo* lipogenesis.

Second, although I make reference to the glucose and lipid "arms" of the insulin-signaling pathway, this is merely shorthand based in part on the largely historical portrayal of these pathways as divergent downstream of Akt. Aside from the evident allosteric and thermodynamic coordination of HGP, DNL, and FAO, insulin appears to exert long-term control over these processes at the transcriptional level as well through FoxO proteins, particularly FoxO1. Indeed, no other insulin-regulated protein downstream of Akt has been so extensively validated as essential to the regulation of both glucose and lipid metabolism. Yet most of the evidence linking FoxO1 to control of glucose and lipid metabolism, though convincing in a phenomenological sense, does not adequately explain how this transcription factor can affect metabolic processes outside of the nucleus. In other words, we do not know which of FoxO1's hundreds of purported target genes are truly essential for fulfilling its clear roles in metabolic control.

In Chapter 4, I describe an unbiased first step in this direction by taking a genetic approach. Specifically, I hypothesize that **determining whether FoxO1 acts as a direct transcription factor or as a transcriptional co-regulator with respect to a particular physiologic parameter will allow us to rule out or rule in subsets of its target genes as responsible for that effect.** To that end, I will describe the L-DBD mouse, which expresses only a DNA binding-deficient form of FoxO1 in its liver. By comparing the L-DBD mouse to L-FoxO1 and control animals, I have determined the primary "mode" of FoxO1's action on hepatic glucose and lipid metabolism. I have then coupled this physiologic information with transcriptomic analysis of each of these mouse models to drastically narrow down the list of candidate target genes for each metabolic process.

Finally, in Chapter 5, I attempt to **integrate** the information learned from these two systems to advance our overall mechanistic understanding of hepatic insulin action and resistance.

Chapter 1

FIGURES

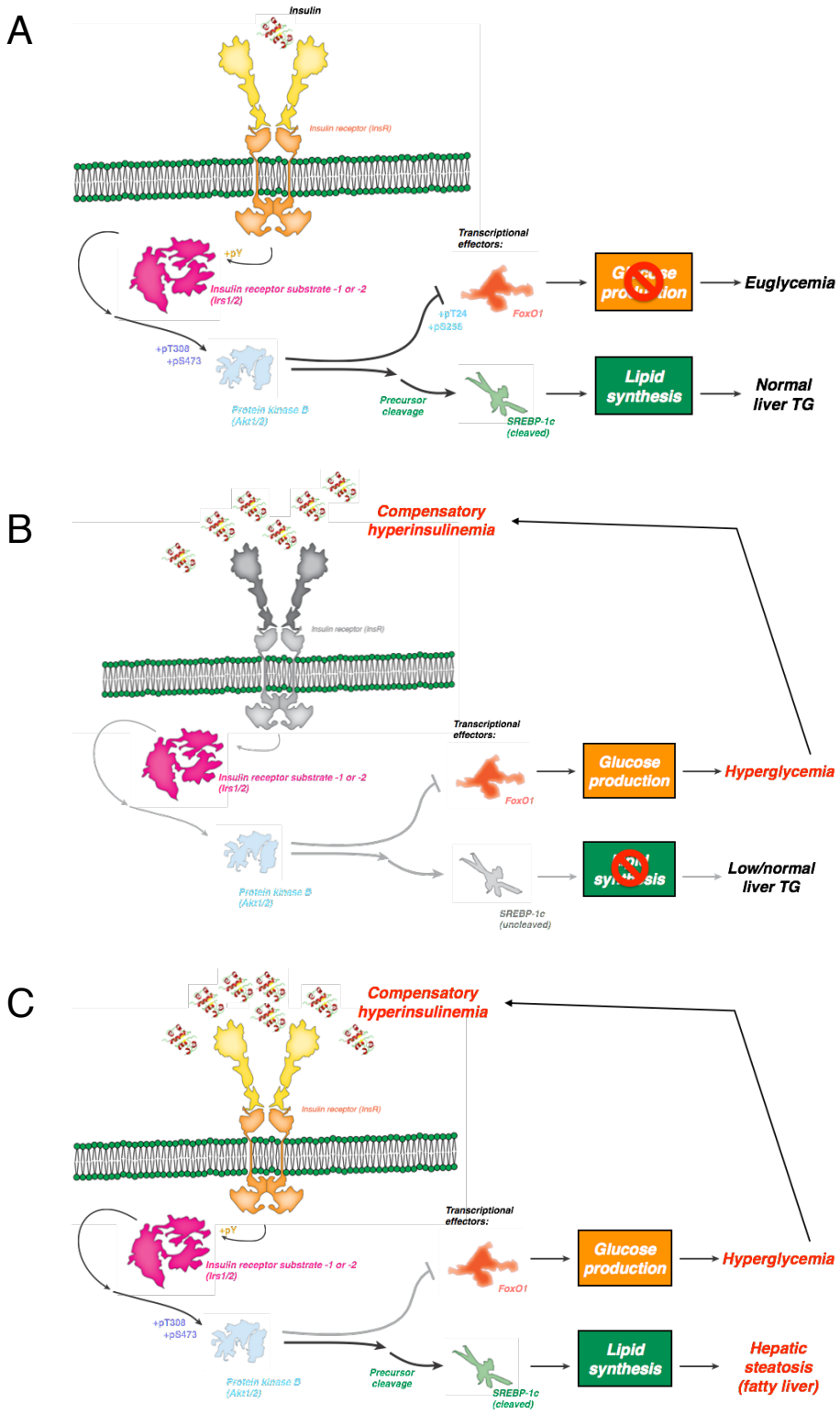

A
Insulin
Insulin receptor (InsR)
+pY
Insulin receptor substrate -1 or -2
(Irs1/2)
+pT308
+pS473
Protein kinase B
(Akt1/2)
Transcriptional
effectors:
FoxO1
+pT24
+pS256
Glucose
production
Euglycemia
Precursor
cleavage
SREBP-1c
(cleaved)
Lipid
synthesis
Normal
liver TG
B
Compensatory
hyperinsulinemia
Insulin receptor (InsR)
Insulin receptor substrate -1 or -2
(Irs1/2)
Protein kinase B
(Akt1/2)
Transcriptional
effectors:
FoxO1
Glucose
production
Hyperglycemia
SREBP-1c
(uncleaved)
Low/normal
liver TG
C
Compensatory
hyperinsulinemia
Insulin receptor (InsR)
+pY
Insulin receptor substrate -1 or -2
(Irs1/2)
+pT308
+pS473
Protein kinase B
(Akt1/2)
Transcriptional
effectors:
FoxO1
Glucose
production
Hyperglycemia
Precursor
cleavage
SREBP-1c
(cleaved)
Lipid
synthesis
Hepatic
steatosis
(fatty liver)

Figure 1.1

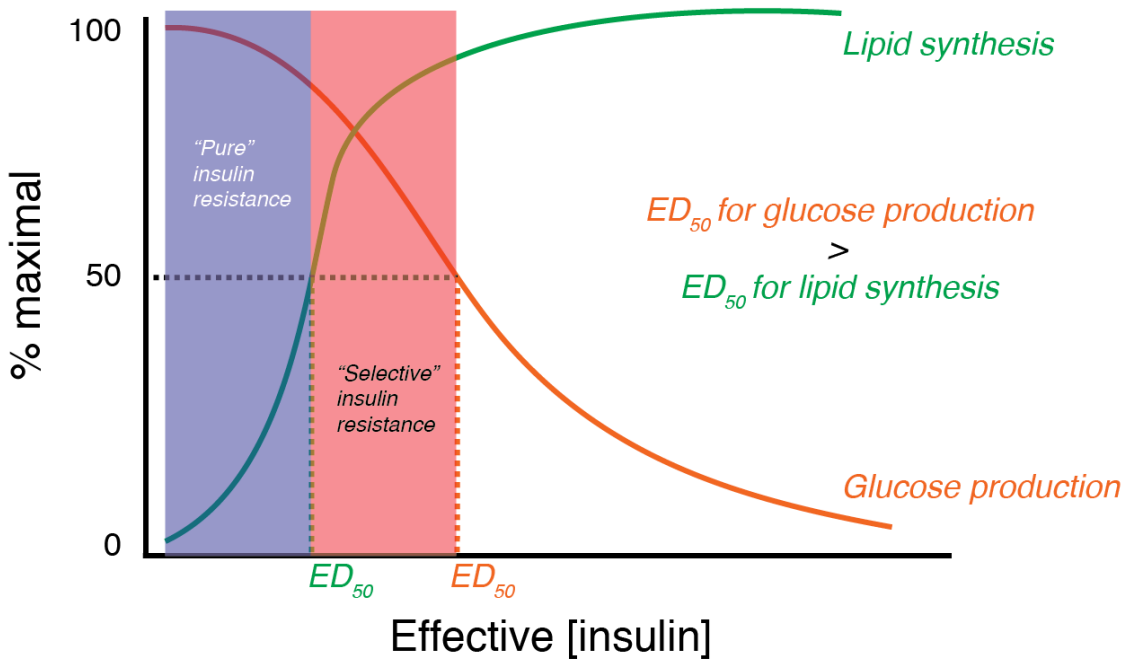


Figure 1.2. Theoretical Continuum of Insulin Action

Effective [insulin] is a general reflection of the amount of InsR on the cell surface.

Figure 1.2

Chapter 2

EXPERIMENTAL PROCEDURES

2.1. *In Vivo* Studies

2.1.1. Mice and Diets

Male and female C57Bl6/J mice aged over 8 weeks were used for all experiments in Chapter 3. Male mice aged 12-20 weeks were used for all experiments in Chapter 4 with the exception of L-FoxO1,3,4 mice and associated Cre- controls, aged 8-10 weeks. L-FoxO1 mice have been previously described [145]. Heterozygous DBD knock-in mice were generated by two previous postdoctoral fellows in the lab, Tadahiro Kitamura and Alex Banks, through homologous recombination by recombinase-mediated cassette exchange (RMCE) [157, 320] (Fig. 2.1). Breeding for the L-DBD colony was begun by Kyoichiro Tsuchiya before the author joined the lab. All L-FoxO1, L-DBD, and respective Cre- control mice were bred on to the C57Bl6/J background. Genotyping was performed by Thomas Kolar; primer sequences are listed in Table 2.1. Mice were weaned at 3 weeks of age to standard (chow) diet (Harlan; 4% fat). Western-type diet (Harlan; 21% anhydrous milk fat, 34% sucrose, 0.2% cholesterol) was fed to animals as indicated beginning at 6 weeks of age for 10 weeks. L-FoxO1,3,4 mice and Cre- controls, all on a mixed genetic background, were furnished by Rebecca Haeusler and Samuel Lee. The Columbia University IACUC approved all animal procedures.

2.1.2. Metabolic Testing

Overnight fasts were conducted for 16 hr, from 17:00 to 09:00. Mice to be re-fed were then given *ad libitum* access to chow from 09:00 to 13:00. Blood glucose measurements were made from tail vein blood using OneTouch glucose monitor and strips, immediately before sacrifice for terminal procedures (Lifescan). Intraperitoneal glucose and pyruvate tolerance tests were performed in overnight-fasted mice using a dose of 2 g/kg dextrose (*aq*) or sodium pyruvate (*aq*). Insulin tolerance test was performed in 5-hr-fasted mice using a dose of 0.8 U/kg Novolog insulin. Oral lipid tolerance test (OLTT) and TG secretion experiments were performed in mice fasted for 5 h. OLTT was performed using olive oil administered orally at 10 µL/g body weight. TG secretion was measured following intraperitoneal injection of Poloxamer 407 (*aq*) at 10 µL/g. In both cases tail vein blood was collected at indicated time points and TG content measured by colorimetric assay (Wako). Tolerance tests were performed by Ana Flete-Castro.

2.1.3. In Vivo *Insulin Signaling*

Mice for *in vivo* insulin signaling assessment were fasted for 5 hr, anesthetized, and injected in the inferior vena cava with 0.5 mU/g NovoLog insulin (Novo Nordisk). After 3 min, samples of liver, soleus muscle, and epididymal white adipose tissue were snap frozen using liquid nitrogen.

2.1.4. Biometrics and Indirect Calorimetry

Body composition of *ad libitum*-fed adult male mice was performed via MRI (Bruker Optics) by Ana Flete-Castro and percent composition was calculated based on total body weight. Indirect calorimetry and measurement of activity was performed as previously described [157, 321] using a TSE Labmaster Platform (TSE Systems). Mice were acclimated to metabolic cages for 24 h before beginning measurements. Measurements were carried out for 72 h and averages were calculated over the three light cycles and three dark cycles of that period. Illustration of cumulative food intake over 24 h reflects the second day of measurement.

2.1.5. Tissue Sample Collection

Mice were sacrificed using CO_2 in accordance with the laboratory animal protocol. Blood was drawn by cardiac puncture immediately after sacrifice. After being allowed to clot, bloods were centrifuged and serum withdrawn. Measurements of serum insulin were made by ELISA (Mercodia) and lipids by colorimetric assays (Wako for non-esterified fatty acids and cholesterol, Thermo for TG), respectively. Tissue samples were removed within 3 min following sacrifice and snap frozen on liquid nitrogen except for liver samples used for histology, which were preserved in formalin. Histology procedures were performed by the Pathology Core in the Naomi Berrie Diabetes Center.

2.1.6. Hepatic Lipid Measurement

Hepatic lipids were extracted from ~50 mg snap-frozen tissue samples using a modified method of Folch [322]. Briefly, tissue samples were homogenized in PBS via bead mill and were then extracted successively in 2:1 chloroform:methanol and then 86 chloroform:14 methanol:1 water. The hydrophobic

phases of each extraction were pooled in glass scintillation vials and blown to dryness under N_2 gas. Dried lipids were then resuspended and solubilized in 8.5:1.5 chloroform:Triton X-100. Solubilized lipids were then blown to dryness again and resuspended in water. TG and cholesterol content were assayed colorimetrically and normalized to sample weight.

2.2. Primary Hepatocyte Studies

2.2.1. Primary Hepatocyte Isolation and Culture

Primary hepatocytes were isolated from anesthetized mice using a collagenase perfusion protocol. A vascular clip was placed over the supradiaphragmatic inferior vena cava. A 24G¾ Exel Safelet catheter was then inserted into the inferior vena cava at the level of the left renal vein and connected to a peristaltic pump to infuse 40 mL perfusion solution (HBSS with 0.5 mM EGTA) following cutting of the portal vein. Next we infused 100 mL collagenase solution (Medium 199 containing 1% BSA, 20 mM HEPES, and type 4 collagenase [Worthington] at 3 mg/g body weight). The liver was then excised and finely minced in plating medium (Medium 199 with 10% FBS, and antibiotics [100 U/mL penicillin/streptomycin (Invitrogen), 50 μg/mL gentamicin (Sigma)]). The hepatocyte suspension was filtered through a 100-μm nylon mesh (BD). Following a series of low-speed centrifugations followed by medium replacements, cells were plated at a density of 400,000/mL on collagen-coated cultureware (BD). 90 min after plating, cells were washed with PBS and plating medium was replaced. For most applications, 3 h later cells were washed twice again with PBS and incubated overnight in serum-free medium (Medium 199 supplemented with 1% fatty acid-free BSA and antibiotics). For cells treated with the chronic hyperinsulinemia (CHI) protocol in Chapter 3, medium was supplemented with 100 nM Novolog insulin at every step from plating onward, including during overnight serum starvation.

2.2.2. Glucose Production Assay

For glucose production assay, cells were washed twice with PBS and serum-free medium was replaced with glucose production medium (glucose- and phenol red-free DMEM supplemented [Sigma] with 1% BSA, 3.3 g/L sodium bicarbonate, 20 mM calcium lactate, 2 mM sodium pyruvate, and antibiotics). Cells

were incubated with 100 μM 8-chlorophenylthio-cAMP (Sigma) with or without 1 μM dexamethasone (Sigma) or vehicle for 5 hr. In experiments in which acute insulin was employed, cells were pre-treated for 1 h with insulin before adding glucose-production cocktails. For experiments with S961 (Novo Nordisk) or Akti-1/2 (Akt inhibitor VIII, Sigma), inhibitor or vehicle (DMSO for Akti-1/2) was added at the indicated concentrations 30 min before the insulin pre-treatment and was continued throughout the experiment. At indicated time points, aliquots of medium were sampled, centrifuged, and glucose content was measured via peroxidase-glucose oxidase assay (Sigma) and normalized to protein content.

2.2.3. De Novo *Lipogenesis*

Following overnight serum starvation, medium was changed to serum-free medium with or without 10 nM insulin. For experiments with S961 (Novo Nordisk), inhibitor was added at the indicated concentrations 30 min before the insulin pre-treatment and was continued throughout the experiment. After 2 hr, the medium was spiked with 0.6 μCi/mL [1,2-^{14}C]-acetic acid (PerkinElmer Life Sciences) and incubated for an additional 3 hr. Lipids were extracted twice using 3:2 hexane:isopropanol and pooled extracts were dried in glass scintillation vials under N_2 gas. For measurement of total DNL, lipids were resuspended in 2:1 chloroform:methanol and were then counted using a liquid scintillation counter (PerkinElmer). For specific measurement of TG synthesis, dried lipids were resuspended in 2:1 chloroform:methanol and transferred onto TLC plates using a SpotOn TLC Sample Applicator (Analtech). TLC was performed using a mobile phase of 70:30:1 hexane:diethyl ether: acetic acid. Areas of silica containing TG, as identified by staining with iodine vapor, were scraped into glass scintillation vials and counted as above. In both cases, CPM readings were normalized to total cellular protein.

2.2.4. Fatty Acid Oxidation

Primary hepatocytes were cultured overnight in plating medium (*i.e.*, not serum starved). Cells were washed three times with PBS and incubated for 4 hr in Medium 199 supplemented with 1.5% fatty acid-free BSA, 0.2 mM unlabeled oleic acid, and 1 μCi/mL [1-^{14}C]-oleic acid. Media from each well were transferred to glass Erlenmeyer flasks sealed with rubber plugs containing a suspended center well

(Kontes) holding grade 4 filter paper (Whatman) alkalinized with 1N potassium hydroxide. 70% (v/v) perchloric acid was then injected into each flask followed by agitation of the flasks at room temperature for 1 hr. Radiolabeled CO_2 content of each filter paper was then assessed by scintillation counting and normalized to total cellular protein after correcting for specific activity of the original labeling medium in each well.

2.3. Gene and Protein Expression Analyses

2.3.1. mRNA Studies

Samples of frozen liver (~10 mg) were homogenized in QiaZOL (Qiagen) using an electric homogenizer (Fisher Scientific, Model 500). Lysates were extracted with chloroform and the aqueous phase precipitated with 70% ethanol. Primary hepatocytes were lysed in Buffer RLT (Qiagen) supplemented with 10 μL/mL β-mercaptoethanol. Samples were processed using the RNeasy Mini Kit (Qiagen) following manufacturer's instructions, including on-column digestion with DNase-I (Qiagen). RNA concentrations were measured using a NanoDrop Spectrophotometer (Thermo). 1 μg of RNA was reverse transcribed using the GoScript Reverse Transcription System (Promega) following manufacturer's instructions. cDNAs were diluted 1:10 and RT-PCR was performed using a DNA Engine Opticon 2 System (Bio-Rad) with SYBR Green (Promega). Primers were synthesized by Invitrogen; sequences are available in Table 2.2. Gene expression levels were normalized by TATA-binding protein (*Tbp*) using the $2^{-\Delta\Delta Ct}$ method.

2.3.2. Western Blotting

Frozen livers (~50 mg) were homogenized in or primary hepatocytes directly lysed in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM sodium chloride, 10% glycerol, 2% NP-40, 1 mM EDTA, 20 mM sodium fluoride, 30 mM sodium pyrophosphate, 0.2% SDS, 0.5% sodium deoxycholate) supplemented with protease/phosphatase inhibitors (Cell Signaling). Protein concentration was assessed by bicinchoninic acid assay against a standard solution of albumin (Sigma). Electrophoresis was performed using homemade 8% polyacrylamide gels at 125 V for 2 h in a running buffer of 25 mM Tris base, 0.192 mM glycine, and 0.1% SDS. Precision Plus Protein Kaleidoscope Standards (Bio-Rad) were used as

molecular weight marker. Gels were transferred at 4ºC to PVDF membranes (Bio-Rad), sandwiched between Whatman paper, at 100 V for 2 h in a transfer buffer of 25 mM Tris base, 0.192 mM glycine, and 10% methanol. Following transfer, membranes were stained with a solution of Ponceau Red S and cut into strips covering appropriate molecular weight ranges. The membrane strips were then destained in TBS-T (1X Tris-buffered saline with 0.05% Tween-20 [Sigma]) and blocked for 1 h at room temperature in blocking buffer (TBS-T with 5% nonfat dry milk [Parmalat]). Incubation with primary antibody was carried out overnight at 4ºC in a solution of 2.5% BSA in TBS-T. A list of antibodies used is available in Table 2.3. The next morning, membrane strips were washed several times in TBS-T followed by incubation with secondary antibody (1:2000 in blocking buffer). Following several additional washed with TBS-T, membrane strips were treated with Pierce Enhanced Chemiluminescent (ECL) Western Blotting Substrate (Thermo) and were exposed to radiography film (Fisher). Films were developed using an X-OMAT Developer (Kodak). Densitometric analysis was performed using ImageJ software (NIH).

2.3.3. Luciferase Assay

Luciferase assays were performed by Dr. Michihiro Matsumoto, a former postdoctoral fellow in the laboratory. HEK293 cells were transiently transfected with plasmids encoding either *Foxo1*^{wt}, *Foxo1*^{dbd}, or empty vector as well as 3X IRE-luc reporter plasmid or empty vector using Lipofectamine 2000 (Invitrogen) in DMEM supplemented with 10% FBS. 36 h after transfection of plasmids, media was changed to serum-free DMEM/PCSM. 12 h after serum starvation, cells were lysed and luciferase assay was performed using the Dual Luciferase Reporter Assay System (Promega) in a Monolight 310 luminometer (PharMingen).

2.4. RNA-Seq

2.4.1. Physical Procedures

mRNA samples from three fasted and three re-fed mice of each genotype (control, L-FoxO1, L-DBD) and purified as described above were submitted to the Columbia University Molecular Pathology Core for assay using an Agilent Bioanalyzer 2100. All mRNA samples submitted demonstrated RNA integrity

number (RIN) values of greater than 8.0. Samples were then submitted to the Columbia Genome Center (CGC). The CGC prepared the RNA-seq library via poly(A) pulldown using TruSeq RNA Sample Prep Kit v2 (Illumina). Libraried cDNA was then sequenced using a HiSeq 2000/2500 sequencing system (Illumina) at a read depth of 30 million over 100 bp fragments.

2.4.2. In Silico *Analysis*

Sequence alignment, statistical analysis, and heat mapping were performed by Dr. Bin Fang of the University of Pennsylvania. Statistical significance was defined more stringently by $q < 0.05$ or less stringently by $p < 0.05$. Gene-ontology analysis was performed using DAVID Bioinformatics Database, http://david.abcc.ncifcrf.gov/.

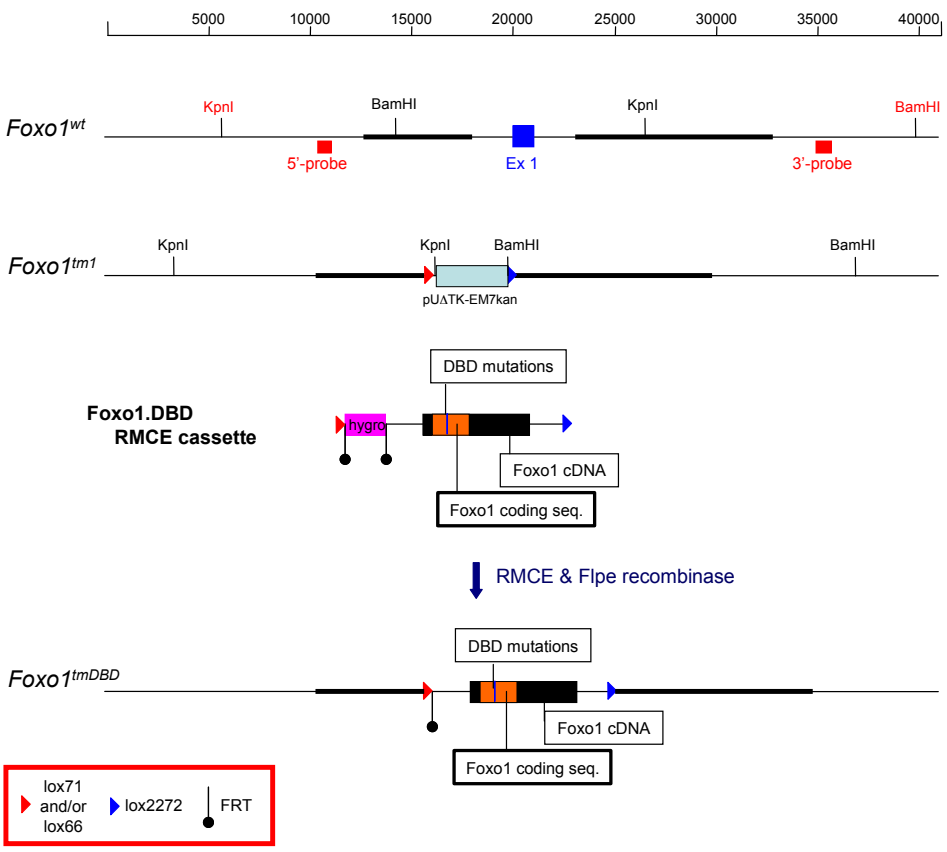


Figure 2.1. RMCE Strategy for Generating *Foxo1^{dbd}*

Figure was generated by Tadahiro Kitamura and Alex Banks.

Figure 2.1

Sections 2.6

CHAPTER 2 TABLES

Allele	Forward	Reverse
Foxo1 (flox)	GCTTAGAGCAGAGATGTTCTCACATT	CCAGAGTCTTTGTATCAGGCAAATAA
Foxo1 (dbd)	TGCACATGTCTCCATACTTGTTA	TGTAATCACACTCTGCCGAGGT
Foxo1 (het)	ACTTCCAGTTCAACATCAGCCG	TTCCCGCTTCAGTGACAACGTC
Cre (univ.)	ACCTGAAGATGTTCGCGATTATCT	ACCGTCAGTACGTGAGATATCTT

Table 2.1. Primers Sequences Used for Genotyping

All sequences are written as 5' → 3'.

Table 2.1

Gene	Forward	Reverse
Acaca	GGCCAGTGCTATGCTGAGAT	TATCACACAGCCAGGGTCAA
Cyp27a1	GCCTCACCTATGGGATCTTCA	TCAAAGCCTGACGCAGATG
Cyp7a1	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA
Cyp7b1	GCATGGCCCTGAAATTCTT	AGTGAGCCACAGAATGCAAA
Cyp8b1	GCCCACAGCCTTCAAGTATG	CGACCAGCTTGAAGTCGAAG
Enho	ATGGCCTCGTAGGCTTCTTG	GGCAGGCCCAGCAGAGA
Fasn	CTGACTCGGCTACTGACACG	TGAGCTGGGTTAGGGTAGGA
Foxo1 (total)	GCGTGCCCTACTTCAAGGATAA	TCCAGTTCCTTCATTCTGCACT
Foxo1 (wt)	GAAGAATTCAATTCGCCAC	CTGCACTCGAATAAACTTGC
Foxo1 (dbd)	GAAGGCTTCAATTCGCCGC	CTGCACTCGAATAAACCTGC
G6pc	GTCTGGATTCTACCTGCTAC	AAAGACTTCTTGTGTGTCTGTC
Gck	TCGGGAGTCAGGAACATCTC	AAGAAGGGACAAAGCCAGGT
Hsd3b5	GCTCTTGGAAACCACAAGGAAC	GACAATCCTCTGGCCAAGAAAC
Igfbp1	AGATCGCCGACCTCAAGAAAT	CTCCAGAGACCCAGGGATTTT
Irs2	TCCAGAACGGCCTCAACTAT	AGTGATGGGACAGGAAGTCG
Pck1	CCTGGAAGAACAAGGAGTGG	AGGGTCAATAATGGGGCACT
Pklr	TCGAAAGTGGAAAGCTTCGT	ATGGGGTGCAACTAGGTCAG
Ppargc1a	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTC
Rgs16	GGGCTCACCACATCTTTGAC	TTGGTCAGTTCTCGGGTCTC
Scd1	CATCATTCTCATGGTCCTGCT	CCCAGTCGTACACGTCATTTT
Srebp1c	GAAGCTGTCGGGGTAGCGTCT	CTCTCAGGAGAGTTGGCACCTG
Tbp	CCCTATCACTCCTGCCACAC	ACGAAGTGCAATGGTCTTTAGG

Table 2.2. Primers Sequences Used for qPCR

All sequences are written as 5' → 3'.

Table 2.2

Protein Target (+ epitope)	Species/Clonality	Manufacturer	Product no.
β-Actin (13E5)	R mAb	Cell Signaling	4970S
Akt	R pAb	Cell Signaling	9272S
p-Akt (T308)	R mAb	Cell Signaling	13038S
p-Akt (S473)	R mAb	Cell Signaling	4060S
Fasn (C20G5)	R mAb	Cell Signaling	3180S
FoxO1 (C29H4)	R mAb	Cell Signaling	2880S
p-FoxO1 (S256)	R pAb	Cell Signaling	9461S
Gck	S pAb	N/A*	N/A*
Gsk3β (27C10)	R mAb	Cell Signaling	9315S
p-Gsk3β (S9)	R mAb	Cell Signaling	9322S
InsRβ (MA-20) p-	M mAb	Novus Biologicals	06349-64
InsRβ (Y1150/1)	R mAb	Cell Signaling	3024S
IRS1 (D23G12)	R mAb	Cell Signaling	3407S
IRS2 (L1326)	R mAb	Cell Signaling	3089S
mTOR (7C10)	R mAb	Cell Signaling	2983S
p-mTOR (S2448)	R mAb	Cell Signaling	5536S
SREBP-1c (2A4)	M mAb	Novus Biologicals	NB600-582

Table 2.3. Primary Antibodies Used for Western Blotting

M = mouse, R = rabbit, S = sheep; mAb = monoclonal, pAb = polyclonal

*Gck antibody was a gift of Mark Magnuson (Vanderbilt University).

Table 2.3

Chapter 3

MODELING PURE VS. SELECTIVE INSULIN RESISTANCE IN PRIMARY HEPATOCYTES

3.1. Introduction

Our hypothesis for the genesis of selective IR in liver suggests that it is at least in part hepatocyte autonomous. We therefore aim to recapitulate selective IR by modeling CHI in primary mouse hepatocytes as described previously [247, 295, 305]. The ability to demonstrate that this model works would substantiate the idea that CHI *per se* can contribute to the development of selective IR, rather than CHI merely representing an epiphenomenon coinciding with selective IR.

The kinetics of CHI-induced InsR downregulation in cultured hepatocytes is well described [284, 285, 288, 323-326]. Incubation of primary hepatocytes with 100-nM insulin results in a ~50% decrease in InsR numbers by 24 h that remains steady up to 72 h. Removal of insulin from the culture medium after 24 h results in a nearly complete rebound in InsR number to the level of untreated cells within hours [285]. InsR downregulation can be demonstrated in primary hepatocytes chronically exposed even to lower, more physiologic insulin concentrations [324].

Precedent also exists to suggest the possibility of recapitulating selective IR in isolated cells in response to CHI. For example, a model of CHI in primary rat hepatocytes that resulted in significantly increased insulin-stimulated *de novo* lipogenesis compared to control cells in spite of significantly decreased InsR binding and insulin-stimulated uptake of 3-aminoisobutyrate [325]. Increased lipogenesis in this system may be due to persistent activation of the SREBP-1c pathway despite markedly reduced insulin-stimulated PI3K activity [295]. These studies, though consistent with our hypothesis, do not speak directly to the dual effects of CHI on glucose and lipid production; our objective is therefore to address this outstanding question.

3.2. Results

3.2.1. Model of Chronic Hyperinsulinemia Recapitulates Selective IR

We treated mouse primary hepatocytes with or without 100-nM insulin (“chronic hyperinsulinemia,” henceforth CHI) for 24 h followed by acute (30 min) treatment with or without 10-nM insulin. CHI treatment results in an approximately 50% reduction in InsR protein levels, indicating InsR downregulation (Fig. 3.1). In keeping with reduced InsR signaling, insulin-stimulated phosphorylation of Akt at Thr 308 and Ser 473

is markedly reduced, although the basal level of Akt phosphorylation at both sites is higher than in control (Fig. 3.1). This is consistent with findings in the published literature.

As Akt is generally considered the “branch point” between the glucose and lipid arms of hepatic insulin action [213], we assessed major downstream indices of insulin action on these processes. Phosphorylation of FoxO1, as indicated by an upward electrophoretic mobility shift, parallels that of its insulin-responsive kinase, Akt, as expected (Fig. 3.1). That this electrophoretic mobility shift in FoxO1 is completely lost in CHI-treated cells is consistent with the hypothesis that the glucose arm has been rendered resistant to the acute effects of insulin. On the other hand, cleavage of SREBP-1c, a surrogate measure of the activation of the lipid arm, appears to be relatively more intact in the CHI-treated cells (Fig. 3.1). That is, although SREBP-1c cleavage appears to lose much of its acute responsiveness to insulin as well, the absolute amount of cleaved SREBP-1c is comparable between insulin-treated control cells and CHI-treated cells treated acutely with or without insulin (Fig. 3.1). The preservation of cleaved SREBP-1c levels in the CHI-treated cells is likely attributable to increased levels of precursor (uncleaved) SREBP-1c that had accumulated in response to high-dose insulin over the 24-h exposure period (Fig. 3.1). CHI-treated primary hepatocytes interestingly exhibited sharp decreases in protein levels of IRS1/2 (Fig. 3.1 and data not shown). We confirmed these findings in rat Fao hepatoma cells as well (data not shown).

Although these indices of insulin action are consistent with our expectations, it is necessary to test functional readouts of both arms of the insulin-signaling pathway. We therefore performed glucose production (GP) assays in mouse hepatocytes subjected to the CHI paradigm described above (Fig. 3.2A). As expected, treatment with a cell-permeable cAMP analogue (CPT-cAMP) resulted in a ~35% induction of GP over 5 h that was completely reversed by concomitant treatment with insulin. On the other hand, although CHI treatment did not affect maximal induction of GP by cAMP, the inhibitory effect of insulin was completely lost. qPCR analysis of key enzymes involved in GP were congruent with the glucose-production findings (Fig. 3.2B-C). mRNA levels of *G6pc* and *Pck1* were induced 30- and 15-fold, respectively, by treatment with cAMP; this effect was, as expected blocked by co-treatment with insulin. CHI-treated cells consistently exhibited an attenuated response of *G6pc* and *Pck1* expression to cAMP treatment, but again even this blunted response was completely unresponsive to insulin (Fig. 3.2B-C).

Thus, we can state that CHI treatment does indeed induce insulin resistance in the glucose arm of the pathway.

In order to determine whether the IR evoked by CHI is truly selective, we assessed *de novo* lipogenesis (DNL) in the context of the CHI model (Fig. 3.3A). CHI treatment tended to increase basal lipogenesis by 23%. Nevertheless, acute insulin treatment still significantly induced DNL by 38%, although this was decreased relative to the effect of acute insulin in control cells (53%). Expression of *Srebp1c* and *Gck* were both increased at baseline in CHI-treated cells, although there was no significant effect of acute insulin treatment on expression of these genes in either control or CHI-treated cells (Fig. 3.3B-C). These observations were confirmed at the protein level as well (Fig. 3.1). Overall, CHI treatment renders primary hepatocytes incapable of inhibiting cAMP-induced GP even as they remain able to activate lipogenesis in response to the same dose of insulin, hence selective IR.

3.2.2. Variable InsR Antagonism Results in a Spectrum of IR

The recapitulation of selective IR in response to CHI treatment is consistent with the empirical findings in hyperinsulinemic humans and mice. The *ex vivo* model of CHI, however, is associated with certain limitations. First, the reduction in InsR protein levels by ~50% represents only a snapshot of InsR impairment that does not allow us to understand at which point the two metabolic arms actually diverge. Next, CHI involves exposure of the cells to insulin as a matter of course. Thus, before IR sets in, cells would respond to the treatment as they would an acute bolus of insulin. This is reflected, for example, in the higher baseline phosphorylation of Akt and protein levels of precursor SREBP-1c and Gck, and may therefore mask subtle defects in the acute response of DNL to insulin. Lastly, CHI-induced IR may actually represent only one particular even of selective IR and we therefore aimed to take a parallel approach to investigating selective IR.

We have therefore taken an acute approach to manipulating the insulin sensitivity of hepatocytes by employing S961, a peptide competitive inhibitor of InsR derived from insulin [327]. By treating cells with a range of concentrations of S961, we expect to generate a spectrum of insulin resistance that will allow us to better visualize the differential insulin sensitivities of the glucose and lipid arms [327, 328]. To this

end, we have treated mouse primary hepatocytes with or without S961 for 30 min prior to and then along with acute treatment with insulin. S961 treatment dose-dependently inhibits the phosphorylation of IRS1, Akt (Thr 308), GSK3β (Ser 9), and FoxO1 in response to insulin (Fig. 3.4A). On the other hand, there is no discernible effect on either total or cleaved SREBP-1c levels (Fig. 3.4A). As expected, there is also no effect on total InsRβ levels (Fig. 3.4A). By plotting the percentage of maximal insulin action on Akt or GSK3β phosphorylation as a function of the logarithm of antagonist concentration, we can calculate the IC_{50} of S961 as an index of the relative IR of these components of the proximal insulin-signaling pathway (Fig. 3.5). The calculated IC_{50} for the response of phosphorylation of Akt Thr 308 to 10^{-8} M insulin is -9.06 (0.87 nM), for Akt Ser 473 is -8.57 (2.69 nM), and for GSK3β Ser 9 is -9.01 (0.98 nM) (Fig. 3.5).

Antagonism of InsR also results in dose-dependent defects in the insulin responsiveness of glucose production (Fig. 3.4B). Acute treatment of cells with 10^{-8} M insulin results in a 31% reduction of cAMP-stimulated GP. Co-treatment with S961 at 10^{-6} and 10^{-7} M S961 completely blocks this effect of insulin, while 10^{-8} M S961 allows for a 9% reduction of GP by insulin. Treatment with 10^{-9} and 10^{-10} M S961 allowed for 21% and 24% reductions of glucose production, respectively, by insulin. Thus, even at an antagonist concentration 100-fold lower than that of insulin, the cells still are unable to completely suppress GP in response to insulin. Treatment even with the maximal dose of S961 did not affect maximal GP in response to cAMP. By plotting the percentage of maximal insulin action on GP as a function of the logarithm of antagonist concentration, we can calculate the IC_{50} of S961 as an index of the relative IR of the glucose arm (Fig. 3.6). The calculated IC_{50} for the response of the glucose arm to 10^{-8} M insulin is -8.63 (2.3 nM).

The insulin-responsive lowering of mRNA levels of *G6pc* and *Pck1* is likewise dose-dependently inhibited by S961 (Fig. 3.4C). cAMP-induced expression of *G6pc* and *Pck1* were decreased by 96% and 92%, respectively, by insulin. Surprisingly, *G6pc* mRNA decreased by about a third in response to insulin even at the highest concentration of S961, and was suppressed maximally by insulin in the presence of 10^{-9} M S961. *Pck1* expression, on the other hand, was not decreased by insulin in the presence of 10^{-6} or 10^{-7} M S961, although, as for *G6pc*, was fully suppressed at 10^{-9} M S961. The calculated IC_{50} for

suppression of cAMP-induced *G6pc* and *Pck1* expression by 10^{-8} M insulin are -7.60 (25.1 nM) and -7.79 (16.2 nM), respectively (Fig. 3.7).

Variable InsR antagonism resulted in a distinctly different dose-response curve for DNL (Fig. 3.4E). Treatment with 10^{-8} M insulin stimulated DNL by 67% in control hepatocytes. This effect was fully suppressed by S961 at 10^{-6} and 10^{-7} M but treatment with even the highest dose of S961 did not alter basal DNL. Treatment with 10^{-8} M S961 permitted a 29% increase in DNL in response to insulin, roughly 44% of the maximal response. At 10^{-9} and 10^{-10} M the rates of DNL exceeded even that in the insulin-treated control cells, rising by about 78% in each case. The slightly higher rates of DNL in these cells was not, however, statistically significant compared to insulin-only control. The calculated logarithmic IC_{50} for S961 inhibition of DNL in response to 10^{-8} nM insulin, an index of the relative IR of the lipid arm, is -8.07 (8.51 nM) (Fig. 3.6).

We can directly compare the IC_{50} values obtained for GP and DNL, as each represents the relative IR of its respective metabolic process within the same experimental context (Fig. 3.6). Again, the IC_{50} for GP was calculated as -8.63 (*i.e.*, $10^{-8.63}$ M, or 2.3 x 10^{-9} M S961) while that for DNL is -8.07 (*i.e.*, $10^{-8.07}$ M, or 8.5 x 10^{-9} M S961). Thus, an InsR antagonist concentration of nearly four times greater than that needed to half-maximally suppress insulin's effect on GP is required to half-maximally suppress insulin's effect on DNL. This outcome strongly supports our hypothesis that modulation of effective InsR number *per se* can produce selective IR.

3.2.3. Inhibition of Akt1/2 Results in Pure IR

Previous studies have suggested that Akt represents the most distal element of the InsR signaling cascade that unites insulin's effects on glucose and lipid metabolism. We therefore tested the effect of inhibition of Akt on GP and DNL in primary hepatocytes. Akti-1/2 is a chemical inhibitor of Akt that prevents its phosphorylation. Accordingly, Akti-1/2 treatment completely blocked phosphorylation of Akt in response to insulin, and thereby also markedly decreased phosphorylation of FoxO1, GSK3β, and mTOR without affecting upstream IRS2 (Fig. 3.8A). Of note, treatment with the inhibitor was without notable effect on SREBP-1c cleavage. Akti-1/2 treatment also blocked the ability of insulin to decrease GP in

response to cAMP (Fig. 3.8B). Similar to the effect of CHI treatment, Akt inhibition decreased the maximal cAMP-induced expression of *G6pc* and *Pck1* (Fig. 3.8C-D). Although Akti-1/2 prevented the lowering of *G6pc* expression to normal by insulin, it did allow for an approximately 50% repression, while Akti-1/2 did not prevent the lowering of *Pck1* expression to basal levels by insulin (Fig. 3.8C-D). Finally, Akti-1/2 treatment completely abrogated insulin's induction of DNL (Fig. 3.8E). These data support the idea that Akt signaling unites insulin's effects on GP and DNL in the context of primary hepatocytes, and thus interfering with its action recapitulates a form of pure IR [90].

3.3. Discussion

3.3.1. Overview of Findings

Classical biochemistry teaches that insulin acts in the liver to shift its anabolic activity away from glucose production and toward glycogen synthesis and lipogenesis. Accordingly, patients with inactivating mutations in *Insr* cannot suppress glucose production or stimulate lipogenesis in response to insulin. A persistent conundrum facing clinical endocrinology, however, has been the selective IR exhibited by so many garden-variety type 2 diabetic patients. This study supports the idea that pure and selective IR lie along a single spectrum in which decreasing InsR activation differentially alters glucose and lipid metabolism because of inherent differences in the relative insulin responsiveness of each process. Specifically, as we have shown using a peptide competitive inhibitor of InsR, a greater degree of intact InsR is required to properly halt HGP than to stimulate DNL. Thus, at intermediate levels of InsR activation, DNL can proceed while HGP fails to respond. At very low levels of intact InsR, neither process can go forward.

This loss of InsR may stem from downregulation due to the chronic hyperinsulinemia that is common to those in the run-up to T2DM. Indeed, modeling of CHI in primary hepatocytes shows that these cells can be rendered selectively resistant to insulin's lowering of GP while remaining able to engage in insulin-stimulated DNL. In other words, the co-occurrence of CHI and selective IR in garden-variety pre-diabetic patients may not merely represent an epiphenomenon; rather, the two may be etiologically linked.

3.3.2. Qualitative and Quantitative Impact of CHI on InsR

As discussed extensively in Chapter 1, CHI has been shown to induce IR. That CHI may precede the development of hepatic IR is supported by the general consensus that IR in skeletal muscle and WAT, the combination of which would induce compensatory CHI, precedes hepatic IR by many years in the natural history of T2DM [67-71, 329]. This is manifested clinically in the observation that post-prandial hyperglycemia, which in part reflects failure of glucose disposal in muscle, precedes fasting hyperglycemia, a reflection of HGP [69, 71, 329]. Much evidence supports the idea that CHI-induced IR is due to downregulation of InsR in the hepatocyte, thereby producing a quantitative defect in insulin signaling. Indeed, a similar study in rat hepatocytes demonstrated CHI treatment resulting in a ~50% reduction in InsR number affected only the maximal rates of insulin-stimulated lipogenesis and glycogen synthesis and not their inherent sensitivities to insulin (*i.e.*, ED_{50}) [285]. The studies described here are concordant with this paradigm, as *ex vivo* modeling of CHI in primary mouse hepatocytes also results in a ~50% reduction in InsR protein levels. Of note, specific binding of insulin to membrane preparations of liver from obese humans was also decreased by about 50% relative to non-obese controls, further supportive of the physiologic relevance of this model [289].

It is important to note that this thesis has only shown a decrease in *total* hepatocellular InsR levels, however, which does not necessarily reflect the relative contributions of intracellular *vs.* cell-surface receptors. Indeed, cell-surface InsR may even represent a minority of total cellular InsR levels [288, 323, 326, 330] but even this pool of InsR has been shown to be reduced in diabetic and non-diabetic obese livers [288]. Moreover, insulin binding in the short term can reversibly alter subcellular localization of InsR [330-333] even if not total InsR number [291], adding another layer of complexity to the problem and perhaps accounting for some of the observed variability in the degree of InsR downregulation between different studies. Yet another intriguing, albeit controversial possibility behind InsR downregulation is the activation of a cell-surface protease following CHI exposure that cleaves the extracellular α domain of InsR, thereby abolishing the ability of the residual receptor to bind to insulin [334]. Corroborating the potential clinical relevance of this mechanism is the observation that diabetic patients exhibit elevated levels of soluble InsR in their circulation [335].

The fact that insulin can and does alter both cellular InsR content and localization may help to explain why pulsatile rather than continuous delivery of insulin to the liver leads to a more robust acute insulin response and prevents desensitization [277, 336-339], even in primary hepatocytes [333]. Prolonged infusion even of supraphysiologic doses of insulin in humans does not result in loss of insulin responsiveness if supplied in a pulsatile fashion; in fact, this may even *enhance* insulin sensitivity [340]. These observations dovetail with the empirical finding that pancreatic β cells of patients with type 2 diabetes as well as obese non-diabetic patients exhibit a more continuous, or at least less regularly pulsatile, release of insulin, underscoring the "chronic" aspect of CHI [341-345], although some conflicting evidence exists [346]. Moreover, first-degree relatives of T2DM patients have been found to show impaired pulsatility in insulin secretion, further suggesting a primary role of altered insulin secretion in the pathogenesis of selective IR [347]. That incretin-modulating drugs augment insulin secretion in a pulsatile fashion [348, 349], as opposed to the continuous effect of sulfonylureas [350], may help to explain their ability to improve insulin sensitivity as opposed merely to insulin secretion [4]. Taken together these findings suggest that, although patients developing IR are hyperinsulinemic, insulin levels *per se* do not need to be all that high to induce IR. In other words, a loss of normally pulsatile insulin delivery experienced by these patients, even if not resulting in frank hyperinsulinemia, would still be tantamount to CHI in terms of inducing InsR desensitization.

Previous studies also suggest that CHI induces post-binding defects that contribute to the overall cellular IR, a possibility that is not mutually exclusive with a coincident wholesale downregulation of InsR. For example, in primary hepatocytes isolated from diabetic patients, not only were insulin binding per surface area and total InsR protein levels decreased but so was insulin-stimulated InsR kinase activity even when normalized for amount of insulin bound [288]. In other words, the defect in InsR function is disproportionate to the extent of InsR downregulation [351-355]. This is also reflected in the findings of this study, as CHI-treated cells show a complete loss of Akt phosphorylation in response to acute insulin despite the reduction of InsR by only about 50% (Fig. 3.1). Similarly, acute insulin treatment completely curtails the hormone's ability to suppress *G6pc* and *Pck1* expression (Fig. 3.2B-C). Also consistent with a qualitative effect of CHI on InsR function, treatment with increasing doses of S961, which does not alter

total InsR levels at least in the acute setting (Fig. 3.4A), resulted in roughly proportional changes in InsRβ and Akt phosphorylation (Fig. 3.4A, Fig. 3.5).

It is also important to consider potential confounding effects of the high doses of insulin used in this study. First, CHI treatment may risk "specificity spillover" through activation of the IGF-1 receptor (Igf1R) by insulin. This possibility may not be as problematic in our system as in other cell types, however, as adult liver and isolated primary hepatocytes have been found to express little or no Igf1R [356, 357], although it may be induced in regenerating liver [358]. Second, hepatocytes may contain spare receptors for insulin. One study in isolated rat hepatocytes found that only 35% of maximal insulin binding was required for full stimulation of glycogen synthesis [359], although other studies in primary hepatocytes [285] and whole liver [64] did not find evidence of spare receptors. The presence of spare InsR would be problematic for our study in two ways. First, it suggests *a priori* that the ~50% reduction we observe in total InsRβ protein levels is not sufficient to explain the defects in Akt phosphorylation and inhibition of GP. Potential explanations for this apparent discrepancy include (i) that some portion of the InsRβ detected in Fig. 3.1A has been internalized and/or (ii) that post-binding defects in InsR (*e.g.*, reduced kinase activity) may account for a greater share of the loss of insulin action than reduced receptor binding *per se*. Direct assessment of insulin binding to InsR in our model of CHI would address these possibilities. A second potential drawback of the presence of spare receptors is that it might mask subtle defects in acute insulin action, especially as we have chosen to use somewhat supraphysiologic 10 nM insulin as our standard dose for acute treatment. For example, although on average we found that insulin lost the ability to significantly blunt GP following CHI treatment, the data from the three individual experiments demonstrated some heterogeneity; some wells responded better to acute insulin inhibition of GP than others. This is reflected in the residual ~12% average decrease in GP in response to insulin even in CHI-treated cells. The single-well data in insulin-inhibited GP between control and CHI may have been tighter had we used a lower dose of insulin. In the experiment illustrated in Fig. 3.8B, 1 nM insulin was able to reduce cAMP-induced GP by about 20%.

Finally, CHI treatment, although resulting in IR in the longer term, activates insulin signaling over the first several hours of treatment. Thus, it is possible that the acute effects of CHI treatment may reset

the baseline for later measures of insulin action. For example, we found that CHI treatment blunted maximal cAMP-induced expression of *G6pc* and *Pck1*. Similarly, as discussed in Section 3.3.4, we found basal IRS1 and IRS2 levels to be decreased following CHI. IRS1 and IRS2 are known to be positively regulated by FoxO proteins, suggesting that their decrease may reflect inactivation of FoxO by insulin [145, 360]. These concerns would endorse waiting longer than 24 h for any residual acute effects of insulin to dissipate. However, mouse primary hepatocytes rapidly begin to lose their hepatocyte character following culture *ex vivo*. We found, for example, that maximal cAMP-induced GP was reduced by over half 48 h after isolation *vs.* the usual 24 h (data not shown). We therefore have attempted to strike a balance between preserving the integrity of the isolated hepatocytes and ensuring sufficient time to engender IR. Nevertheless, although we cannot prove that residual acute effects of insulin account for the peculiar observations listed above, we have reason to believe that this is not the case. Despite decreases in cAMP-induced *G6pc* and *Pck1* expression, basal (untreated) expression of these genes is not decreased on average in CHI-treated cells, and in some experiments was even increased (Fig. 3.2B-C). More importantly, both basal and maximal cAMP-induced GP are identical in control and CHI-treated cells (Fig. 3.2A). Finally, despite the decrease in IRS1/2 protein levels, *Irs2* mRNA was unchanged in the basal or stimulated states (data not shown). It is therefore likely that these observations genuinely reflect the CHI-induced IR state.

3.3.3. Mechanism of Insulin-Stimulated DNL in Primary Hepatocytes

Although the topic of cell autonomous *vs.* non-autonomous effects in DNL (as well as GP) will be discussed more extensively in Chapters 4-5, it merits an introduction here. A key finding of these studies is the preservation of DNL in the face of CHI-induced IR, consistent with our expectations based on garden-variety clinical IR. However, our system on its face does not completely support the model that compensatory hyperinsulinemia in insulin resistance drives DNL through a preservation of insulin signaling through the Akt → mTORC1 → SREBP-1c branch of the pathway [213, 215, 217, 218, 276]. In our studies, SREBP-1c expression and cleavage do not generally correlate well with observed rates of DNL, probably because SREBP-1c cleavage is reported not to be particularly sensitive to insulin in

isolated mouse hepatocytes (see Chapter 5). Indeed, we found quite a variable responsiveness of SREBP-1c cleavage in response to acute insulin treatment (*cf.* Fig. 3.1 *vs.* Fig. 3.4A and Fig. 3.8A).

It is important to bear in mind in the discussion of selective IR that even though we refer to the lipid arm as remaining "sensitive" to insulin, it may well not be as sensitive as in a healthy liver. Lipogenesis is *relatively* more sensitive to insulin than is GP, and in selective IR operates under the aegis of hyperinsulinemia. Even though we found that SREBP-1c cleavage is not especially sensitive to insulin in our system, we did consistently find an increase in both SREBP-1c mRNA and precursor protein levels following CHI treatment (Fig. 3.1, 3.3C). This finding has been previously reported in rat primary-hepatocyte models of CHI [209, 295]. Thus, the steady-state level of cleaved SREBP-1c is likely higher than in control cells, especially as basal SREBP-1c cleavage appeared higher following CHI treatment. This is consistent with our finding of higher mRNA and protein levels of key SREBP-1c lipogenic target gene *Fasn* (Fig. 3.1, 3.3C). So, even if the acute insulin responsiveness of SREBP-1c cleavage and activity is decreased, it is still expected to remain high overall relative to insulin-stimulated FoxO1 phosphorylation and effects on GP. On the other hand, acute inhibition of insulin signaling, either directly by S961 treatment or indirectly by Akt inhibition, may uncouple DNL from SREBP-1c expression, as these treatments hindered DNL without systematically affecting SREBP-1c cleavage. We suspect that, due to the far shorter time course of these experiments *versus* the CHI model, changes in basal SREBP-1c expression would not be expected to play a dominant role in DNL. However, we cannot make this statement conclusively as we did not measure insulin-stimulated mRNA expression of SREBP-1c and its targets in S961- or Akti-1/2-treated cells.

Taken together with the CHI data (and data from Chapter 4, to be discussed at greater length in Chapters 4-5), we speculate that, although both "chronic" (*i.e.*, CHI) and "acute" (*i.e.*, S961, Akti-1/2) inhibition of InsR signaling betray selective IR, the mechanisms underlying the relative preservation of insulin signaling to DNL may be distinct. We therefore have formulated a hypothesis regarding our DNL results in CHI-treated *vs.* control cells that separates out effects on basal (*i.e.*, chronic insulin-stimulated) *vs.* acute insulin-stimulated DNL. In the case of acute insulin treatment (and the effects of S961 or Akti-1/2 on it), we see no differences in expression of classic lipogenic genes (Fig. 3.3B-D), consistent with

previous observations in primary mouse hepatocytes. Thus, the increase in DNL likely occurs via posttranslational mechanisms (see Chapters 1 and 5). If this hypothesis is true, then even these posttranslational mechanisms of insulin-stimulated DNL are relatively more sensitive to insulin than those inhibiting GP (also likely to be largely posttranslational, as discussed in Chapter 5).

On the other hand, as stated above, long-term treatment with high-dose insulin increases mRNA and protein levels of SREBP-1c, Fasn, and Gck, and decreases mRNA levels of *G6pc*. This essentially represents the Brown and Goldstein model of CHI-driven increased DNL [276]. The increased expression of lipogenic genes in the basal state may account for the increased basal DNL of CHI-treated cells. That the levels of these genes still trend higher than control in the acute insulin-stimulated state therefore may also explain the persistent trend of higher rates of DNL even with insulin treatment. This possibility is concordant with our finding that the increase in DNL in response to acute insulin is only slightly decreased in CHI-treated cells (38%) to that in control cells (53%). This suggests that the posttranslational effect of acute insulin treatment is nearly normal in CHI-treated cells.

In summary, we propose a model whereby CHI (*e.g.*, in the post-absorptive state) increases basal expression of lipogenic genes and proportionally increases rates of DNL. Moreover, CHI does not impair acute insulin-stimulated DNL (*e.g.*, in the postprandial state) relative to GP even on top of the increase in basal DNL. This model is oversimplified, as it does not account for other mediators of DNL in response to fasting and feeding in whole liver. For instance, cultured hepatocytes are exposed to a constant ambient level of glucose that is uncoupled from insulin levels. *In vivo*, however, an influx of insulin would normally be preceded by an increase in glucose levels, which in turn could drive lipogenic gene expression through ChREBP independently of SREBP-1c [63, 202]. In the case of selective IR, therefore, decreased control of HGP would be expected to increase ChREBP-driven lipogenesis. Carbohydrates can also directly drive lipogenesis via allosteric activation of glycolytic enzymes including glucokinase [180, 361, 362]. In our system, increased *Gck* expression in CHI would be expected to further accentuate ChREBP signaling as well [232]. Thus, even though there may be a slight decrement in the ability to increase DNL in response to acute insulin post-CHI treatment, this may not manifest *in vivo* due

to increased carbohydrate-driven lipogenesis [202, 325]. Indeed, even in LIRKO mice, re-fed expression levels of several lipogenic genes are normal or nearly so due to ChREBP and other factors [202].

In spite of these caveats, however, our finding that CHI-induced IR spares DNL even in isolated hepatocytes represents an important conceptual step forward in our understanding of the connection between IR and hepatic steatosis. That is, our findings suggest that processes autonomous to the hepatocyte can contribute to steatosis independently of alterations in WAT-liberated FFA, namely both transcriptional and posttranslational changes in insulin-stimulated DNL [48]. Although previous studies investigating expression of lipogenic genes in primary hepatocytes suggested this possibility, these studies did not generally measure DNL directly [209, 213, 218, 295]. On the other hand, older studies that also provide evidence that DNL continues unabated in the face of CHI did not correlate these data with lipogenic gene expression or compare them to GP [285, 288, 325].

Overall, the idea that the development of steatosis requires some participation of hepatic insulin signaling is consistent with data indicating that humans with pure IR due to *Insr* mutations do not develop fatty liver even though their fasting FFA levels may be somewhat elevated [65]. Nevertheless, although the fractional contribution of DNL to hepatic TG is increased in IR states, the contribution of re-esterified WAT released by lipolysis is still significant, especially in the fasting state [48, 363]. For instance, mice lacking hormone-sensitive lipase (HSL), which are unable to liberate FFA from WAT, show increased hepatic insulin sensitivity and reduced hepatic TG [364, 365]. Thus, the combination of selective IR in WAT and liver may together drive the development of NAFLD [48]. The potential mechanisms of selective IR will be discussed further in Chapter 5.

Section 3.4

CHAPTER 3 FIGURES

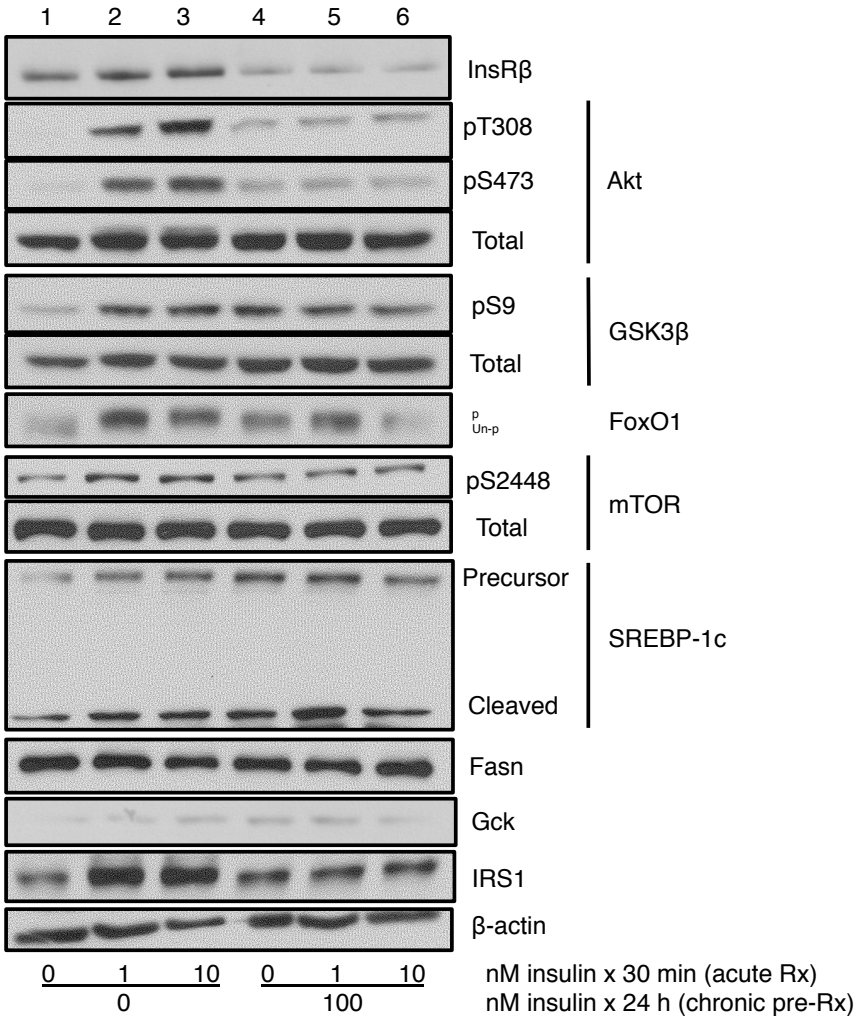

1 2 3 4 5 6
InsRβ
pT308
pS473
Akt
Total
pS9
GSK3β
Total
p
Un-p
FoxO1
pS2448
mTOR
Total
Precursor
SREBP-1c
Cleaved
Fasn
Gck
IRS1
β-actin
0 1 10 0 1 10
0 100
nM insulin x 30 min (acute Rx)
nM insulin x 24 h (chronic pre-Rx)

Figure 3.1

Figure 3.1. Modeling Chronic Hyperinsulinemia in Primary Hepatocytes

Western blots demonstrating alterations in InsR signaling cascade components in response to acute and/or chronic insulin treatment. Each lane contains equal amounts of protein pooled from three independent experiments.

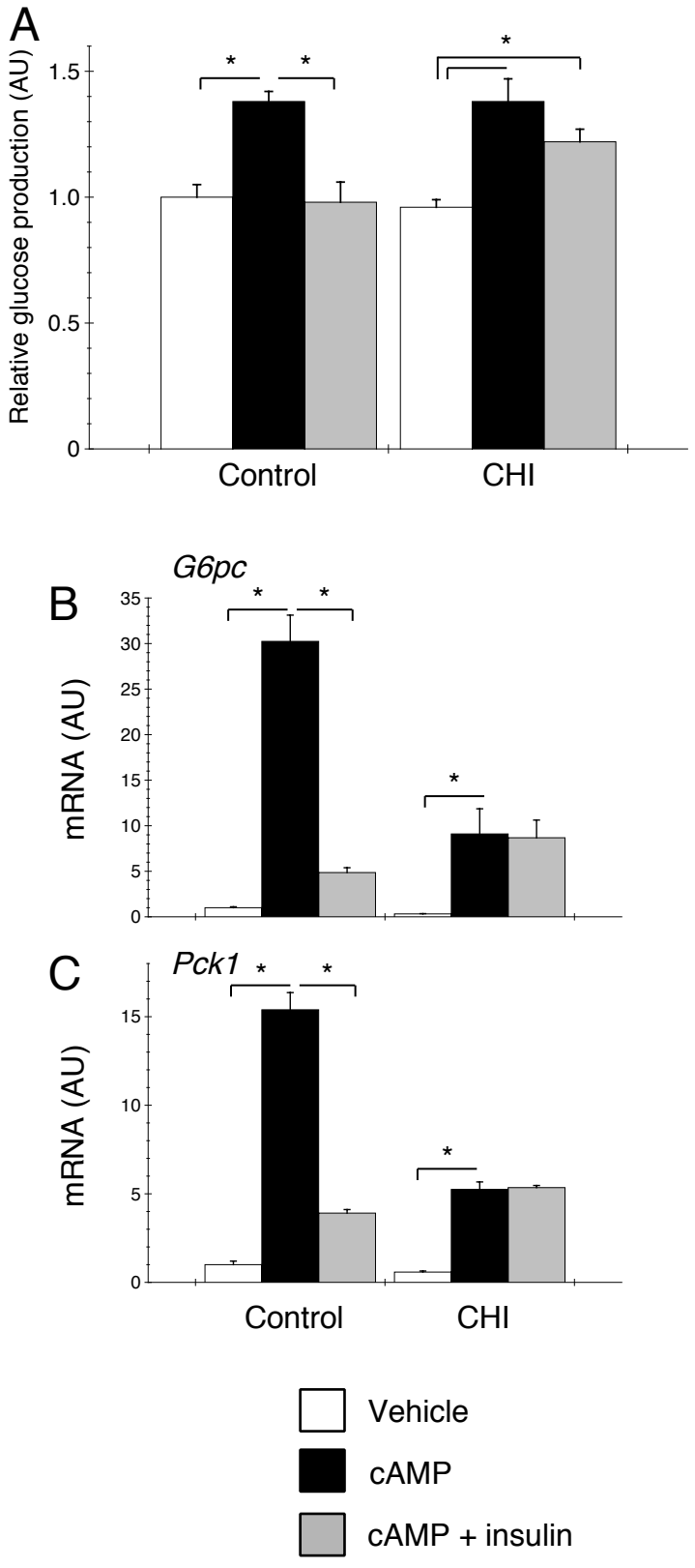

A
Relative glucose production (AU)
1.5
1.0
0.5
0
*
*
*
Control
CHI
G6pc
B
mRNA (AU)
35
30
25
20
15
10
5
0
*
*
*
C
Pck1
mRNA (AU)
15
10
5
0
*
*
*
Control
CHI
Vehicle
cAMP
cAMP + insulin

Figure 3.2

Figure 3.2. Effect of CHI Treatment on Glucose Production

Relative glucose production (A) and gene expression (C-D) in control or CHI-treated primary hepatocytes incubated for 5 h with vehicle, 100 μM CPT-cAMP, or 100 μM CPT-cAMP + 10 nM insulin. Data are normalized to vehicle-treated control. In (A) data are mean ± SEM of three independent experiments performed in quadruplicate; in (B) data are mean ± SEM of one representative experiment of three performed in triplicate.

* $p < 0.05$ by Bonferroni's post-hoc analysis following two-way ANOVA.

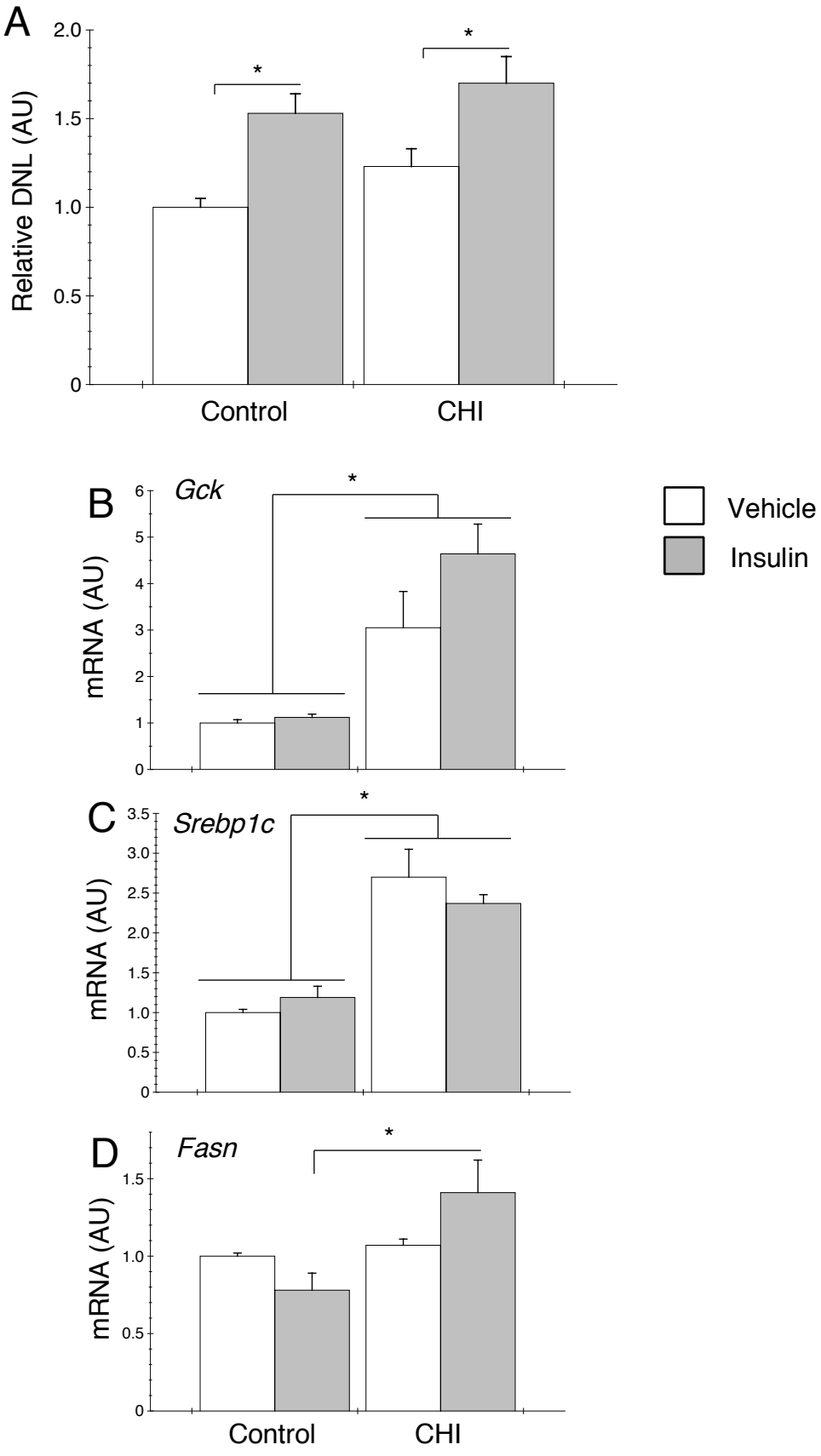

A
Relative DNL (AU)
2.0
1.5
1.0
0.5
0
*
*
Control
CHI
B
Gck
mRNA (AU)
6
5
4
3
2
1
0
*
C
Srebp1c
mRNA (AU)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
D
Fasn
mRNA (AU)
1.5
1.0
0.5
0
*
Control
CHI
Vehicle
Insulin

Figure 3.3

Figure 3.3. Effect of CHI Treatment on *De Novo* Lipogenesis

(A) *De novo* lipogenesis in control or CHI-treated primary hepatocytes. Following overnight serum starvation cells were treated with vehicle or 10-nM insulin for 2 hr and then ^{14}C-labeled acetic acid was added for an additional 3 hr. Data are presented as mean ± SEM normalized to vehicle-treated control of five independent experiments performed in triplicate.

(B-D) Gene expression in control or CHI-treated primary hepatocytes incubated for 5 h with vehicle or 10 nM insulin. Data are presented as mean ± SEM normalized to vehicle-treated control and are representative of three independent experiments performed in triplicate.

* $p < 0.05$ by Bonferroni's post-hoc analysis following two-way ANOVA.

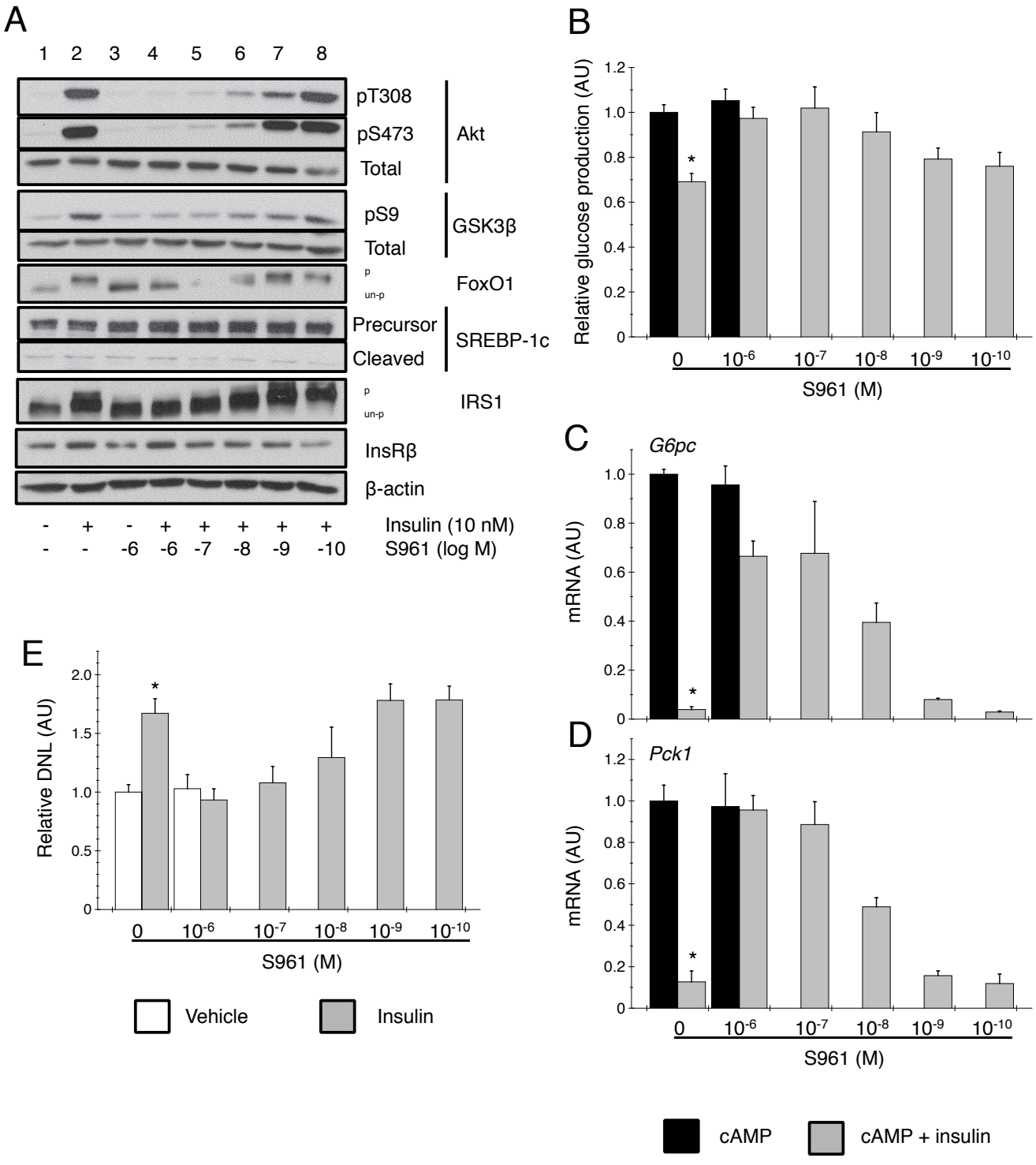

A
1 2 3 4 5 6 7 8
pT308
pS473
Akt
Total
pS9
GSK3β
Total
p
un-p
FoxO1
Precursor
SREBP-1c
Cleaved
p
un-p
IRS1
InsRβ
β-actin
- + - + + + + + Insulin (10 nM)
- - -6 -6 -7 -8 -9 -10 S961 (log M)
B
Relative glucose production (AU)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
0 10-6 10-7 10-8 10-9 10-10
S961 (M)
C
G6pc
mRNA (AU)
1.0
0.8
0.6
0.4
0.2
0
*
D
Pck1
mRNA (AU)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
0 10-6 10-7 10-8 10-9 10-10
S961 (M)
cAMP
cAMP + insulin
E
Relative DNL (AU)
2.0
1.5
1.0
0.5
0
*
0 10-6 10-7 10-8 10-9 10-10
S961 (M)
Vehicle
Insulin

Figure 3.4

Figure 3.4. Dose-Response Study of InsR Antagonism

(A) Western blots demonstrating alterations in InsR signaling cascade components in response to treatment with 10 nM insulin and/or the indicated concentration of S961. Each lane represents pooled samples of equivalent amounts of lysate from three independent experiments.

(B-D) Relative glucose production (B) and gene expression (C) in primary hepatocytes incubated for 5 h with 100 μM CPT-cAMP with or without 10 nM insulin and the indicated concentrations of S961.

(E) *De novo* lipogenesis in control or S961-treated primary hepatocytes. Following overnight serum starvation cells were treated with or without S961 and with or without insulin for 2 hr and then ^{14}C-labeled acetic acid was added for an additional 3 hr.

Data are normalized to insulin-/S961-untreated control and represent mean ± SEM of three independent experiments performed in triplicate. * $p < 0.05$ *vs.* vehicle-treated control by Bonferroni's post-hoc analysis following two-way ANOVA.

A

S961 conc. (M)	Akt pT308 (% max)	Akt pS473 (% max)	Gsk3β pS9 (% max)
0	100	100	100
10^{-6}	-6.7	-7.2	-4.6
10^{-7}	-4.1	-4.7	3.2
10^{-8}	12.5	12.2	21.8
10^{-9}	45.2	78.2	49.6
10^{-10}	131.0	131.1	82.2

B

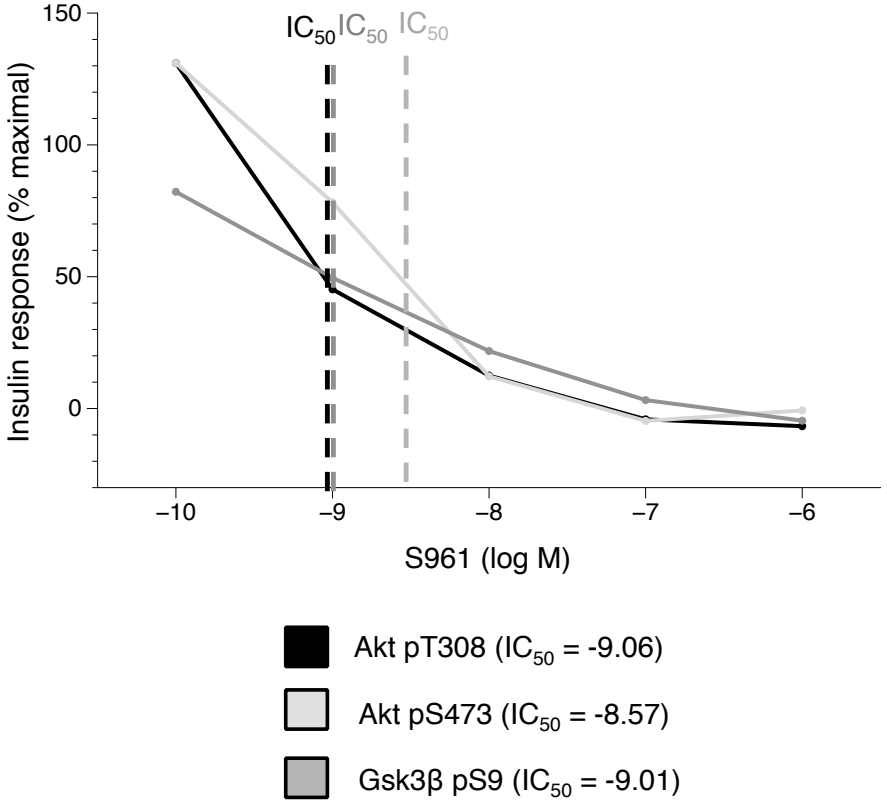


Figure 3.5

Figure 3.5. Relative Insulin Responsiveness of Akt and GSK3β Phosphorylation

(A) % maximal insulin response of phosphorylation of Akt T308, Akt S473, and GSK3β S9 as a function of S961 concentration. Figures are calculated from densitometric analysis of the Western blots shown in Fig. 3.4A as the percentage of the departure from baseline at the indicated S961 concentration relative to the maximal change with insulin in the absence of antagonist.

(B) Graphical representation of calculations in (A) for Akt and GSK3β phosphorylation IC_{50} is calculated as $\log_{10}$ of the S961 concentration at which each dose-response curve intercepts 50% maximal insulin response. Data are derived from densitometric analysis of Fig. 3.4A.

A

S961 conc. (M)	Glucose prod. (% max)	Lipogenesis (% max)
0	100	100
10^{-6}	7.4	-11.6
10^{-7}	-9.5	10.5
10^{-8}	23.8	44.6
10^{-9}	65.2	121.0
10^{-10}	76.0	124.5

B

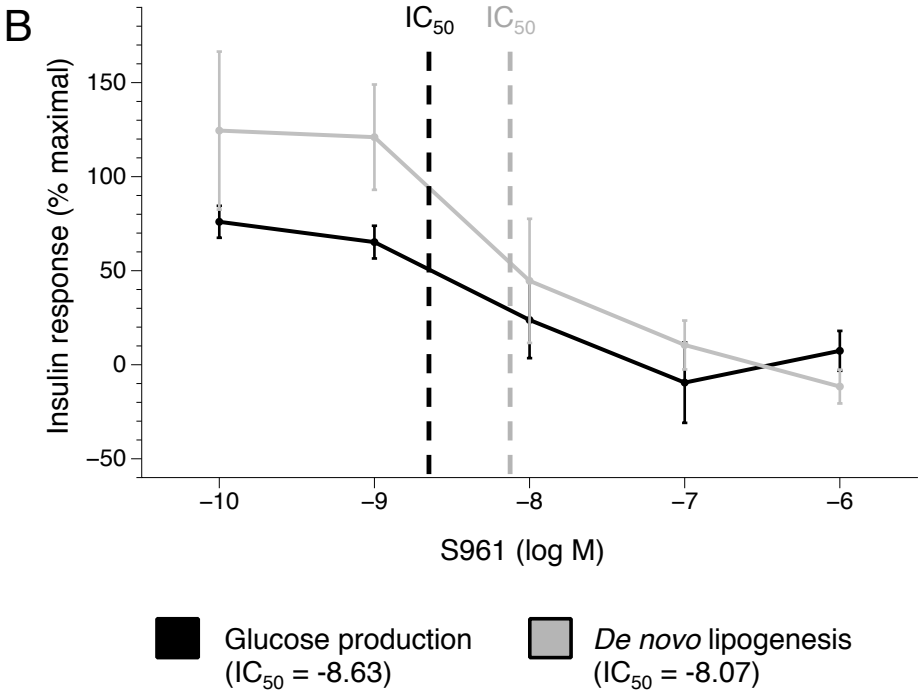


Figure 3.6

Figure 3.6. Relative Insulin Responsiveness of GP and DNL

(A) % maximal insulin response of Akt T308 phosphorylation, glucose production, and *de novo* lipogenesis as a function of S961 concentration. Figures are calculated from the data in Fig. 3.2 as the percentage of the departure from baseline at the indicated S961 concentration relative to the maximal change with insulin in the absence of antagonist.

(B) Graphical representation of calculations in (A) for glucose production and *de novo* lipogenesis. IC_{50} is calculated as $\log_{10}$ of the S961 concentration at which each dose-response curve intercepts 50% maximal insulin response. Data are derived from Fig. 3.2 and represent mean ± SEM of three independent experiments performed in triplicate.

A

S961 conc. (M)	Glucose prod. (% max)	*G6pc* exprn. (% max)	*Pck1* exprn. (% max)
0	100	100	100
10^{-6}	7.4	34.3	5.0
10^{-7}	-9.5	33.7	13.3
10^{-8}	23.8	63.1	58.0
10^{-9}	65.2	95.5	96.4
10^{-10}	76.0	100.7	100.9

B

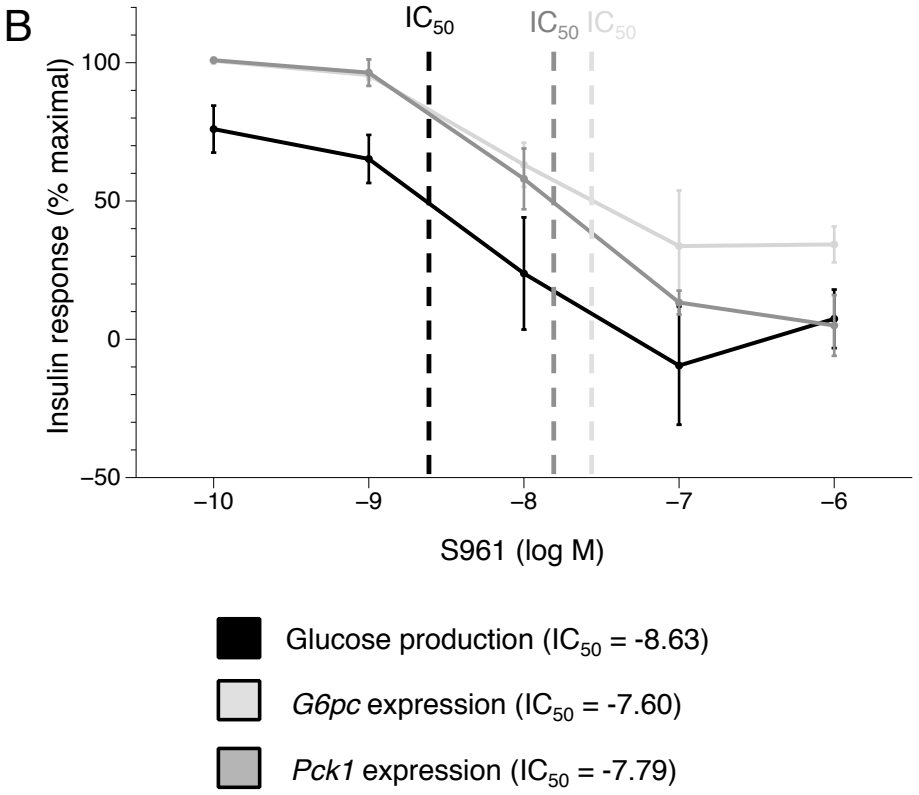


Figure 3.7

Figure 3.7. Relative Insulin Responsiveness of GP and gene expression

(A) % maximal insulin response of glucose production and expression of *G6pc* and *Pck1* as a function of S961 concentration. Figures are calculated from the data in Fig. 3.2 as the percentage of the departure from baseline at the indicated S961 concentration relative to the maximal change with insulin in the absence of antagonist.

(B) Graphical representation of calculations in (A) for glucose production and gene expression. IC_{50} is calculated as $\log_{10}$ of the S961 concentration at which each dose-response curve intercepts 50% maximal insulin response. Data are derived from Fig. 3.2 and represent mean ± SEM of three independent experiments performed in triplicate.

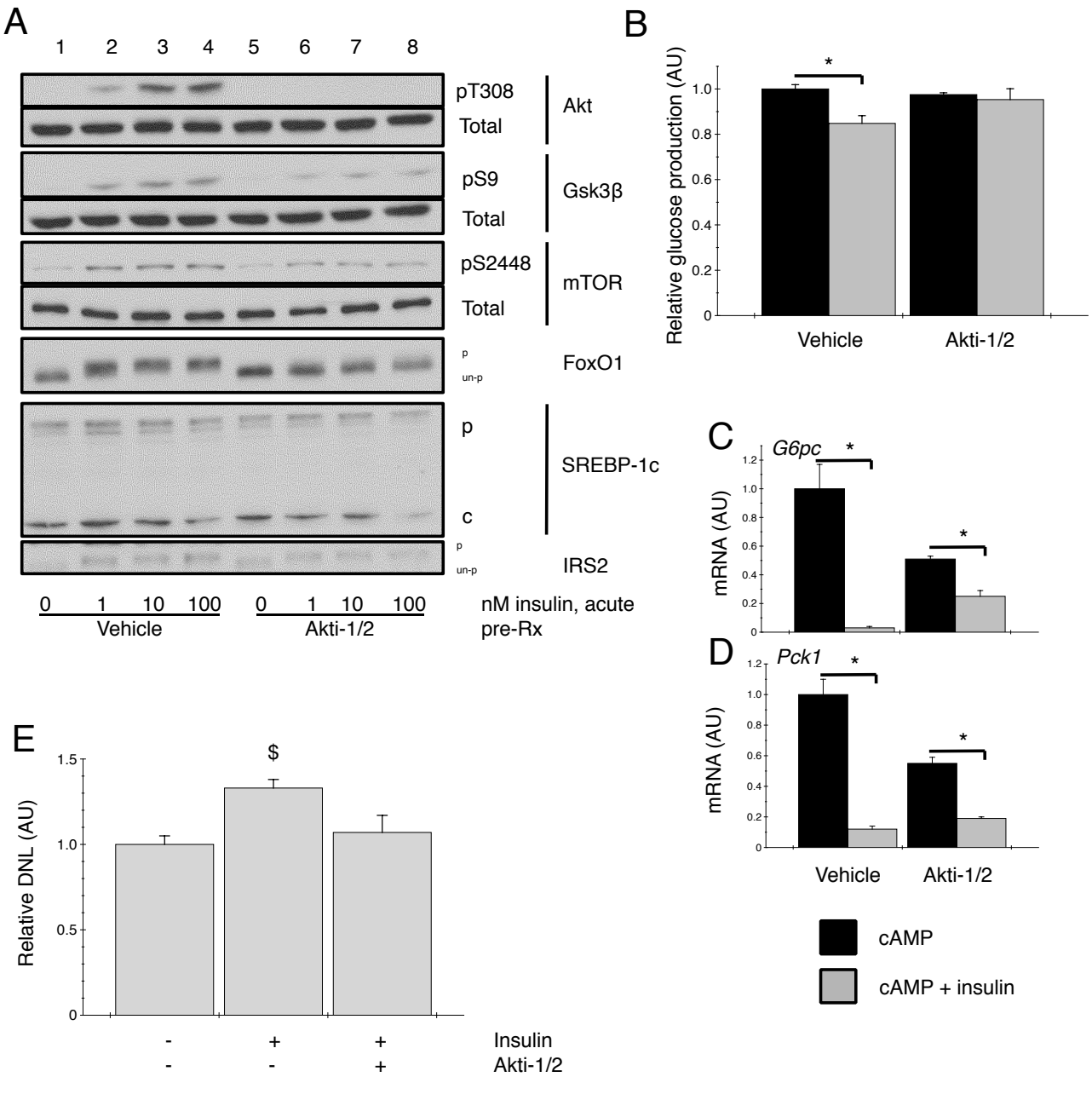

A
1 2 3 4 5 6 7 8
pT308
Total
Akt
pS9
Total
Gsk3β
pS2448
Total
mTOR
p
un-p
FoxO1
p
c
SREBP-1c
p
un-p
IRS2
0 1 10 100 0 1 10 100
Vehicle
Akti-1/2
nM insulin, acute pre-Rx
B
Relative glucose production (AU)
Vehicle
Akti-1/2
C
G6pc
mRNA (AU)
D
Pck1
mRNA (AU)
Vehicle
Akti-1/2
cAMP
cAMP + insulin
E
Relative DNL (AU)
$
Insulin
Akti-1/2

Figure 3.8

Figure 3.8. Effect of Akt Inhibition on Glucose Production and Lipogenesis

(A) Western blots demonstrating alterations in InsR signaling cascade components in response to treatment with 0, 1, 10, or 100 nM insulin with or without pre-treatment with 5 μM Akti-1/2. Blots are representative of three independent experiments. Data are representative of mean ± SEM of three independent experiments.

(B-D) Relative glucose production (B) and gene expression (C-D) in primary hepatocytes pre-treated with Akti-1/2 or vehicle and incubated for 5 h with 100 μM CPT-cAMP with or without 1 nM insulin. Data are representative of of mean ± SEM three independent experiments performed in triplicate.

(E) Relative *de novo* lipogenesis in primary hepatocytes pre-treated with Akti-1/2 or vehicle and incubated for 5 h with or without 1 nM insulin. Data are mean ± SEM of one representative experiment of two performed in triplicate.

* $p < 0.05$ by Bonferroni's post-hoc analysis following two-way ANOVA. $ $p < 0.05$ relative to control by Tukey's post-hoc analysis following one-way ANOVA.

Chapter 4

MECHANISTIC STUDY OF HEPATIC FOXO1 FUNCTION

4.1. Introduction

As discussed in Chapter 1, several mechanisms have been proposed to explain FoxO1's effects on hepatic glucose and lipid metabolism. While these models, even if all true, are not mutually exclusive, some may trump others in controlling a specific metabolic process; their relative control strengths have never been simultaneously tested. If we are to push forward in developing therapeutics aimed at FoxO1, which by itself represents a relatively poor drug target in liver, it will be of paramount importance to understand which of its many target genes would be most suited to pharmacologic manipulation. We therefore took an unbiased approach to parsing out which of FoxO1's multiple actions are most directly responsible for its observed phenotypic outputs.

An under-appreciated aspect of FoxO1 biology that we believe an ideal inroad into our question is its ability to act both dependently and independently of direct binding to its DNA consensus sequence, the insulin-response element (IRE). In other words, FoxO1 can act both as a transcription factor ("TF mode") and as a transcriptional co-regulator ("CR mode"). Crystallographic analysis of FoxO1 bound to an IRE has identified two residues within its forkhead domain – Asn 208 and His 212 – as essential for sequence-specific DNA binding [366]. Alteration of these residues to Ala and Arg, respectively, produces a mutant that retains proper nucleocytoplasmic partitioning in response to insulin but is incapable of binding to the IRE [147, 367]. Importantly, this mutant FoxO1 is not merely a hypomorphic variant of wild-type FoxO1; microarray analysis of a carcinomatous cell line has revealed a distinct subclass of genes that are induced to a greater extent by overexpressed FoxO1-HRAA than by wild-type FoxO1 [368].

Thus, a logical step toward better understanding of FoxO1 action is to take a genetic approach in parsing out the metabolic effects of hepatic FoxO1 that proceed via its action as a transcription factor *versus* those in which FoxO1 plays a co-regulatory role. We have accomplished this goal by generating the L-DBD mouse, whose liver expresses only DNA binding-deficient FoxO1, and comparing it to control and L-FoxO1 mice completely lacking FoxO1 in the liver. By complementing metabolic phenotyping studies with transcriptomic analysis by RNA-seq, we can effectively home in on a uniquely delimited pool of candidate genes whose differences in expression among the three groups best correlate with observed phenotypic differences.

The pattern of differential findings between genotypes – whether in terms of metabolism or gene expression – implies the mechanistic basis of FoxO1's regulation of those processes. For example, a process significantly altered in L-FoxO1 mice but not in L-DBD mice must require FoxO1 to act in CR mode. We therefore could suppose that the process is most dominantly controlled by gene(s) whose expression would be regulated by FoxO1 independent of DNA binding. In this way our strategy builds upon previous studies that have compared control mice only to those completely lacking FoxO1 in the liver; such an approach can only yield information as to whether FoxO1 plays a net activating or inhibiting role in a given process. Using these insights, we can evaluate the relative importance of previously proposed means of FoxO1 action as well as potentially uncovering novel mechanisms to boot.

4.2. Results

4.2.1. Generation and Analysis of L-DBD Mice

We have developed a mutant allele of *Foxo1* (*Foxo1*^{dbd}) containing the N208A and H215R mutations described above in addition to the naturally occurring variant K219R (Fig. 4.1A). We confirmed that FoxO1-DBD, unlike wild-type FoxO1, is incapable of driving luciferase activity from a reporter-gene construct containing canonical FoxO1 consensus binding sites (Fig. 4.1B). We have then employed recombinase-mediated cassette exchange (RMCE) to insert a cDNA cassette encoding *Foxo1*^{dbd} into the *Foxo1* locus by homologous recombination. These transgenic mice therefore are of the genotype *Foxo1*$^{wt/dbd}$. Homozygosity for alleles encoding FoxO1-DBD results in embryonic lethality. Although we did not determine the cause, we presume that it results from arteriovenous malformations, as in the case of global *Foxo1* knockout [369].

To dissect the role of the transcriptional vs. co-regulatory functions of FoxO1 in liver, we introduced the *Foxo1*^{dbd} allele in mice bearing a floxed allele of *Foxo1* (*Foxo1*^{fl}). This breeding strategy (Fig. 4.1C) generates mice in which each cell contains one copy of *Foxo1*^{fl} and one copy of *Foxo1*^{dbd} (*Foxo1*$^{fl/dbd}$) henceforth termed DBD-het, as well as *Foxo1*$^{fl/fl}$ mice as controls. By breeding controls and DBD-hets with mice bearing a Cre recombinase transgene driven by the liver-specific α_1-antitrypsin (AT) promoter we can generate two additional genotypes for study, as illustrated in Fig. 4.1C. First, the AT-

Cre^{+} *Foxo1*$^{fl/fl}$ (L-FoxO1) mouse lacks all FoxO1 specifically in liver. Second, the AT-Cre^{+} *Foxo1*$^{fl/dbd}$ (henceforth, L-DBD) mouse lacks all floxed (*i.e.*, wild type) FoxO1 in liver, thus leaving only DNA binding-deficient FoxO1-DBD. In all non-hepatocyte cells the mice have one allele of *Foxo1*^{fl} and one allele of *Foxo1*^{dbd}, as do DBD-het mice.

Quantitative RT-PCR with allele-specific primers demonstrated the generation of the desired genotypes (Fig. 4.1D). Western blot analysis verified the absence of FoxO1 protein in liver extracts from L-FoxO1, but of the retention of about half of the normal complement of FoxO1 in L-DBD mice (Fig. 4.1E), indicating that L-DBD mice express purely DNA binding-deficient FoxO1 in liver.

4.2.2. Metabolic Features of Heterozygous Foxo1^{dbd} *Mice and Hepatocytes*

To rule out extra-hepatic metabolic effects of *Foxo1*^{dbd} heterozygosity *per se*, we compared adult male controls (*Foxo1*$^{fl/fl}$) and DBD-hets (*Foxo1*$^{fl/dbd}$) with mice heterozygous for a null allele of *Foxo1* (*FoxO1*$^{fl/-}$; henceforth, FoxO1-het) (Fig. 4.1C-D, 2E). We found no differences in fasting or re-fed glucose or insulin levels, glucose, pyruvate, or insulin tolerance tests, body weight and composition (Fig. 4.2A-D, Table 4.1), or in the expression of known hepatic FoxO1 target genes following an overnight fast (Fig. 4.2E). These data are consistent with prior findings in FoxO1-het [142, 370]. Primary hepatocytes from control, FoxO1-het, and DBD-het mice showed no impairment of basal or cAMP/dexamethasone-stimulated glucose production (Fig. 4.3A-B) in spite of decreased *G6pc* expression in mice of both heterozygous genotypes (Fig. 4.3C), although *Igfbp1* and *Ppargc1a* expression were normal (Fig. 4.3D-E). Thus, we conclude that *Foxo1*^{dbd} heterozygosity *per se* does not result in a metabolic phenotype that might confound the interpretation of data from the L-DBD mouse.

4.2.3. Metabolic Characterization of L-DBD Mice

We analyzed the metabolic features of adult L-DBD male mice. They gained weight at the same rate as L-FoxO1 and control mice (Fig. 4.4A and data not shown), and showed no differences in body composition (Fig. 4.4B). Indirect calorimetry revealed no significant differences in food intake, energy expenditure, oxygen consumption, or respiratory quotient (RQ) (Fig. 4.4C-G), further suggesting that

Foxo1^{dbd} heterozygosity alone does not perturb whole-body metabolism. Likewise, there were no differences between L-DBD and control mice in glucose or insulin levels following an overnight fast or a 4-hr re-feed, whereas L-FoxO1 mice showed a modest decrease in re-fed insulin levels compared to controls (Fig. 4.5A-B).

L-DBD mice exhibited an enhancement of glucose tolerance (GTT) identical to L-FoxO1 mice (Fig. 4.5C) [144, 145], suggesting that *Foxo1*^{dbd} is effectively a null mutant with respect to glucose tolerance. These results were borne out by pyruvate tolerance tests (PTT), showing superimposable curves in L-FoxO1 and L-DBD mice (Fig. 4.5D) [145]. Intraperitoneal insulin tolerance tests (ITT) on fasted animals failed to reveal differences between control and L-FoxO1, in accordance with previous data [145], but showed a modest enhancement in L-DBD mice (Fig. 4.5E). Quantitative analyses of the areas under the curve (AUC) from experiments on multiple cohorts confirmed these conclusions (Fig. 4.5F). Moreover, qPCR analysis of RNA extracted from livers of overnight-fasted mice showed decreased *G6pc* and *Igfbp1* (Fig. 4.5G). As we have previously reported [145], *Pck1* expression was not significantly decreased in the absence of FoxO1 (Fig. 4.5G). These results indicate that deletion of hepatocellular FoxO1 results in decreased HGP.

FoxO1 regulates insulin sensitivity in part through a homeostatic loop with Akt. That is, we have found that a constitutively nuclear mutant form of FoxO1 is capable of increasing basal Akt phosphorylation, partially through upregulation of *Irs2* expression (see Section 5.3.1) [247, 371]. However, we detected no genotypic differences in the activation of Akt and its substrates, FoxO1 and GSK3β, in liver (Fig. 4.6A), skeletal muscle (Fig. 4.6B), or white adipose tissue (Fig. 4.6C) following intravenous administration of insulin in 5-hr-fasted mice. We saw a subtle decrease of Akt and GSK3β phosphorylation in L-FoxO1 mice in the more physiologic context of re-feeding (Fig. 4.7A-C). However, the effect may be attributable to lower insulin levels in re-fed L-FoxO1 mice since the difference disappeared when we compared mice matched for insulin levels (Fig. 4.7D-F). As a prototypical FoxO1 target gene involved in the regulation of insulin sensitivity, we measured levels of *Irs2* [371], and saw a significant reduction in livers from fasted L-FoxO1 and L-DBD animals (Fig. 4.7G). Taken together, these

data indicate that insulin signaling is similarly preserved in L-FoxO1 and L-DBD mice, and that *Irs2* expression requires FoxO1 to act in transcription factor mode.

4.2.4. Impaired Glucose Production in Hepatocytes from L-DBD Mice

Next, we isolated primary hepatocytes from control, L-FoxO1, or L-DBD mice and assessed their ability to generate glucose from pyruvate and lactate either basally or in the presence of CPT-cAMP and dexamethasone (cAMP/dex). Glucose production nearly doubled in control hepatocytes in a time-dependent manner following the addition of cAMP/dex (Fig. 4.8A-B). In contrast, primary hepatocytes from L-DBD mice showed a nearly 30% decrease in basal and cAMP/dex-stimulated glucose production, similar to L-FoxO1 hepatocytes (Fig. 4.8A-B). Consistent with these findings, L-FoxO1 and L-DBD primary hepatocytes showed a >80% decrease in the effect of cAMP/dex on *G6pc* and a ~40% decrease of *Pck1*, as a result of which the suppressive effect of insulin on both genes was virtually abolished (Fig. 4.8C-D). In contrast, neither L-FoxO1 nor L-DBD hepatocytes showed substantial effects on the expression of *Ppargc1a*, another gene implicated in FoxO1's control of HGP (Fig. 4.8E) [372]. This finding is consistent with data indicating that FoxO1 is required for the effect of *Ppargc1a* on glucose production [145]. Nevertheless, there was a small but significant decrease in the expression of *Ppargc1a* in L-FoxO1 hepatocytes *versus* L-DBD (Fig. 4.8E), mirroring a similar though non-significant trend in fasted liver (Fig. 4.5G), suggesting that *Ppargc1a* is a co-regulatory target of FoxO1.

4.2.5. Hepatic Triglyceride Metabolism in L-DBD Mice

We then examined features of hepatic lipid metabolism in L-DBD mice. We found no differences in circulating levels of FFA, TG, or cholesterol among mice of different genotypes (Table 4.2) [145, 168]. Liver weight was modestly increased in re-fed, but not in overnight-fasted L-FoxO1 mice (Fig. 4.9A). This difference was due at least in part to increased TG content (Figure 4.9B) but surprisingly was not observed in L-DBD mice. There was no difference in liver cholesterol content among genotypes in the fasted or re-fed states (Table 4.2).

We analyzed different aspects of hepatic lipid handling in order to parse out the mechanism underlying differential liver triglyceride content. Oral lipid tolerance tests and hepatic TG secretion were normal (Fig. 4.9C-E). In contrast, β-oxidation of radiolabeled oleic acid decreased by ~40% in L-FoxO1 hepatocytes, and by ~60% L-DBD hepatocytes (Fig. 4.9F). Analysis of *de novo* lipogenesis (DNL) using radiolabeled acetic acid demonstrated a non-significant ~35% increase in ^{14}C incorporation into the TG pool of primary hepatocytes from L-FoxO1 mice under basal as well as insulin-stimulated conditions. Hepatocytes of L-DBD mice showed an even greater increase of ~75% (Fig. 4.9G). The inability of L-DBD hepatocytes to restore lipid oxidation and lipogenesis to their control levels indicates that these cell-autonomous effects require FoxO1 to bind directly to DNA.

To determine the mechanism of the alteration in DNL, we measured expression of several lipogenic genes (Fig. 4.10A-F). There were significant elevations in fasting levels of glucokinase (*Gck*) and stearoyl-CoA desaturase-1 (*Scd1*) in L-FoxO1, but not L-DBD mice compared to controls. On the other hand, fasting levels of pyruvate kinase (*Pklr*), a target of the lipogenic transcription factor Chrebp [373], were significantly lower, while those of acetyl-CoA carboxylase-1 (*Acaca*) were unchanged in L-FoxO1 and L-DBD livers compared to controls. We also measured the expression of four key bile-acid metabolic genes (Fig. 4.10G-J), as we have previously attributed some of FoxO1's ability to regulate hepatic TG to alterations in bile-acid pool composition [168]. This study replicates the decreased expression of *Cyp8b1* and *Cyp27a1* but not of *Cyp7a1* in L-FoxO1 mice. That the expression pattern of these genes in L-DBD mice parallels that of L-FoxO1 mice indicates that this particular role of FoxO1 requires its action as transcription factor.

As our laboratory's work has implicated Gck as a link between FoxO1 and DNL, we also measured its expression in primary hepatocytes in parallel with our DNL assay. Unlike in whole liver, we did not detect any significant differences in *Gck* expression between genotypes or in response to insulin (Fig. 4.11A), although expression did trend higher in L-DBD hepatocytes. For the sake of comparison, *Gck* expression was significantly increased by up to 78 times control levels in primary hepatocytes from L-FoxO1,3,4 mice (Fig. 4.11B). In keeping with higher *Gck* expression, total *de novo* lipogenesis was increased 2.5-fold in vehicle-treated L-FoxO1,3,4 primary hepatocytes compared to control but unlike

control did not further increase with insulin treatment (Fig. 4.11C). Although this is a measure of total DNL rather than specifically of TG synthesis, we have found the two to be interchangeable in this system.

We also sought to determine whether the significant increase of DNL in insulin-treated L-DBD hepatocytes was due to enhanced insulin signaling. However, phosphorylation of Akt (T308) and Gsk3β (S9) in response to insulin was rather decreased in primary hepatocytes from L-FoxO1 and L-DBD mice (Fig. 4.11D). These experiments indicate that loss of FoxO1 function increases lipogenesis and decreases FFA oxidation, and that FoxO1-DBD fails to restore these functions. We conclude that FoxO1 physiologically inhibits these processes in a DNA binding-dependent manner.

4.2.6. Lipid Metabolism in WTD-Fed L-DBD Mice

We have previously demonstrated that FoxO1 ablation increases hepatic TG deposition in mice fed a Western-type diet (WTD) [168]. We therefore placed L-DBD, L-FoxO1, and control mice on WTD for ten weeks and analyzed them in either the *ad libitum*-fed or 5-hr-fasted state. At the completion of the diet there were no significant differences among genotypes in body weight or circulating levels of glucose, insulin, FFA, TG, and cholesterol in either state (Table 4.3). Liver weight increased by ~25% in fed L-FoxO1 and L-DBD mice (Fig. 4.12A), accompanied by a near doubling of liver TG, although this difference did not reach statistical significance owing to large individual variance (Fig. 4.12B). Histologic examination of liver sections taken from these mice confirmed the presence of hepatic steatosis in L-FoxO1 and L-DBD mice (Fig. 4.12C). These findings were accompanied by coordinate increases in levels of mRNA encoding *Fasn*, *Gck*, and *Scd1* (Fig. 4.12D-G).

Finally, we analyzed whether FoxO1-DBD modified the effects of WTD feeding on insulin signaling in liver and primary hepatocytes. Fasting levels of p-Akt (Thr 308 and Ser 473) and p-GSK3β (S9) were uniformly increased in WTD-fed mice of all genotypes, blunting the increase in response to feeding (Fig. 4.13A-D). This is probably due to hyperinsulinemia [185]. We investigated this process by pre-incubating primary hepatocytes with insulin as a surrogate of *in-vivo* hyperinsulinemia (Fig. 4.11C), as previously described. Following this treatment, basal (*i.e.*, "fasted") phosphorylation levels of Akt and GSK3β increased relative to non-exposed cells, but were not further augmented by acute insulin

treatment (“fed” state). As in fed livers, L-FoxO1 and L-DBD hepatocytes exhibited a trend toward lower levels of Akt and GSK3β phosphorylation following acute insulin challenge. Thus, it appears that FoxO1-DBD does not exert independent effects on insulin signaling in this context.

4.2.7. Transcriptomic Profiling of L-DBD Mice

To dissect the mechanistic basis of FoxO1’s actions, we first investigated the phenotypic consequences of *Foxo1*^{dbd} hemizygosity, then turned to transcriptomic analyses to understand changes in gene expression that correlate genotypically with metabolic parameters. We performed RNA-seq analysis from liver samples from fasted or re-fed Control, L-FoxO1, and L-DBD mice. We found considerable overlap with respect to genes significantly altered in L-FoxO1 and L-DBD livers *versus* control (Fig. 4.14A). Thus, it appears that the largest share of FoxO1 target genes are regulated by direct DNA binding of FoxO1 (*i.e.*, as a transcription factor). Nevertheless, there is indeed a small but distinct pool of target genes that are differentially altered in L-FoxO1 *vs.* L-DBD mice – that is, genes toward which FoxO1 acts in a co-regulatory manner (Fig. 4.14A-B, 4.15B-C). Analysis of the relative differences in the expression of these genes indicates that the FoxO1-DBD acts both as a co-activator and co-repressor toward these target genes in roughly equal measure in both the fasted and re-fed states (Fig. 4.14B, 4.15B-C). In some cases, the ostensible mechanism of FoxO1’s regulation of a particular target gene appears to vary depending on feeding state (data not shown). We validated the expression patterns of selected genes (*i.e.*, toward which FoxO1 acts as co-regulator *vs.* transcription factor) by qPCR (Fig. 4.15A-C).

The complete phenotypic concordance between L-FoxO1 and L-DBD mice in terms of glucose production indicates that *Foxo1*^{dbd} represents a complete loss-of-function allele and that FoxO1’s role in promoting hepatic glucose production proceeds via a mechanism requiring direct binding to DNA. This allows us to focus our transcriptomic analysis on genes that are significantly altered in both L-FoxO1 and L-DBD mice relative to control but not to each other as particularly attractive candidates for the major effectors of FoxO1’s influence on hepatic glucose production (Fig. 4.14A). In our RNA-seq data, 297 genes are significantly different in fasted L-FoxO1 mice versus control and 307 are different between fasted L-DBD and control. However, those that are significantly altered relative to control but *not* to each

other in both of these sets number only 159. Thus, through our study of the L-DBD mouse, we have approximately halved the number of candidate genes that we would have obtained based on merging glucose-tolerance and gene-expression data from control vs. L-FoxO1 alone.

We can apply a similar line of reasoning to the “lipid arm” of hepatic metabolism. We have shown that, unlike their L-FoxO1 littermates, chow-reared L-DBD mice retain normal control of hepatic triglyceride metabolism in the re-fed state. This demonstrates a relevant metabolic function for FoxO1 as a transcriptional co-regulator rather than as a transcription factor. Based on our transcriptomic analysis (Fig. 4.14A), we can cut down a list of candidate genes from 275 for refed Control vs. L-FoxO1 only to 44, those significantly altered in refed L-FoxO1 vs. control and L-DBD, but not in control vs. L-DBD. If the more important changes occur instead in the fasting state, our analysis cuts down the list of candidates from 298 (L-FoxO1 vs. control alone) to 31. We have therefore simplified our list of theoretical candidates by over 80% by first gating the analysis on the basis of FoxO1’s mode of action with respect to hepatic TG metabolism.

Gene ontology analysis indicates that the genes that differ significantly between L-FoxO1 and L-DBD livers vary in functional categorization between the fasted and re-fed states (Table 4.4). In the fasted state, the ontological categories most highly altered by the presence of FoxO1-DBD relate to metabolism of steroids, particularly steroid hormones. On the other hand, in the refed state, those most affected by FoxO1-DBD relate to both innate and adaptive immune processes. Selected candidate genes will be discussed in Section 4.3.5.

4.3. Discussion

4.3.1. Overview of Findings

This study demonstrates that the pleiotropic effects of FoxO1 on hormone- and nutrient-dependent gene expression require binding to DNA. There is another, less recognized mode of action, whereby FoxO1 engages in non-DNA-based interactions with components of the transcriptional complex to regulate gene expression. Examples of this mode of action include FoxO1 binding to Maml1 and Csl, central elements in Notch signaling [370, 374]. However, the present study indicates that this mode of action does not

contribute to the main cell-autonomous effects of FoxO1 on hepatic glucose and lipid metabolism. In fact, we show that reconstitution of a FoxO1 DNA-binding-deficient allele in liver of mice that lack endogenous FoxO1 fails to restore HGP, lipogenesis, and FFA oxidation. While the conclusion that FoxO1 controls HGP by binding to consensus sites on target promoters was predicted by previous work [152, 372] the finding of increased lipogenesis in L-DBD mice is surprising, as the inhibition of this process by FoxO1 is more easily reconciled with a co-repressor function [375]. Another important finding of the present study is the heretofore-unrecognized effect of FoxO1 ablation, mimicked by the DBD mutant, to reduce FFA oxidation [158, 166, 167, 245].

The segregation of different functional outputs of a transcription factor on the basis of DNA binding-dependent *vs*. independent actions has been observed in other contexts. For instance, it appears to be a feature of bHLH transcription factors, including Hand2 and Scl [376, 377]. Prior evidence in this vein exists for FoxO1 as well. For instance, FoxO1-DBD can suppress myogenic differentiation of C2C12 myoblasts as efficiently as FoxO1-WT [374]. Likewise, constitutively nuclear FoxO1-DBD retains the ability to enhance basal phosphorylation of Akt in liver [247] (see Section 5.3.1). Overall, FoxO1 has been shown to engage in protein-protein interactions with at least a dozen distinct transcriptional regulators, although the physiologic significance of many of these interactions has not previously been validated [378].

4.3.2. FoxO1 Regulation of Hepatic Glucose Metabolism

The centrality of FoxO1 in insulin's control of HGP is beyond question. This study further cements this certainty, as we have found that hepatocyte-specific deletion of FoxO1 results in enhanced glucose tolerance due to decreased HGP. As mentioned earlier, however, despite the robust reproducibility of this result, the mechanism underlying it is relatively unknown. The well-known regulation of *G6pc* expression by FoxO1, and perhaps also of *Fbp2*, *Pdk4*, *Ppargc1a*, and *Pck1*, has been essentially taken for granted as the explanation.

Despite the appealingly straightforward nature of this hypothesis, however, multiple lines of evidence suggest that *G6pc* and *Pck1* expression may not be directly responsible for FoxO1's empirical

role in HGP. Complete loss of *G6pc* or of *Pck1* in the liver, far more dramatic than the decrease in expression due to FoxO1 deficiency, do not result in a physiologic impairment in glucose production due to indirect actions of other tissues on this process, for example through FFA flux from adipose tissue and vagal control by the CNS, as well as compensatory increases in glucose production by intestine and kidney [379, 380]. Moreover, while concomitant knockout of all three FoxO proteins in the liver results in a further enhancement of glucose tolerance, it is not accompanied by any apparent defect in *Pck1* expression [144], and thus we can assume that it is not responsible for FoxO1-associated HGP. Real-time hyperinsulinemic-euglycemic clamp studies have cast doubt on the temporal correlation between physiologic changes in *G6pc*/*Pck1* expression/activity and the blunting of HGP by insulin [94, 114]. Finally, type 2 diabetic patients, a group exhibiting inappropriately high FoxO1 activity, do not necessarily exhibit increased hepatic expression of *G6pc* or *Pck1* despite inappropriately high HGP [381]. Taken together, these observations indicate that although the InsR → FoxO1 axis is crucial to proper regulation of HGP, the precise mechanism linking FoxO1 to HGP remains unclear [2].

In this study we have taken a first step by demonstrating that L-DBD mice phenocopy L-FoxO1 mice in terms of glucose-metabolic parameters. That is, direct binding of FoxO1 to the IRE is required for its regulation of HGP. Of note, *G6pc* expression was reduced similarly in L-DBD and L-FoxO1 mice, thus still not allowing us to rule out the importance of this gene to overall HGP. The fact that *G6pc* expression does correlate well with FoxO-associated HGP while *Pck1* expression does not implies that the effect of FoxO1 deficiency on glucose metabolism may reflect more on alterations in glycogen metabolism than in gluconeogenesis, consistent with hyperinsulinemic-euglycemic clamp data in L-FoxO1 mice as well as the finding that L-FoxO1 livers engage in inappropriately high glycogen storage [2, 145]. Moreover, although the significant enhancement of pyruvate tolerance exhibited by L-FoxO1 and L-DBD mice suggests a primary change in gluconeogenesis, this effect may also result from increased glycogen synthesis from G6P and/or decreased glycogenolysis in the absence of FoxO1. In this case, low *G6pc* expression would prevent the conversion of G6P to releasable glucose. The *in vivo* change in *G6pc* expression is only about 25%, casting doubt on its physiologic primacy and raising the possibility that other contributors are necessary for the effect. Nevertheless, a potential limitation of this study is that it did not assess glycogen

content in L-DBD livers, although it is expected that they would demonstrate inappropriately high glycogen storage similar to L-FoxO1 mice. Excess glycogen in L-DBD livers may account for the slight, albeit non-significant elevation in liver weights in L-DBD mice in spite of their normal TG levels.

A pertinent negative highlighted by this study is PGC-1α, which we show here to be a *bona fide* co-regulatory target of FoxO1. That is, although L-FoxO1 primary hepatocytes express significantly lower levels of *Ppargc1a* when stimulated with cAMP/dex, gene expression is completely normal in L-DBD hepatocytes (Fig. 4.8E) – despite the inability of FoxO1-DBD to activate glucose production above the level of L-FoxO1 cells (Fig. 4.8A-B). Thus, although its importance as a FoxO1 co-activator has been much touted [155, 372], FoxO1-mediated HGP actually proceeds largely independently of PGC-1α. That L-DBD primary hepatocytes also show deficient FAO suggests that FoxO1's regulation of the process does not proceed through PGC-1α regulation of mitochondrial oxidation [145, 174].

We also can consider candidate genes brought to the fore by RNA-seq. As mentioned earlier, the conclusion that FoxO1 regulation of HGP requires it to act in TF mode means that we can eliminate nearly half of the genes significantly altered in the absence of FoxO1 in fasted liver. The remaining list of 159 genes includes several intriguing possibilities. Perhaps the most obvious possibility is *Pdk4* [134], which inhibits the pyruvate dehydrogenase complex to spare pyruvate for glucongeogenesis and has already been shown to be regulated by FoxO1 [154]. Another logical candidate, *Nr0b2*, which encodes small heterodimer partner (SHP), also has already been implicated in FoxO1 control of HGP [2]; it acts as a nuclear co-repressor to block FoxO1-associated *G6pc, Pck1*, and *Pdk4* gene expression through negative feedback [382-384]. In fact, we have already studied *Nr0b2* in the context of its negative regulation of bile-acid metabolic enzymes *Cyp7a1* and *Cyp8b1* and shown that its expression is decreased in WTD-fed L-FoxO1 livers [168], suggesting another level of coordination of glucose and lipid metabolism by FoxO1. Finally, *Slc37a4*, which encodes the G6P transporter that carries G6P from the cytosol into the ER lumen where it can be acted upon by G6Pase [385], is decreased nearly by half in both L-FoxO1 and L-DBD samples. This is consistent with decreased glucose release and conceptually is reconcilable with a cell-autonomous model of FoxO1 action. Our laboratory has previously found that

Slc37a4 mRNA is significantly decreased in L-FoxO1,3,4 livers even though it is not strongly regulated by fasting/feeding [253].

4.3.3. FoxO1 Regulation of Hepatic Lipid Metabolism

The most notable physiologic parameter influenced by hepatic FoxO1 in its co-regulatory capacity is triglyceride metabolism. To wit, L-FoxO1 liver TG levels were significantly higher than controls following re-feeding; L-DBD mice, on the other hand, had normal re-fed liver TG levels (Fig. 4.9B). Therefore, we can conclude that the overall regulation of liver TG storage by FoxO1 proceeds via a co-regulatory mechanism rather than through binding to IREs in target-gene promoters. Although our original study of L-FoxO1 mice did not find any significant alteration in liver TG, it focused only on the fasted and *ad libitum* fed states rather than under the strong lipogenic drive of re-feeding [145]. Indeed, a subsequent study did find a trend toward increased liver TG in re-fed L-FoxO1 mice that we now have found to reach statistical significance [241].

We have attempted to dissect the phase of hepatic lipid handling that is defective in L-FoxO1 livers but not in L-DBD. We detected no differences in oral lipid tolerance (*i.e.*, lipid absorption from the gut) or in TG secretion from the liver (Fig. 4.9C-E). *Ex vivo* experiments in primary hepatocytes demonstrate that loss of hepatocellular FoxO1 increases *de novo* lipogenesis and decreases fatty acid oxidation (Fig. 4.9F). These alterations in hepatocellular metabolism would increase liver TG, as observed *in vivo*. Data collected from L-DBD primary hepatocytes, however, appear less straightforward, possibly owing to indirect control on the liver. Fatty acid oxidation by L-DBD cells does not differ from L-FoxO1, indicating a requirement for direct transcriptional activation by FoxO1.

While previous *in vivo* studies of L-FoxO1 mice have not reported abnormalities of ketone bodies [145, 241], a relatively specific marker of β-oxidation, direct *in vivo* measurement in mice lacking all three liver FoxO isoforms (L-FoxO1,3,4) show decreased fatty acid oxidation rates relative to control [245]. These observations suggest that non-cell-autonomous mechanisms compensate for the *ex vivo* defect in fatty acid oxidation in the absence of functional FoxO1, an interpretation supported by the absence of any

difference in oxygen-consumption rate or respiratory quotient between genotypes (Fig. 4.4F-G). However, these apparent compensatory mechanisms are insufficient when all liver FoxO genes are knocked out.

Ex vivo assessment of DNL by measuring the incorporation of radiolabeled acetic acid into TG also revealed a requirement for direct DNA binding (Fig. 4.9G). The rate of DNL in L-FoxO1 primary hepatocytes was approximately 35% greater than control, both in the basal and insulin-stimulated states. It appears that FoxO1-DBD may even stimulate DNL in a gain-of-function capacity beyond the apparent ability of wild-type FoxO1 to inhibit it, as L-DBD primary hepatocytes tended to exhibit greater rates of DNL even than L-FoxO1 hepatocytes (about 75% higher than control). This may result, for example, from FoxO1-DBD sequestration of co-regulators from other FoxO proteins [386-388] and is consistent with previous data documenting increased rates of DNL in L-FoxO1,3,4 mice [245, 253]. Taken together, therefore, cell-autonomous regulation of *de novo* lipogenesis and fatty acid oxidation by FoxO1 requires direct binding to DNA.

It is important to emphasize, however, that L-DBD mice did not phenocopy the exaggerated liver TG levels of the L-FoxO1 mouse in the re-fed state in spite of its inability to properly control *de novo* lipogenesis and fatty acid oxidation in hepatocytes. We can therefore conclude that the predominant mechanism by which FoxO1-DBD regulates TG metabolism in the healthy liver is not solely at the level of cell-autonomous TG deposition or FFA oxidation. Again, we did not detect any differences between L-FoxO1 and L-DBD mice with respect to oral lipid tolerance or TG secretion. However, FoxO1-DBD may regulate non-cell-autonomous mechanisms that impact on lipid synthesis or oxidation. Indeed, several non-hepatocyte-autonomous mechanisms of controlling hepatic lipid metabolism have been identified to date with which hepatic FoxO1 may interact [168, 389-392]. Our laboratory, for example, previously demonstrated an ability of FoxO1 to regulate hepatic TG through altered bile acid signaling [168]. In this study, however, we did not note any discordance between expression of bile acid metabolic enzymes in L-FoxO1 *vs.* L-DBD mice (Fig. 4.10G-J).

As opposed to the chow-fed state, WTD-fed L-DBD mice exhibit the same increase of hepatic steatosis as L-FoxO1 mice, indicating this type of diet does not alter the balance between the DNA binding-dependent and -independent actions of FoxO1. It is possible that the insulin resistance

associated with WTD, which would be expected to lead to unrestrained FoxO1 activity, unmasks the apparent gain of function exhibited by FoxO1-DBD in primary hepatocytes with respect to *de novo* lipogenesis. In keeping with this possibility, both L-FoxO1 and L-DBD livers present lower levels of Akt and GSK3β phosphorylation than controls in spite of similar insulin levels (Fig. 4.13, Table 4.3), suggesting that FoxO1-DBD is less restrained by insulin signaling than wild-type FoxO1. This is consistent with the fact that steatotic liver derives a much greater percentage of its stored lipid from *de novo* lipogenesis than does healthy liver [73, 74, 306]. That dysregulated bile-acid metabolism appears to affect liver TG levels to a greater extent following WTD feeding [168] may also help to explain the lack of difference in liver TG between WTD-fed L-FoxO1 and L-DBD mice, as FoxO1 appears to regulate bile acid metabolism primarily as a transcription factor (Fig. 4.10G-J).

The RNA-seq-derived shortlist of genes that may account for FoxO1's actions on triglyceride metabolism in the re-fed state does not include any classical lipogenic enzymes. It does, however, contain other types of candidates previously linked to hepatic lipid metabolism, such as the fatty acid transporter *Cd36* [393], the secreted peptide adropin (*Enho*) [394], and cell death-inducing DFFA-like effector C (*Cidec*, also known as Fsp27) [395]. Finally, it is important to note that the main effect of FoxO1 on triglyceride metabolism in the re-fed state may be based at least in part on changes in gene expression during fasting. Genes regulated by FoxO1 in a co-regulatory manner that are altered in the fasted state and may contribute to an altered lipogenic response to refeeding include 6-phosphofructo-2-kinase/fructose-2,6,-bisphosphatase (*Fbp2*), described in Chapter 1, and lipin-1 (*Lpin1*) [396]. We believe, however, that glucokinase (*Gck*), a gene already well studied by our laboratory is primarily responsible for FoxO1's effects on hepatic lipid metabolism.

4.3.4. FoxO Regulation of Glucokinase May Underlie Effects on DNL

Data from our laboratory indicate that FoxO proteins are intimately involved in orchestrating the transition between fasting and feeding [253]. One potential mediator of this phenomenon is glucokinase, a central regulator of glucose flux in the liver and previously demonstrated to be repressed by FoxO1 [144, 167]. Compared to controls, expression of *Gck* is significantly increased by over three-fold in the livers of fasted

L-FoxO1 mice as expected but expression of *Gck* in L-DBD livers is intermediate between Control and L-FoxO1 (Fig. 4.10C). (There is no difference in *Gck* expression between groups four hours after re-feeding.) Higher levels of *Gck* expression in the L-FoxO1 mice at the onset of re-feeding may result in a more efficient accumulation of liver triglyceride than in control and L-DBD, as observed [232, 397, 398]. This would also explain the elevated liver TG of L-FoxO1,3,4 mice even relative to L-FoxO1, in line with the relative increment in *Gck* expression in the absence of FoxO3 and FoxO4 [144]. Thus, FoxO1's repression of *Gck* expression in the fasted state – again, when FoxO1 is most active – proceeds at least in part by a co-regulatory mechanism.

A mechanism based on Gck regulation of the fasting-feeding transition may also help to explain the discrepancy between *ex vivo* DNL and liver TG levels. Insulin has both direct and indirect effects on the liver, and therefore primary hepatocytes can only capture some of this complexity. As mentioned above, L-DBD livers partially retain the ability to suppress *Gck* expression in the fasted state while L-FoxO1 livers show a significant increase in fasting *Gck* expression. However, in primary hepatocytes, there is no significant difference between genotypes in *Gck* expression (Fig. 4.11A), although the general pattern of expression interestingly mirrored that of DNL with a trend toward increased *Gck* expression in L-DBD cells. By comparison, *Gck* expression was at least fourfold higher in primary hepatocytes from L-FoxO1,3,4 mice compared to control (Fig. 4.11B and data not shown) and total DNL was accordingly increased as well (Fig. 4.11C). This suggests that the regulation of *Gck* expression by FoxO proteins is multifaceted. FoxO3 and FoxO4 are evidently able to compensate for the loss of a cell-autonomous inhibitory effect of FoxO1. Meanwhile, some external stimulus modulates regulation of *Gck* expression by FoxO1 in particular *in vivo*.

A further potential clue lies in our observation that *Gck* expression does not significantly increase with insulin treatment alone in mouse primary hepatocytes, as has been previously reported. This indicates that the insulin-stimulated (and thus potentially FoxO1-inhibited) *Gck* expression, for example in response to re-feeding, requires the participation of factor(s) beyond hepatic insulin action alone. Supporting this conclusion is the finding that LIRKO mice still experience a significant, albeit attenuated increase in hepatic *Gck* expression in response to feeding [202]. Moreover, blockade of insulin

signaling specifically in the hypothalamus has been shown to depress the induction of liver *Gck* expression in response to portal insulin infusion [399]. Factors altogether distinct from insulin may also be required. We and others have found, for example, that co-treatment of primary hepatocytes with dexamethasone can enable insulin responsiveness of *Gck* expression [400-402]. That normal FoxO1-mediated expression of *Igfbp1* appears to be in part dependent on glucocorticoid signaling opens the door to similar requirements for FoxO1 regulation of *Gck* expression [145], especially as FoxO1 has been shown to be capable of functional interactions with the glucocorticoid receptor, potentially independently of IRE binding [378].

Taken together, these data suggest a model in which FoxO1 inhibits DNL at multiple levels, depicted graphically in Fig. 4.16. First, in a cell-autonomous fashion, FoxO1 represses DNL via a method requiring direct binding to DNA. One possibility lies in the fact that FoxO1 retains the ability to normally regulate expression of *Gck*, unlike *G6pc*, in primary hepatocytes. Work from our laboratory has shown that the ratio of *G6pc* to *Gck* expression is a reliable indicator of the direction of glucose flux (*i.e.*, of gluconeogenesis/glycogenolysis → HGP *vs.* glycolysis → DNL) [253]. Thus, in both L-FoxO1 and L-DBD primary hepatocytes, a defect in *G6pc* expression in the absence of a change in *Gck* would decrease the *G6pc:Gck* ratio. This, in turn, would impede gluconeogenesis, as observed in this study, while increasing the availability of acetyl-CoA for use in DNL, especially in the presence of insulin [253]. Again, in L-FoxO1,3,4 primary hepatocytes, *Gck* expression is frankly increased and *G6pc* decreased, consistent with the dramatic elevation in DNL compared to control [253].

The importance of *G6PC* in the indirect regulation of lipogenesis is illustrated quite starkly in the case of *G6PC* deficiency. Patients with mutations in the gene, termed von Gierke disease (GSD type Ia), demonstrate florid hypertriglyceridemia [403]. Even acute inhibition of G6Pase activity has been shown to result in the development of hepatic steatosis associated with increased DNL [404]. Although the above discussion has called into question the importance of physiologic fluctuations in hepatic *G6pc* expression, the relative importance of transcriptional control of *G6pc* to glucose production may be greater in isolated hepatocytes. This supposition is based on the far greater reduction in *G6pc* expression in FoxO1-deficient hepatocytes *ex vivo* compared to whole liver as well as the absence of extrahepatic tissues (*e.g.*,

intestine, kidney) to contribute to compensatory glucose production. Even if altered expression of *G6pc per se* is not directly responsible, the process of gluconeogenesis is certainly decreased in L-FoxO1 and L-DBD hepatocytes and thus, as in the case of decreased G6Pase action, would be expected to promote increased lipogenesis. Indeed, reducing gluconeogenic flux via liver-specific deletion of *Pck1*, for example, also results in significantly increased liver TG accumulation [379, 405].

Unlike in primary hepatocytes, fasting and feeding regulation of *Gck* expression via FoxO1 can proceed as normal in whole liver. Thus, by the end of an overnight fast, L-FoxO1 livers have accumulated significantly more *Gck* mRNA than controls. At the onset of re-feeding, these livers are thereby better primed for efficient TG synthesis, hence the increase in re-fed liver TG [253]. (During fasting, relatively low rates of DNL are eclipsed by re-esterification of FFAs liberated by WAT lipolysis in terms of overall liver TG levels [363].) On the other hand, L-DBD livers retain a partial ability to suppress *Gck* expression, thus not allowing these livers as much of a "head start" on DNL after re-feeding. That the expression of *Gck* is elevated to the same extent in both L-FoxO1 and L-DBD livers in the WTD-fed state therefore may explain the lack of difference in liver TG levels between these mice, especially given the heightened contribution of DNL to hepatic TG in this state. Evidently the apparent ability of haplosufficient FoxO1-DBD to regulate *Gck* expression in the chow-fed state is lost in the WTD-fed state, thus altering the *G6pc:Gck* ratio similarly in these mice. Given this hypothesis, it would be of interest to assess whether the difference in liver TG levels would be magnified at earlier time points in re-feeding.

Evidence of a co-regulatory action of FoxO1 on *Gck* expression already exists in the literature [179, 406, 407]. For example, in addition to being able to bind directly to the *Gck* promoter, FoxO1 has also been previously reported to disrupt the activation of *Gck* expression by HNF-4α by interfering with the latter's binding to the promoter [179, 406]. This dual ability of FoxO1 to regulate *Gck* expression as both transcription factor and co-regulator may be reflected in the intermediate expression of *Gck* in L-DBD between control and L-FoxO1. However, all such studies have been conducted in cultured cells and therefore the potential contribution of a FoxO1—HNF-4α interaction to liver triglyceride levels *in vivo* remains unknown.

Overall, this model is not necessarily mutually exclusive with other hypotheses regarding FoxO1 control of hepatic TG, such as via modulation of bile acid metabolism [168]. Indeed, this model alone is not sufficient to explain the augmentation in DNL observed in L-DBD primary hepatocytes even relative to L-FoxO1. Thus, it is likely that other mechanisms also come into play. One enticing possibility, as mentioned above, is a partial dominant-negative effect of FoxO1-DBD on FoxO3/4, especially as *Gck* expression trends slightly higher in L-DBD cells.

4.3.5. Other Potential Co-Regulatory Actions of FoxO1

The mechanistically complex nature of FoxO1's regulation of target-gene expression also is not limited to glucose- and lipid-metabolic transcriptional programs. Gene-ontology analysis of transcriptomic data from L-FoxO1 and L-DBD livers has, in fact, uncovered a variety of cellular processes influenced differentially by co-regulatory *versus* direct transcriptional effects of FoxO1. Furthermore, the transcriptomic signature of FoxO1-DBD differs greatly in the fasted and refed states. In the refed state, surprisingly, nearly all of the most significantly altered biological processes relate to the immune system. These include elements both of innate immunity, such as the acute-phase response and Toll-like receptor signaling, as well as adaptive immunity, such as antigen processing and presentation. Although hepatocytes are the source of many acute-phase reactants, including serum amyloid A proteins [408], two of the genes for which are significantly altered in L-FoxO1 *vs.* L-DBD livers, the changes noted in expression levels of class II histocompatibility-complex components are less easily explained. As the RNA samples used for RNA-seq were drawn from whole liver, it is possible that hepatocellular FoxO1 activity influences the population and/or behavior of immune-system cells in the liver [409, 410]. However, it has also been reported that select populations of hepatocytes can induce expression MHC class II under stress conditions and function as antigen-presenting cells [411]. FoxO1 has indeed previously been implicated in the regulation of inflammatory pathways in the hepatocyte [412] and other cell types [413, 414]. Whether these actions of FoxO1 represent co-regulatory activities, and whether they relate in particular to adaptive immunity in the liver, however, have not previously been studied.

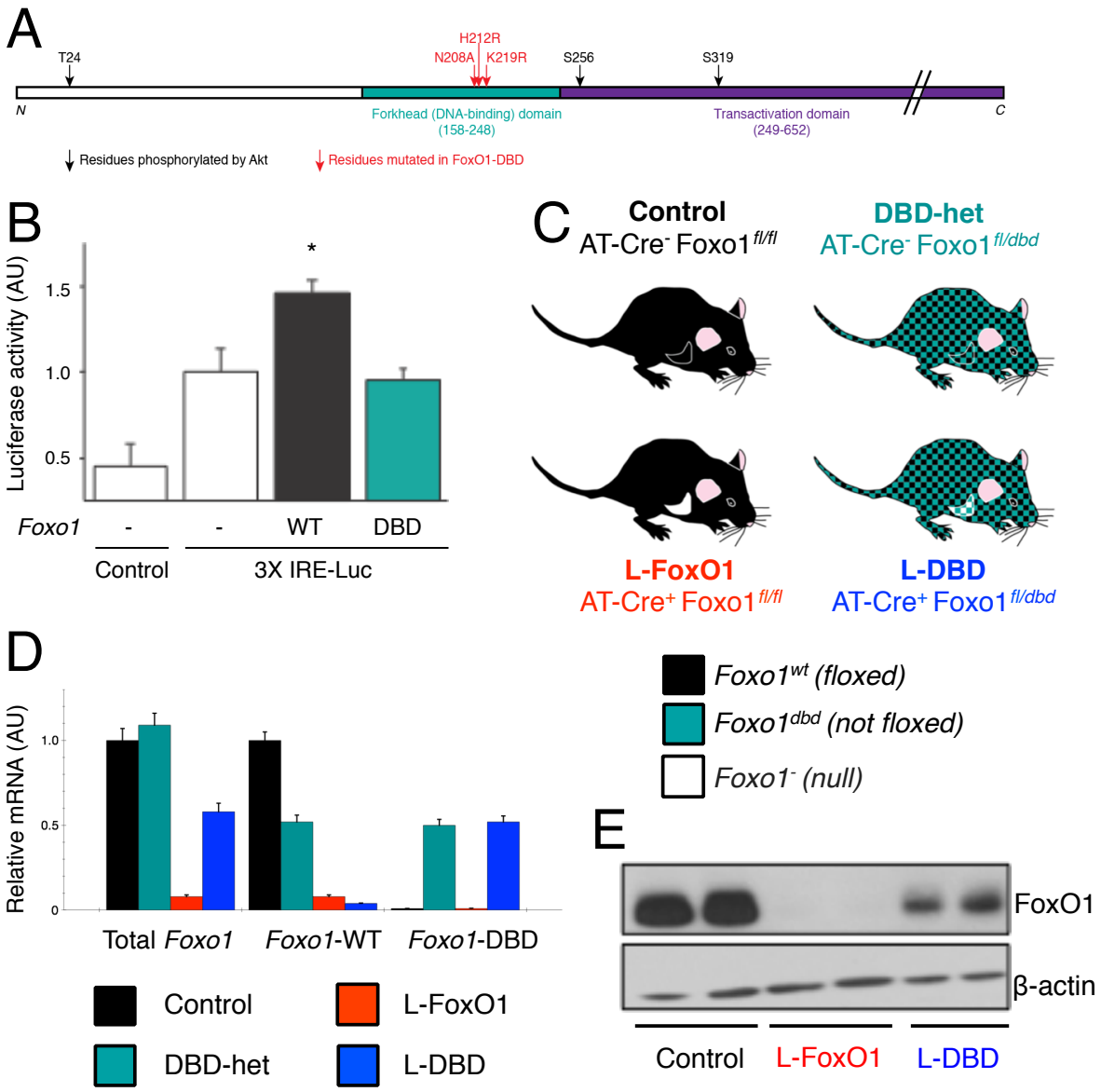
With respect to the fasted state, the majority of significantly different gene-ontological categories relate directly or indirectly to the metabolism of sterols and their hormonal derivatives. As previously mentioned, we already have elucidated FoxO1's role in the regulation of bile acid and cholesterol metabolism [168]. Very little prior evidence exists, however, linking FoxO1 to the metabolism of other sterol derivatives. Nine of the genes significantly altered in L-FoxO1 vs. L-DBD are represented in the KEGG steroid hormone metabolism pathway: *Cyp17a1*, *Cyp2b10*, *Cyp2c70*, *Cyp7b1*, *Hsd17b6*, *Hsd3b5*, *Sc4mol*, *Ugt2b37*, and *Ugt2b38*. A potentially notable candidate gene among these is *Hsd3b5*, 3β-hydroxysteroid dehydrogenase, type 5. The regulation of the expression of this gene by FoxO1 appears multifaceted, for although its expression is increased in L-FoxO1 liver relative to control, it is also strongly repressed in L-DBD liver, suggesting that FoxO1-DBD acts to repress it, perhaps even to a greater extent than can FoxO1-WT. Hsd3b5, which is expressed only in the male liver, is able to catalyze the catabolism of the potent androgen 5α-dihydrotestosterone, but little else is known about its role *in vivo* [415, 416]. Despite the mystery surrounding this gene and its product, however, various lines of evidence suggest a potentially important place for it in the regulation of nutrient metabolism in the liver. For example, in a comparison of the transcriptomic profile of diabetic (*db/db*) *versus* control liver by RNA-seq, *Hsd3b5* was the second most highly downregulated of all transcripts meeting the study's inclusion criteria [417].

If FoxO1 does indeed act as a co-repressor of *Hsd3b5*, it would be expected that heightened FoxO1 activity in the diabetic liver would serve to suppress its expression even more strongly than usual. Evidence of this sort of regulation can be found in a microarray study of livers deficient in Akt1 and Akt2 ("DLKO" mouse) and Akt1/2 as well as FoxO1 ("TLKO" mouse) [167]. *Hsd3b5* was by far the most strongly suppressed gene in the absence of Akt1/2 in the fed state (500-fold decreased relative to control) and was the fourth most highly suppressed in the fasting state (15-fold decreased relative to control). Concomitant deletion of FoxO1, however, completely normalized *Hsd3b5* expression in the fed state; in the fasted state, *Hsd3b5* became the fourteenth most highly *upregulated* gene. Thus, it appears that not only does FoxO1 serve dominantly to inhibit the expression of *Hsd3b5*, but also that FoxO1 mediates this inhibition in a co-regulatory fashion. That *Hsd3b5* expression is evidently so tightly controlled by insulin

signaling via an Akt-FoxO1-dependent pathway substantiates the potential importance of this heretofore-obscure gene in the regulation of hepatic metabolism.

Section 4.4

CHAPTER 4 FIGURES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T24
H212R
N208A
K219R
S256
S319
N
Forkhead (DNA-binding) domain
(158-248)
Transactivation domain
(249-652)
C
Residues phosphorylated by Akt
Residues mutated in FoxO1-DBD
B
Luciferase activity (AU)
1.5
1.0
0.5
*
Foxo1
-
-
WT
DBD
Control
3X IRE-Luc
C
Control
AT-Cre- Foxo1fl/fl
DBD-het
AT-Cre- Foxo1fl/dbd
L-FoxO1
AT-Cre+ Foxo1fl/fl
L-DBD
AT-Cre+ Foxo1fl/dbd
D
Foxo1wt (floxed)
Foxo1dbd (not floxed)
Foxo1- (null)
Relative mRNA (AU)
1.0
0.5
0
Total Foxo1
Foxo1-WT
Foxo1-DBD
Control
L-FoxO1
DBD-het
L-DBD
E
FoxO1
β-actin
Control
L-FoxO1
L-DBD

Figure 4.1

Figure 4.1. Generation and Characterization of the *Foxo1*^{dbd} Allele

(A) Schematic diagram of the FoxO1 primary sequence identifying the residues mutated in *Foxo1*^{dbd}.

(B) Reporter-gene assay in 293 cells transfected with either *Foxo1*^{wt}, *Foxo1*^{dbd}, or empty vector as well as with either 3X IRE-Luc reporter construct or control. Data represent mean ± SEM. * $p < 0.05$ relative to control by Tukey's post-hoc analysis following one-way ANOVA.

(C) Schematic diagram of mouse models used in this study.

(D) Liver qPCR using allele-specific primers for total *Foxo1*, *Foxo1*^{wt} or *Foxo1*^{dbd}. Data represent mean ± SEM.

(E) Western blot of liver extracts from fasted mice.

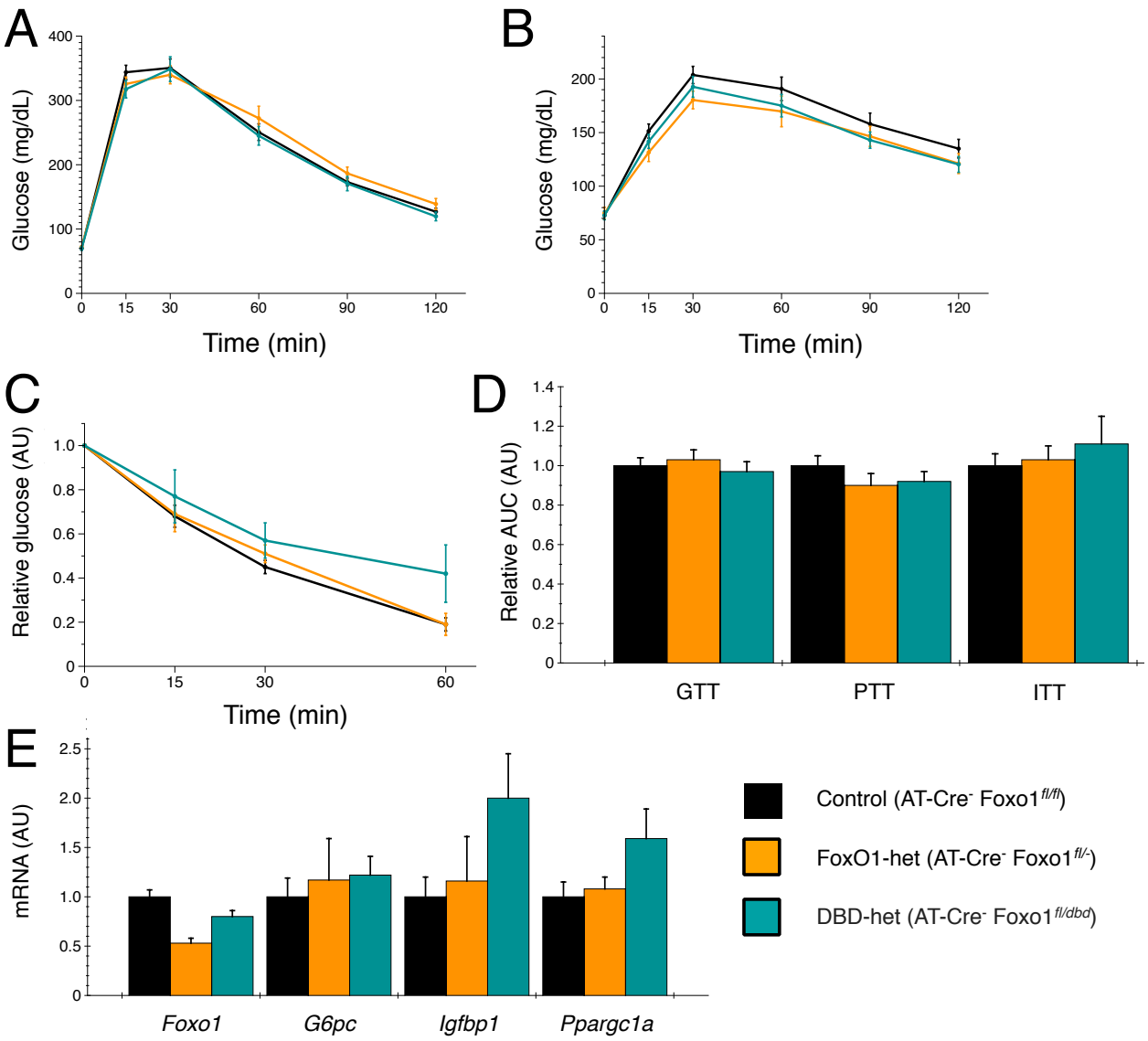

A
Glucose (mg/dL)
Time (min)
B
Glucose (mg/dL)
Time (min)
C
Relative glucose (AU)
Time (min)
D
Relative AUC (AU)
GTT
PTT
ITT
E
mRNA (AU)
Foxo1
G6pc
Igfbp1
Ppargc1a
Control (AT-Cre- Foxo1fl/fl)
FoxO1-het (AT-Cre- Foxo1fl/-)
DBD-het (AT-Cre- Foxo1fl/dbd)

Figure 4.2

Figure 4.2. Metabolic Characterization of FoxO1- and DBD-het Mice

(A-B) Glucose (A) and pyruvate tolerance tests (B) in overnight-fasted mice (N≥7 for all genotypes).

(C) Insulin tolerance test in 5-hr-fasted mice (N = 5-6 for all genotypes).

(D) Quantification of the area under the curve (AUC) for the results in A-C.

(E) Gene expression levels in fasted livers assessed by qPCR.

All mice were reared on a chow diet and studies were performed at 16-20 weeks of age, Data represent mean ± SEM.

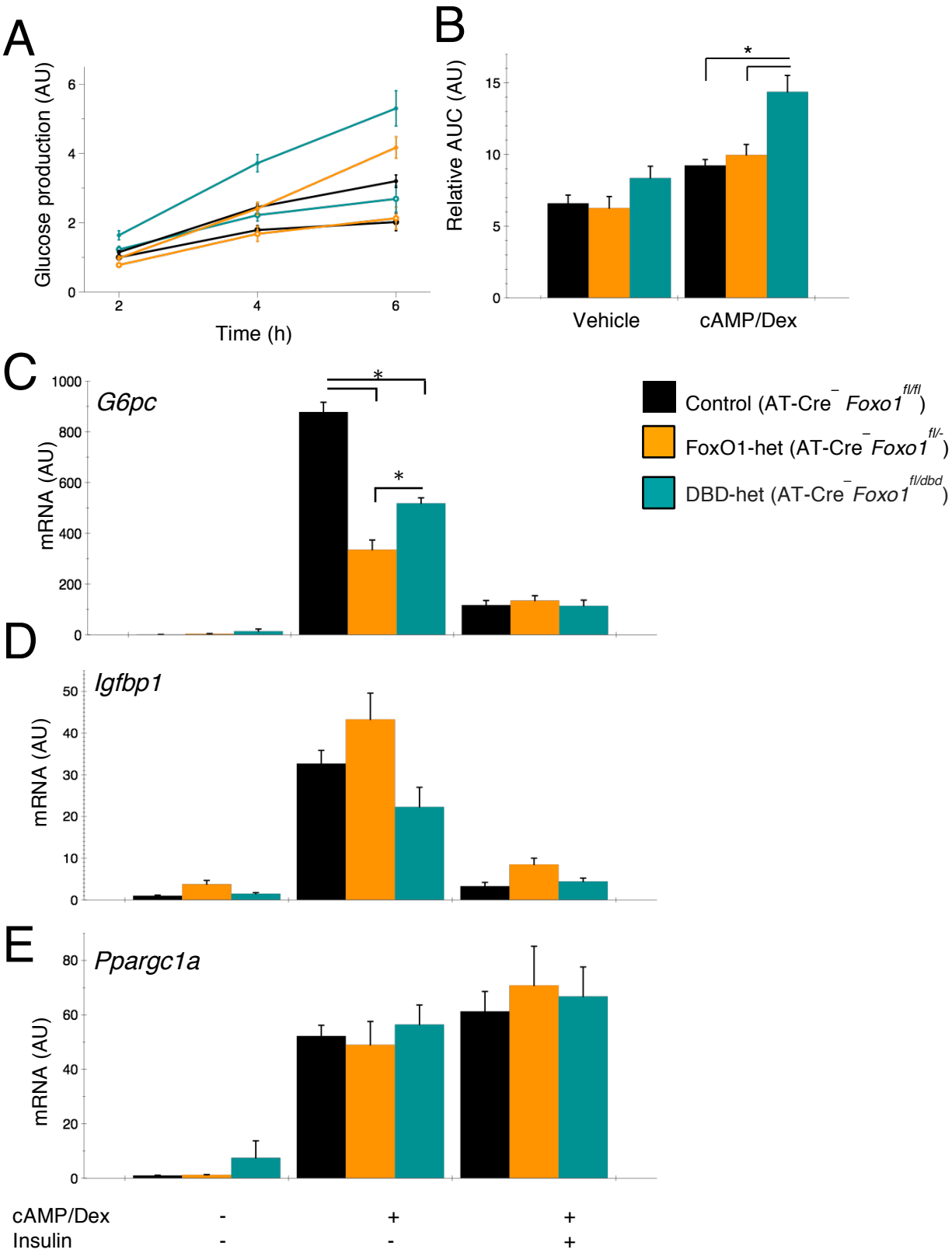

A
Glucose production (AU)
Time (h)
B
Relative AUC (AU)
Vehicle
cAMP/Dex
C
G6pc
mRNA (AU)
Control (AT-Cre⁻ Foxo1fl/fl)
FoxO1-het (AT-Cre⁻ Foxo1fl/-)
DBD-het (AT-Cre⁻ Foxo1fl/dbd)
D
Igfbp1
mRNA (AU)
E
Ppargc1a
mRNA (AU)
cAMP/Dex - + +
Insulin - - +

Figure 4.3

Figure 4.3. Glucose Production in Foxo1- and DBD-Het Primary Hepatocytes

Glucose production assay performed in medium containing either vehicle or 0.1 mM CPT- cAMP + 1 μM dexamethasone (cAMP/dex).

(A) Aliquots of medium were collected at the indicated time points and assayed for glucose content as indicated in the experimental procedures.

(B) Area under the curve quantified from the data in panel A.

(C-E) Expression of fasting-inducible genes in primary hepatocytes treated as either above, or including 100 nM insulin with cAMP/dex.

Data in (A-B) represent mean ± SEM and in (C-E) are representative of three independent experiments performed in triplicate.

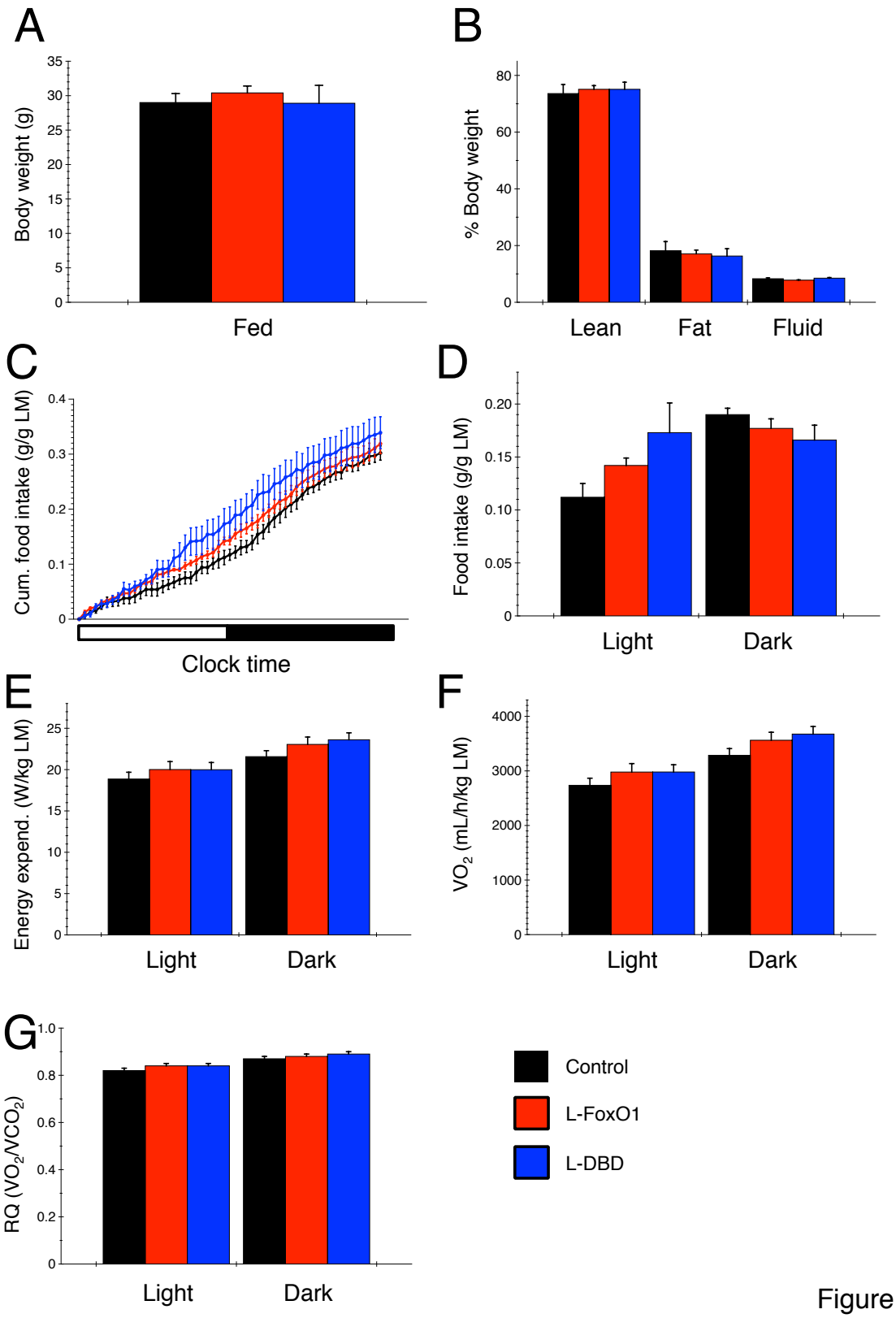

A
Body weight (g)
35
30
25
20
15
10
5
0
Fed
B
% Body weight
80
60
40
20
0
Lean
Fat
Fluid
C
Cum. food intake (g/g LM)
0.4
0.3
0.2
0.1
0
Clock time
D
Food intake (g/g LM)
0.20
0.15
0.10
0.05
0
Light
Dark
E
Energy expend. (W/kg LM)
25
20
15
10
5
0
Light
Dark
F
VO2 (mL/h/kg LM)
4000
3000
2000
1000
0
Light
Dark
G
RQ (VO2/VCO2)
1.0
0.8
0.6
0.4
0.2
0
Light
Dark
Control
L-FoxO1
L-DBD

Figure 4.4

Figure 4.4. Study of Whole-Body Metabolism by Indirect Calorimetry

(A-B) Body weight (A) and body composition (B) in fed mice

(C-D) Cumulative 24-h food intake (C) broken down by light *vs.* dark cycles (D), normalized to lean body weight.

(E) Energy expenditure normalized to lean body weight

(F) Oxygen consumption rate normalized to lean body weight

(G) Respiratory quotient (RQ), calculated as VO_2/VCO_2

N = 5-6 for all genotypes. Data represent mean ± SEM.

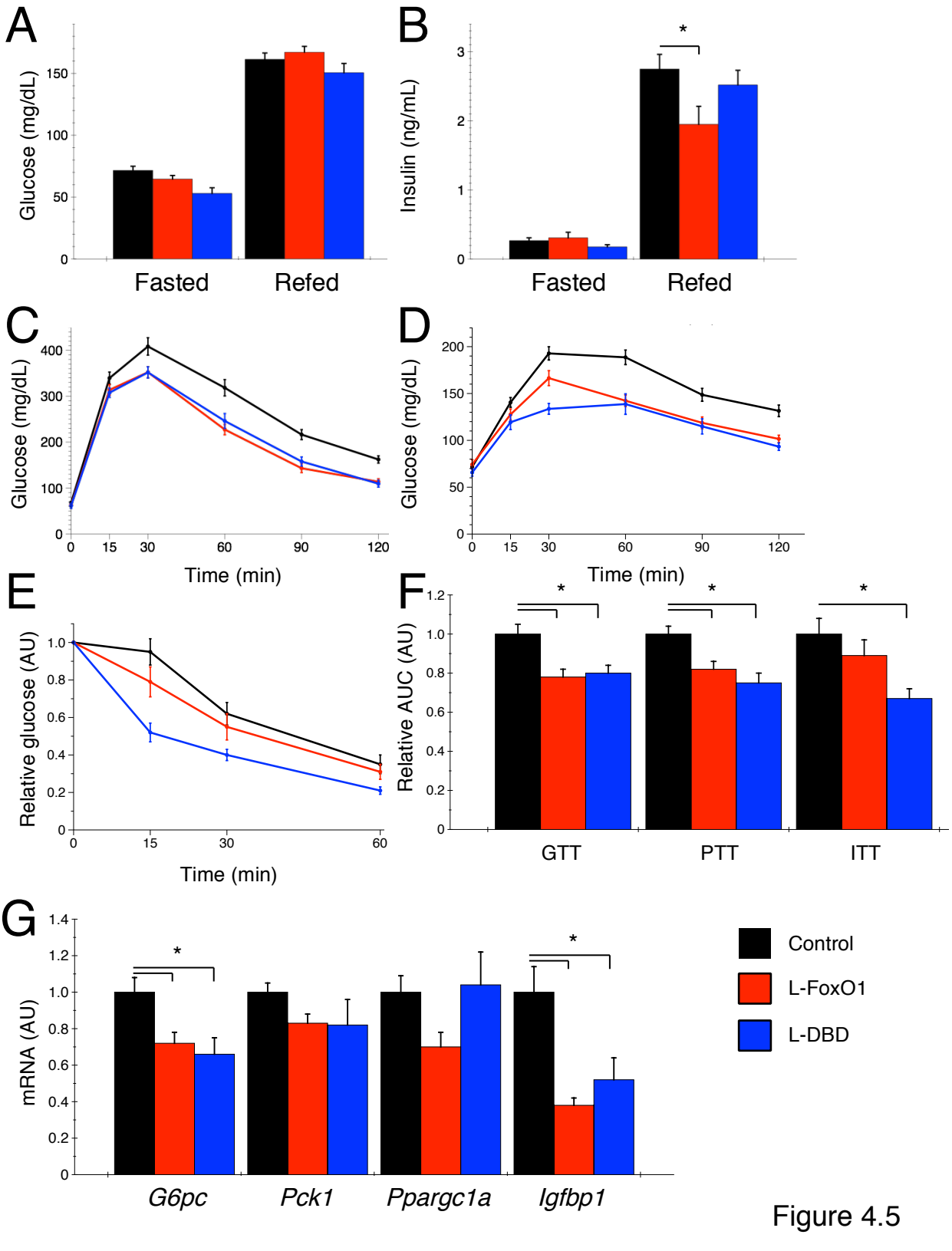

A
Glucose (mg/dL)
150
100
50
0
Fasted
Refed
B
Insulin (ng/mL)
3
2
1
0
*
Fasted
Refed
C
Glucose (mg/dL)
400
300
200
100
0
0
15
30
60
90
120
Time (min)
D
Glucose (mg/dL)
200
150
100
50
0
0
15
30
60
90
120
Time (min)
E
Relative glucose (AU)
1.0
0.8
0.6
0.4
0.2
0
0
15
30
60
Time (min)
F
Relative AUC (AU)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
*
GTT
PTT
ITT
G
mRNA (AU)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
G6pc
Pck1
Ppargc1a
Igfbp1
Control
L-FoxO1
L-DBD

Figure 4.5

Figure 4.5. Glucose Metabolism in L-FoxO1 and L-DBD Mice

(A-B) Glucose (A) and insulin (B) in mice fasted overnight or re-fed for 4 hr.

(C-D) Glucose (C) and pyruvate (D) tolerance tests in overnight-fasted mice.

(E) Insulin tolerance test in 5-hr-fasted mice.

(F) Quantification of the area under the curve (AUC) for the results in (C-E).

(G) Gene expression levels in fasted livers assessed by qPCR.

All mice were reared on a chow diet and studies were performed at 16-20 weeks of age, $N \geq 9$ for all genotypes in all experiments. Data represent mean ± SEM. * $p < 0.05$ by Tukey's post-hoc analysis following one-way ANOVA.

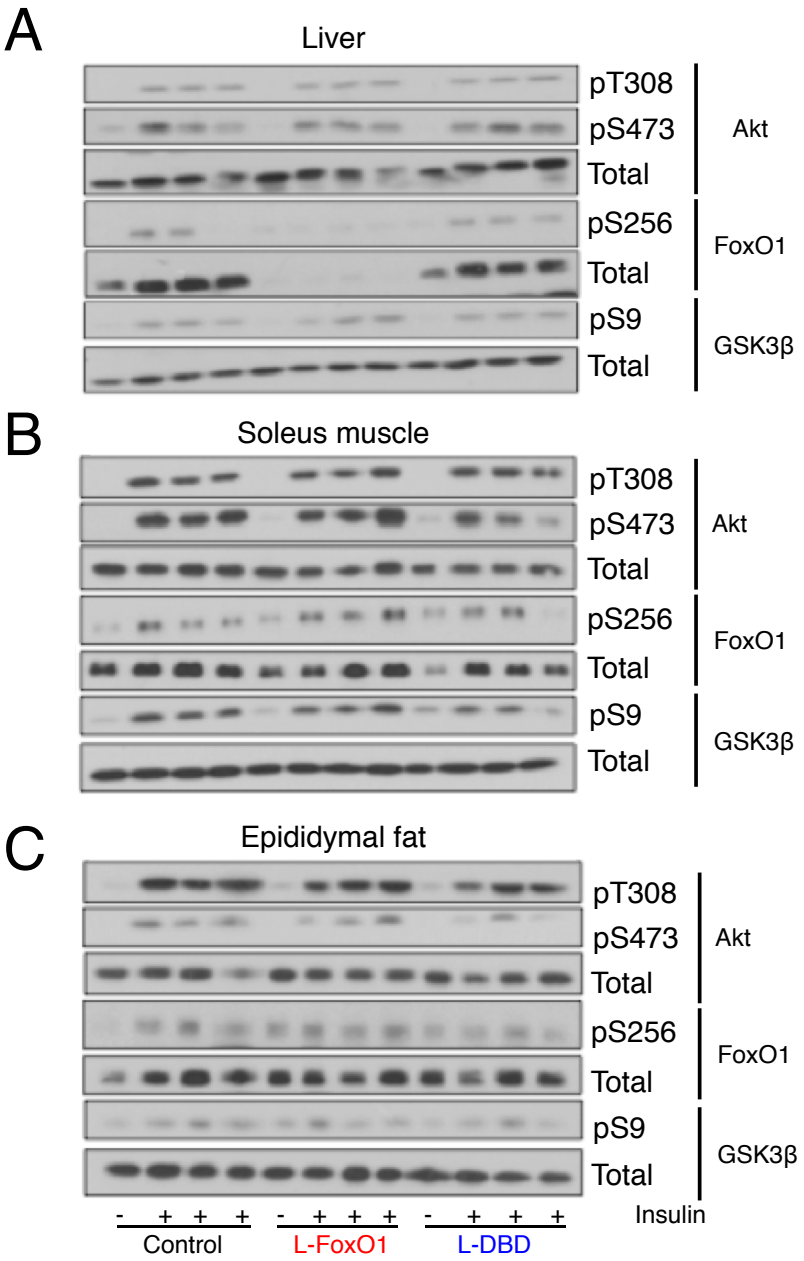

A
Liver
pT308
pS473
Total
Akt
pS256
Total
FoxO1
pS9
Total
GSK3β
B
Soleus muscle
pT308
pS473
Total
Akt
pS256
Total
FoxO1
pS9
Total
GSK3β
C
Epididymal fat
pT308
pS473
Total
Akt
pS256
Total
FoxO1
pS9
Total
GSK3β
- + + + - + + + - + + +
Insulin
Control
L-FoxO1
L-DBD

Figure 4.6

Figure 4.6. Insulin-Dependent Akt and GSK3β Phosphorylation

(A-C) Western blot analysis of insulin-induced protein phosphorylation in liver (A), soleus muscle (B), and epididymal fat (C) after IV saline or insulin injection in overnight-fasted or 4-hr- refed mice (n = 3 for insulin and 1 for saline).

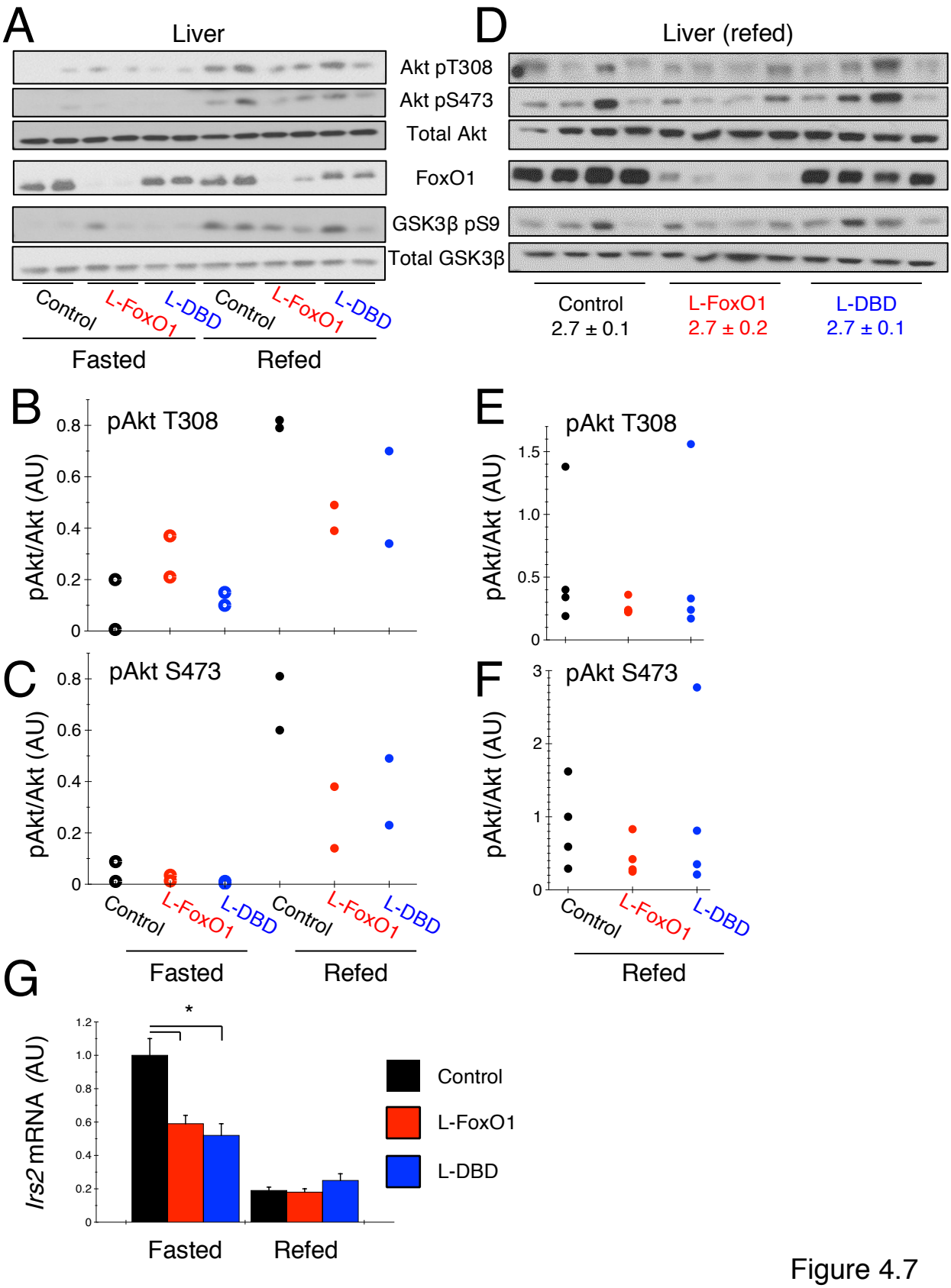

A
Liver
Akt pT308
Akt pS473
Total Akt
FoxO1
GSK3β pS9
Total GSK3β
Control
L-FoxO1
L-DBD
Control
L-FoxO1
L-DBD
Fasted
Refed
D
Liver (refed)
Control
2.7 ± 0.1
L-FoxO1
2.7 ± 0.2
L-DBD
2.7 ± 0.1
B
pAkt T308
pAkt/Akt (AU)
0
0.2
0.4
0.6
0.8
C
pAkt S473
pAkt/Akt (AU)
0
0.2
0.4
0.6
0.8
Control
L-FoxO1
L-DBD
Control
L-FoxO1
L-DBD
Fasted
Refed
E
pAkt T308
pAkt/Akt (AU)
0
0.5
1.0
1.5
F
pAkt S473
pAkt/Akt (AU)
0
1
2
3
Control
L-FoxO1
L-DBD
Refed
G
*
Irs2 mRNA (AU)
0
0.2
0.4
0.6
0.8
1.0
1.2
Control
L-FoxO1
L-DBD
Fasted
Refed

Figure 4.7

Figure 4.7. Hepatic Insulin Signaling

(A) Western blot of insulin-induced protein phosphorylation in fasted or 4 hr-re-fed mice of each genotype. Each lane represents three pooled whole-liver extracts from a single cohort.

(B-C) Densitometric analysis of Akt phosphorylation in Western blots from (A). Each point represents one of the two lanes for each genotype × feeding state combination.

(D) Western blot of insulin-induced protein phosphorylation in 4 hr-refed mice matched for mean insulin concentration, noted below each genotype as mean insulin ± SEM. Each lane represents one liver extract from the indicated genotype.

(E-F) Densitometric analysis of Akt phosphorylation in Western blots from (D). Each point represents one of the corresponding bands in (D).

(G) qPCR analysis of *Irs2* expression in either fasted or 4-hr-re-fed mice of each genotype ($N \geq 7$ for each genotype).

Data represent mean ± SEM. * $p < 0.05$ by Tukey's post-hoc analysis following one-way ANOVA.

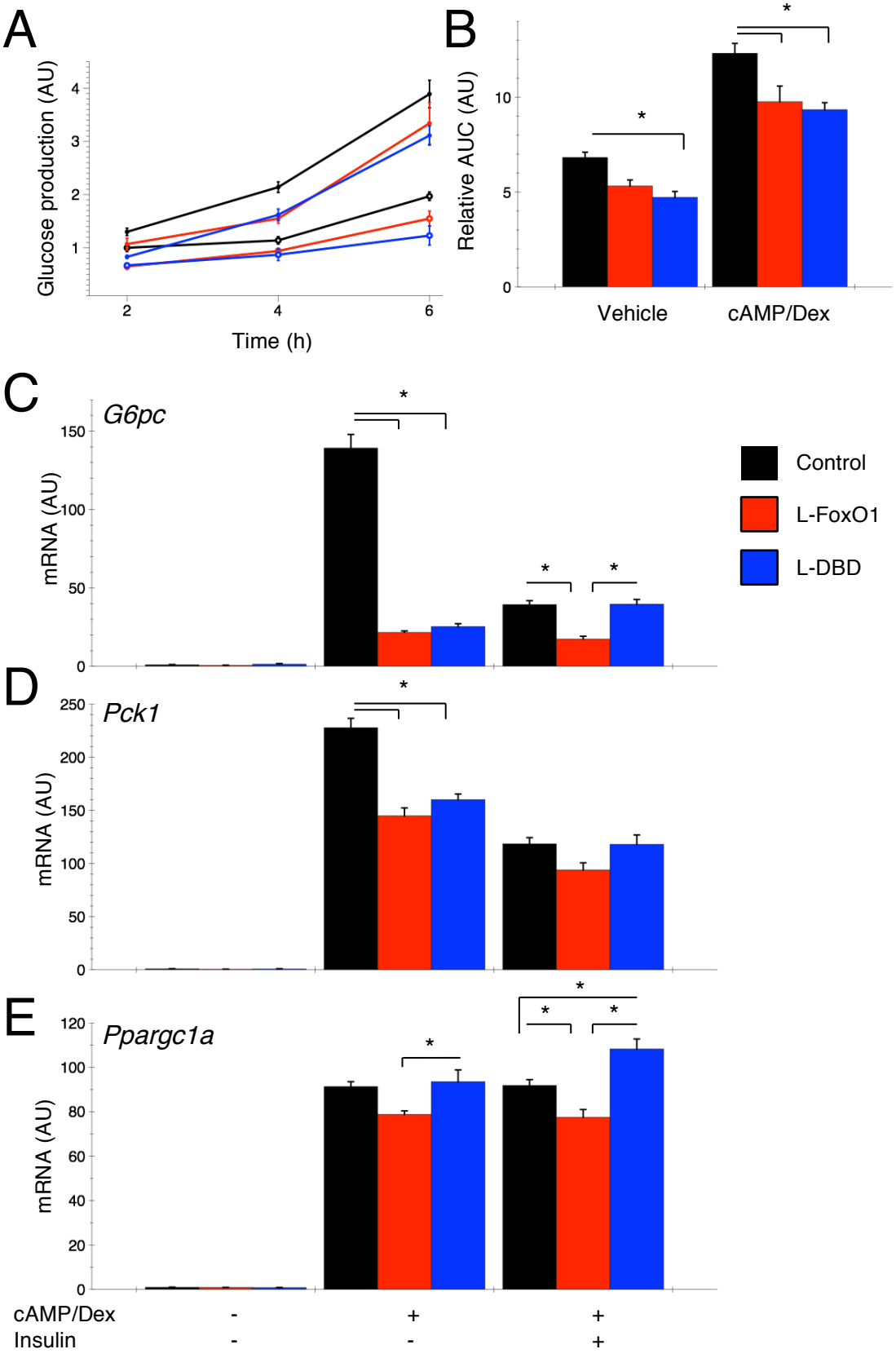

A
Glucose production (AU)
Time (h)
B
Relative AUC (AU)
Vehicle
cAMP/Dex
C
G6pc
mRNA (AU)
D
Pck1
mRNA (AU)
E
Ppargc1a
mRNA (AU)
Control
L-FoxO1
L-DBD
cAMP/Dex
Insulin

Figure 4.8

Figure 4.8. Glucose Production in L-FoxO1 and L-DBD Primary Hepatocytes

(A) Glucose production assays in cells incubated with glucose-production medium supplemented with (filled circles) or without (open circles) 0.1 M CPT-cAMP and 1 μM dexamethasone for 5 hr.

(B) Quantification of the AUC from the data in panel A. Data in (A-B) represent mean ± SEM of three independent experiments performed in triplicate.

(C-E) qPCR of *G6pc, Pck1*, and *Ppargc1a* levels in the presence or absence of cAMP/dex and insulin. Data are presented as mean ± SEM of a representative experiment of three, each performed in triplicate.

* $p < 0.05$ by Tukey's post-hoc analysis following one-way ANOVA.

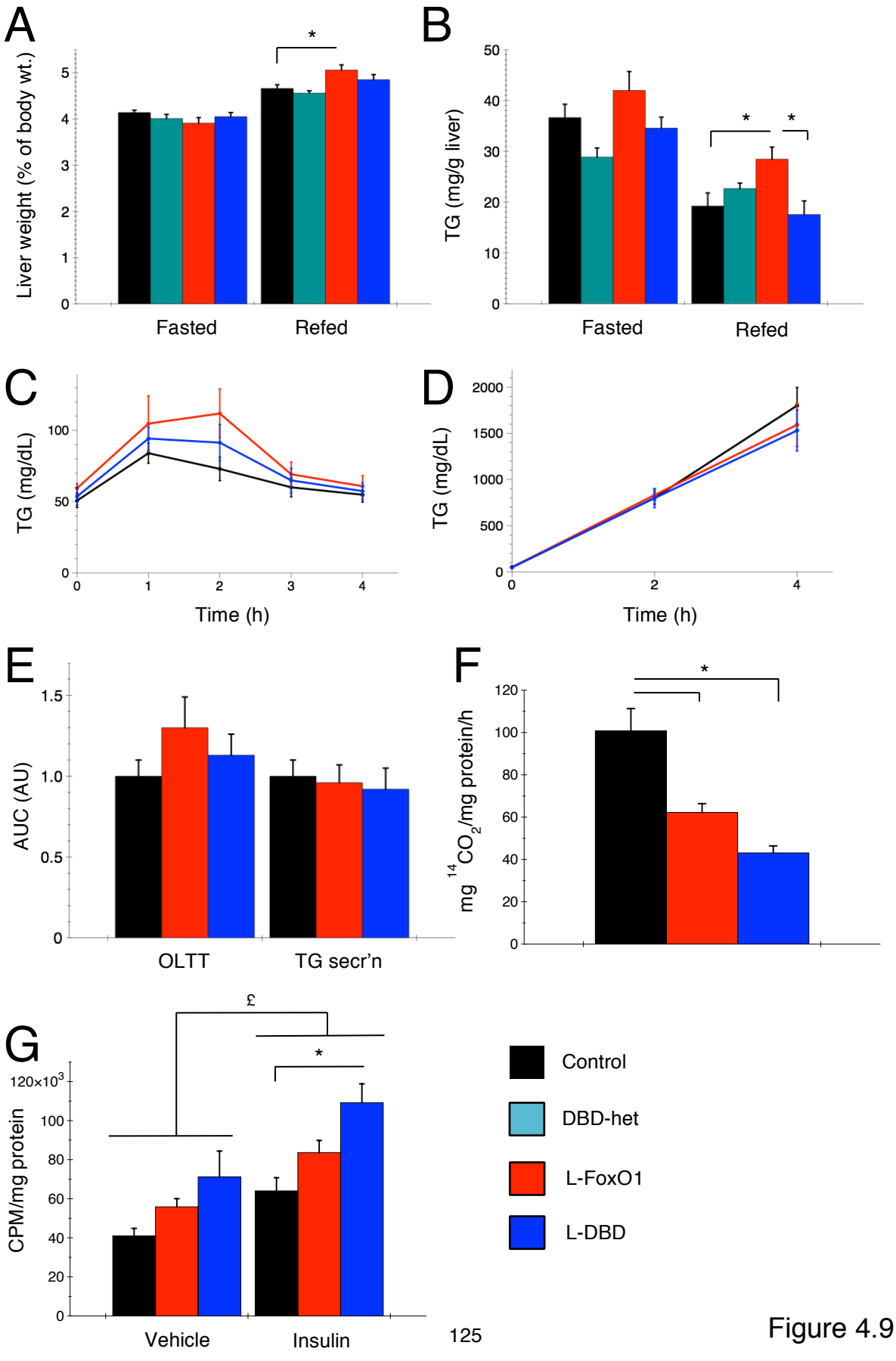

A
Liver weight (% of body wt.)
0
1
2
3
4
5
*
Fasted
Refed
B
TG (mg/g liver)
0
10
20
30
40
50
*
*
Fasted
Refed
C
TG (mg/dL)
0
50
100
0
1
2
3
4
Time (h)
D
TG (mg/dL)
0
500
1000
1500
2000
0
2
4
Time (h)
E
AUC (AU)
0
0.5
1.0
1.5
OLTT
TG secr'n
F
mg $^{14}CO_2$/mg protein/h
0
20
40
60
80
100
120
*
G
CPM/mg protein
0
20
40
60
80
100
120×10^3
£
*
Vehicle
Insulin
Control
DBD-het
L-FoxO1
L-DBD

Figure 4.9

Figure 4.9. Lipid Metabolism in Mice and Primary Hepatocytes

(A) Liver weight relative to body weight. Mice of each genotype were fasted overnight or fasted overnight and refed for 4 hr (N ≥ 10 for each genotype).

(B) Liver TG content in fasted or 4-hr-refed mice, normalized to total liver weight (N ≥ 6 for each genotype).

(C) Oral lipid tolerance test (OLTT) in 5-hr-fasted mice, administered by giving olive oil p.o. and drawing blood at the indicated time points (N = 6-9 for each genotype).

(D) TG secretion assay in 5-hr-fasted mice (N ≥ 5 for each genotype).

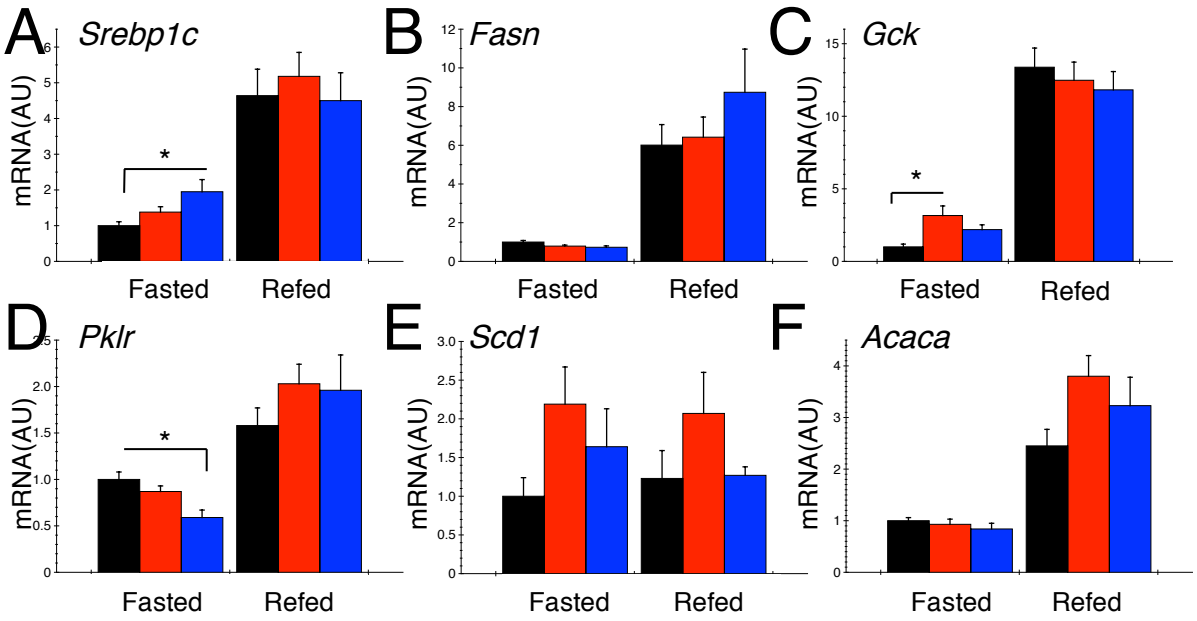
(E) Area under the curve (AUC) of OLTT and TG secretion. Data are normalized to a representative control sample for each procedure.

(F) Fatty acid oxidation in primary hepatocytes from control, L-FoxO1, and L-DBD mice. Data shown as average of three independent experiments performed in triplicate.

Data in (A)-(F) represent means ± SEM. * $p < 0.05$ by Tukey's post-hoc analysis following one-way ANOVA.

(G) *De novo* TG synthesis in primary hepatocytes isolated from control, L-FoxO1, and L-DBD mice. Data shown are mean ± Satterthwaite-corrected SEM of three independent experiments performed in triplicate. £ $p < 0.05$ for main effect as assessed by two-way ANOVA and * $p < 0.05$ using Bonferroni's post-hoc analysis.

Lipogenic genes (A-F)



Bile-acid metabolic genes (G-J)

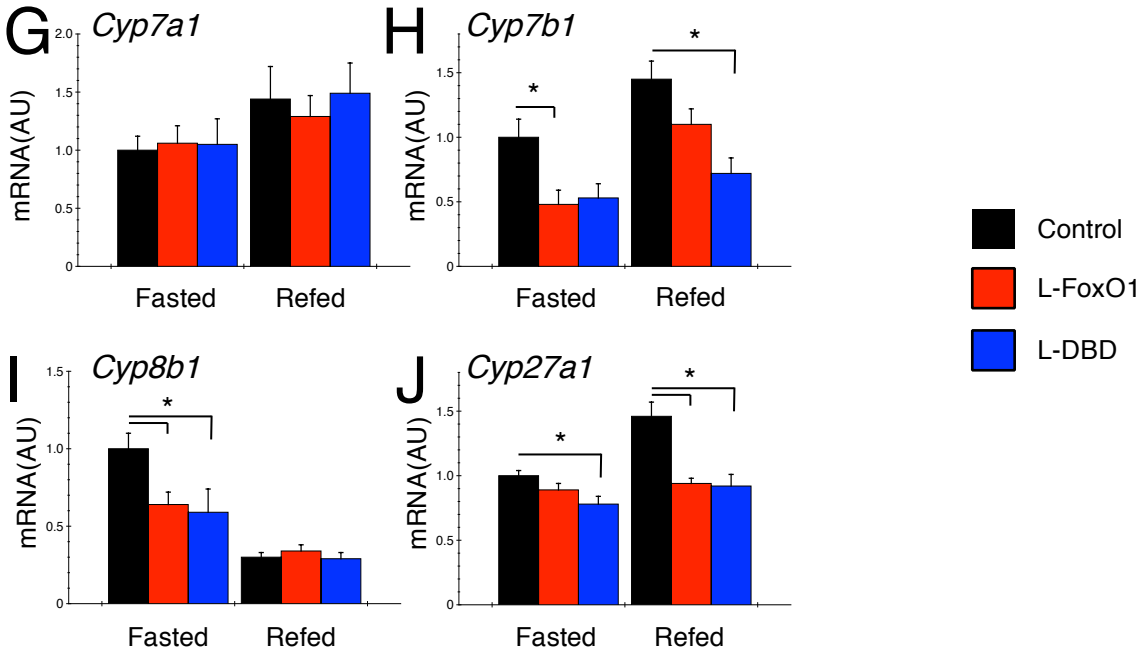


Figure 4.10

Figure 4.10. Expression of Genes Related to Hepatic Lipid Metabolism

(A-F) Expression of lipogenic genes in livers from overnight-fasted or 4-hr-re-fed mice

(G-J) Expression of bile-acid metabolic genes in livers from overnight-fasted or 4-hr-re-fed mice.

Data represent mean ± SEM, $N \geq 7$ for each genotype. * $P < 0.05$ as assessed by Tukey's post-hoc analysis following one-way ANOVA.

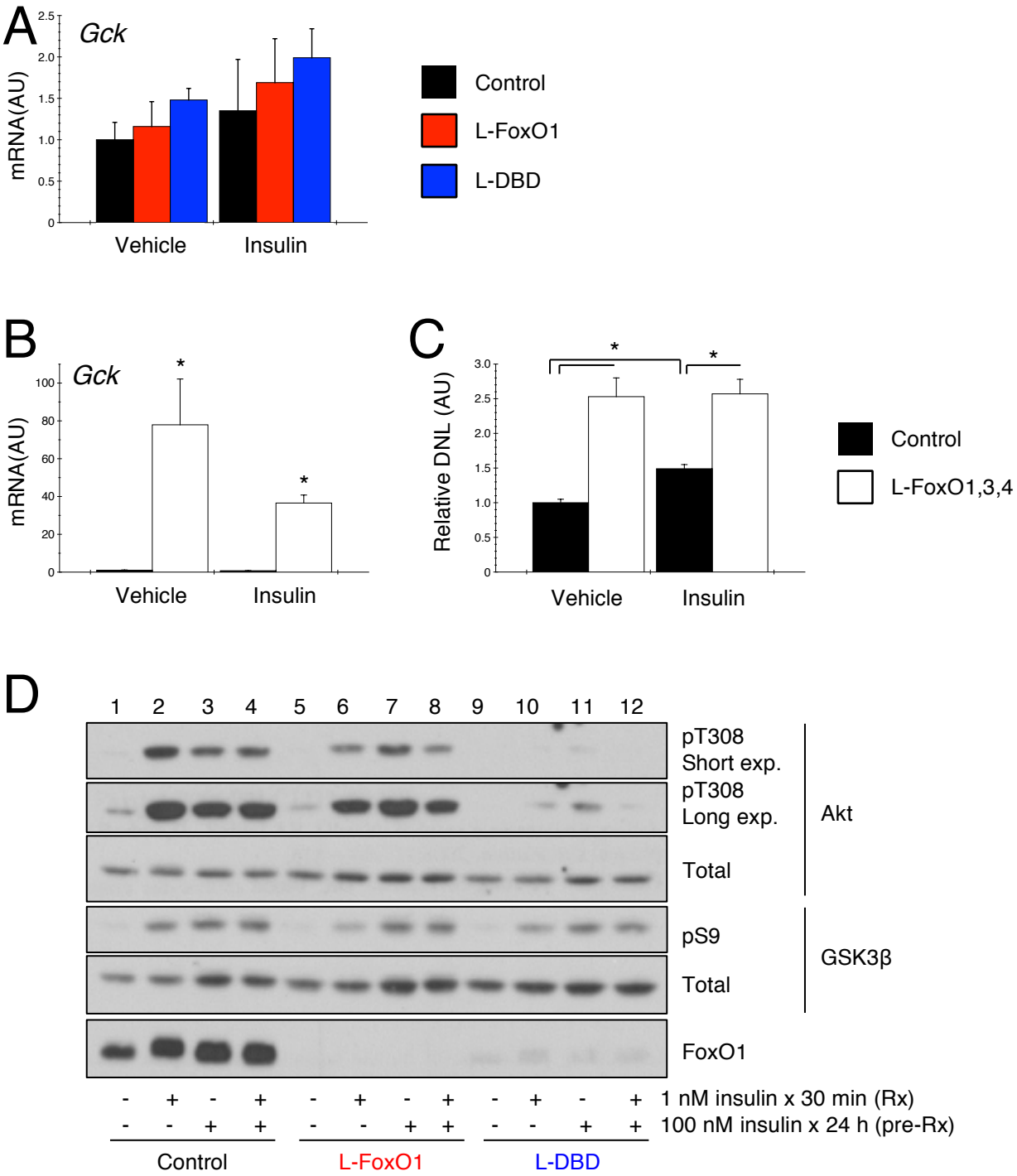

A
Gck
mRNA(AU)
2.5
2.0
1.5
1.0
0.5
0
Vehicle
Insulin
Control
L-FoxO1
L-DBD
B
Gck
mRNA(AU)
100
80
60
40
20
0
*
*
Vehicle
Insulin
C
Relative DNL (AU)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
Vehicle
Insulin
Control
L-FoxO1,3,4
D
1 2 3 4 5 6 7 8 9 10 11 12
pT308
Short exp.
pT308
Long exp.
Total
Akt
pS9
Total
GSK3β
FoxO1
- + - + - + - + - + - + 1 nM insulin x 30 min (Rx)
- - + + - - + + - - + + 100 nM insulin x 24 h (pre-Rx)
Control
L-FoxO1
L-DBD

Figure 4.11

Figure 4.11. Studies on the Mechanism of Lipogenesis in Primary Hepatocytes

(A-B) Gene expression in primary hepatocytes of indicated genotype serum-starved overnight and then treated for 5 h with or without 10 nM insulin. Data in (A) represent the mean of two (insulin) or three (vehicle) independent experiments performed in triplicate ± Satterthwaite-corrected SEM and in (B) are mean ± SEM representative of two independent experiments performed in triplicate. * $p < 0.05$ by unpaired, two-tailed student's *t* test.

(C) *De novo* lipogenesis in primary hepatocytes isolated from control and L-FoxO1,3,4 mice. Data presented are mean ± SEM representative of two independent experiments performed in triplicate. * $p < 0.05$ by Bonferroni's post-hoc test following two-way ANOVA.

(D) Insulin signaling in primary hepatocytes treated with saline or with 1 nM insulin for 30 min following treatment for 24 hr with saline or 100 nM insulin. Data are representative of three independent experiments.

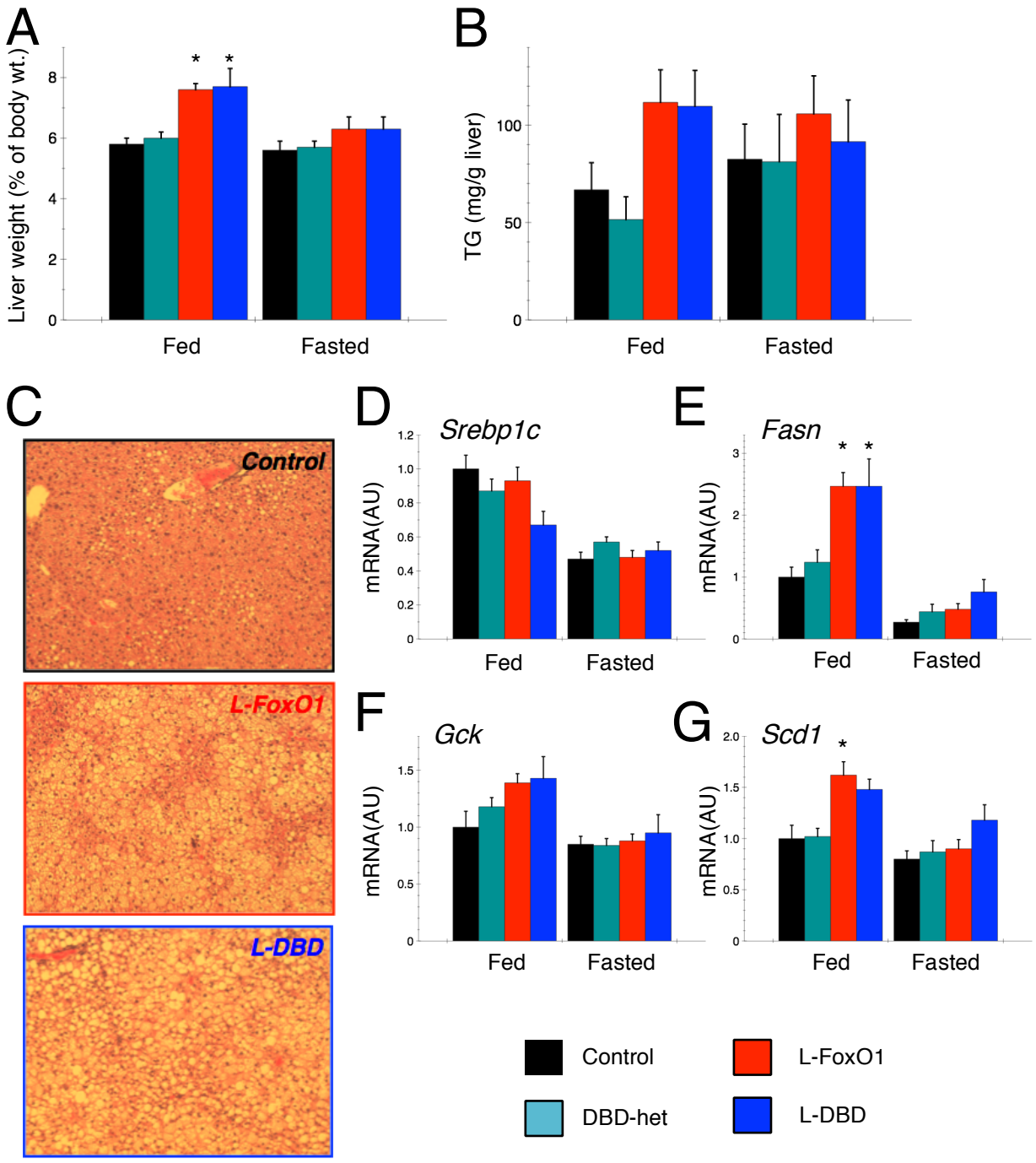

A
Liver weight (% of body wt.)
0
2
4
6
8
* *
Fed
Fasted
B
TG (mg/g liver)
0
50
100
Fed
Fasted
C
Control
L-FoxO1
L-DBD
D
Srebp1c
mRNA(AU)
0
0.2
0.4
0.6
0.8
1.0
1.2
Fed
Fasted
E
Fasn
mRNA(AU)
0
1
2
3
* *
Fed
Fasted
F
Gck
mRNA(AU)
0
0.5
1.0
1.5
Fed
Fasted
G
Scd1
mRNA(AU)
0
0.5
1.0
1.5
2.0
*
Fed
Fasted
Control
L-FoxO1
DBD-het
L-DBD

Figure 4.12

Figure 4.12. Metabolic Characterization of Mice on Western-Type Diet

(A-B) Liver weight relative to body weight (A) and liver TG content (B) in 5-hr-fasted or *ad libitum*-fed mice.

(C) Hematoxylin and eosin staining of liver sections from WTD-fed mice.

(D-G) qPCR measurements of hepatic *Srebp1c, Fasn, Gck*, and *Scd1* in *ad libitum*-fed or 5-hr-fasted mice.

Data represent means ± SEM ($N \geq 7$ for each genotype). * $p < 0.05$ by Tukey's post-hoc analysis following one-way ANOVA.

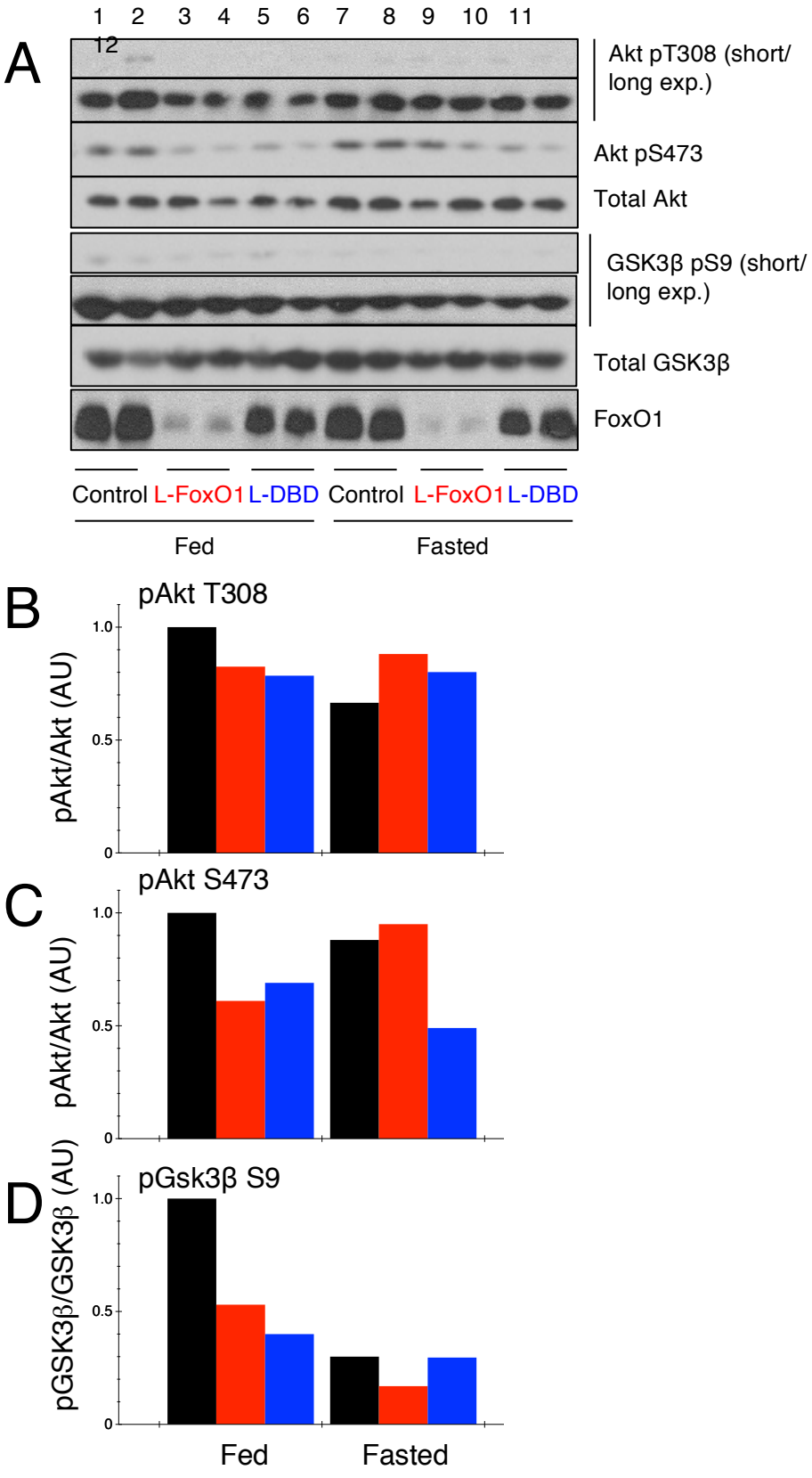

A
1 2 3 4 5 6 7 8 9 10 11 12
Akt pT308 (short/ long exp.)
Akt pS473
Total Akt
GSK3β pS9 (short/ long exp.)
Total GSK3β
FoxO1
Control L-FoxO1 L-DBD Control L-FoxO1 L-DBD
Fed
Fasted
B
pAkt T308
pAkt/Akt (AU)
1.0
0.5
0
C
pAkt S473
pAkt/Akt (AU)
1.0
0.5
0
D
pGsk3β S9
pGSK3β/GSK3β (AU)
1.0
0.5
0
Fed
Fasted

Figure 4.13

Figure 4.13. Studies of Liver Insulin Signaling in WTD-Reared Mice

(A) Western blot of livers from *ad-libitum* fed or 5-hr-fasted mice. Each lane represents pooled liver homogenate from three mice of the same cohort.

(B-D) Densitometric analysis of phospho-Akt T308 (B), S473 (C) and GSK3β S9 (D). Bars represent the average value of the two lanes for each group and are calculated as the ratio of phosphorylated to total protein and normalized to the fed control.

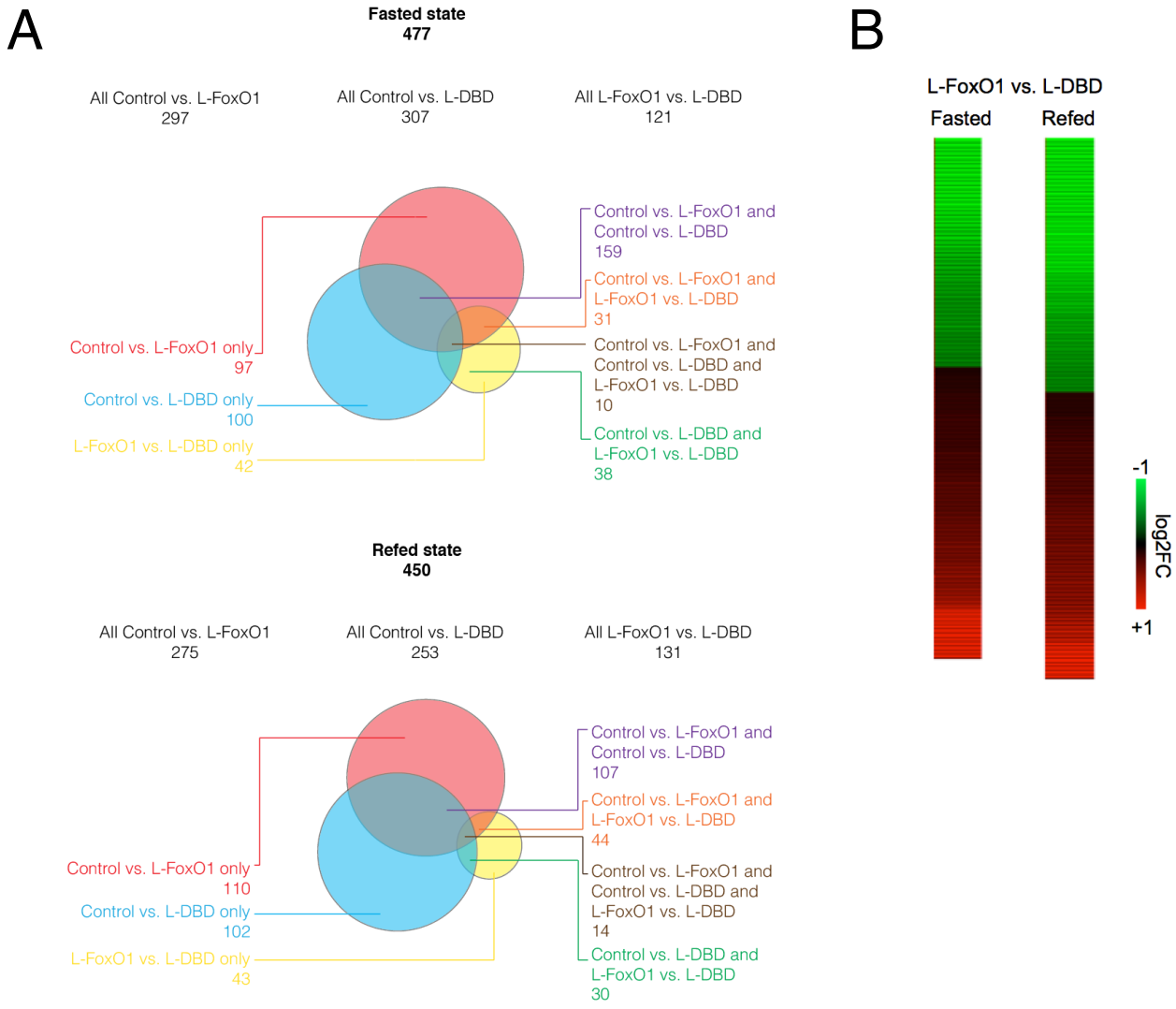

A
Fasted state
477
All Control vs. L-FoxO1
297
All Control vs. L-DBD
307
All L-FoxO1 vs. L-DBD
121
Control vs. L-FoxO1 only
97
Control vs. L-DBD only
100
L-FoxO1 vs. L-DBD only
42
Control vs. L-FoxO1 and
Control vs. L-DBD
159
Control vs. L-FoxO1 and
L-FoxO1 vs. L-DBD
31
Control vs. L-FoxO1 and
Control vs. L-DBD and
L-FoxO1 vs. L-DBD
10
Control vs. L-DBD and
L-FoxO1 vs. L-DBD
38
Refed state
450
All Control vs. L-FoxO1
275
All Control vs. L-DBD
253
All L-FoxO1 vs. L-DBD
131
Control vs. L-FoxO1 only
110
Control vs. L-DBD only
102
L-FoxO1 vs. L-DBD only
43
Control vs. L-FoxO1 and
Control vs. L-DBD
107
Control vs. L-FoxO1 and
L-FoxO1 vs. L-DBD
44
Control vs. L-FoxO1 and
Control vs. L-DBD and
L-FoxO1 vs. L-DBD
14
Control vs. L-DBD and
L-FoxO1 vs. L-DBD
30
B
L-FoxO1 vs. L-DBD
Fasted
Refed
-1
log2FC
+1

Figure 4.14

Figure 4.14. Transcriptomic Analysis of Livers by RNA-Seq

(A) Genotypic comparisons of the numbers of genes significantly altered ($p < 0.05$) in the fasted (upper) and re-fed (lower) states.

(B) Heat map illustrating the magnitude of differences in genes significantly altered in L-FoxO1 *vs.* L-DBD mice in the fasted (left) and re-fed (right) states.

Mechanistic predictions based on RNA-seq:

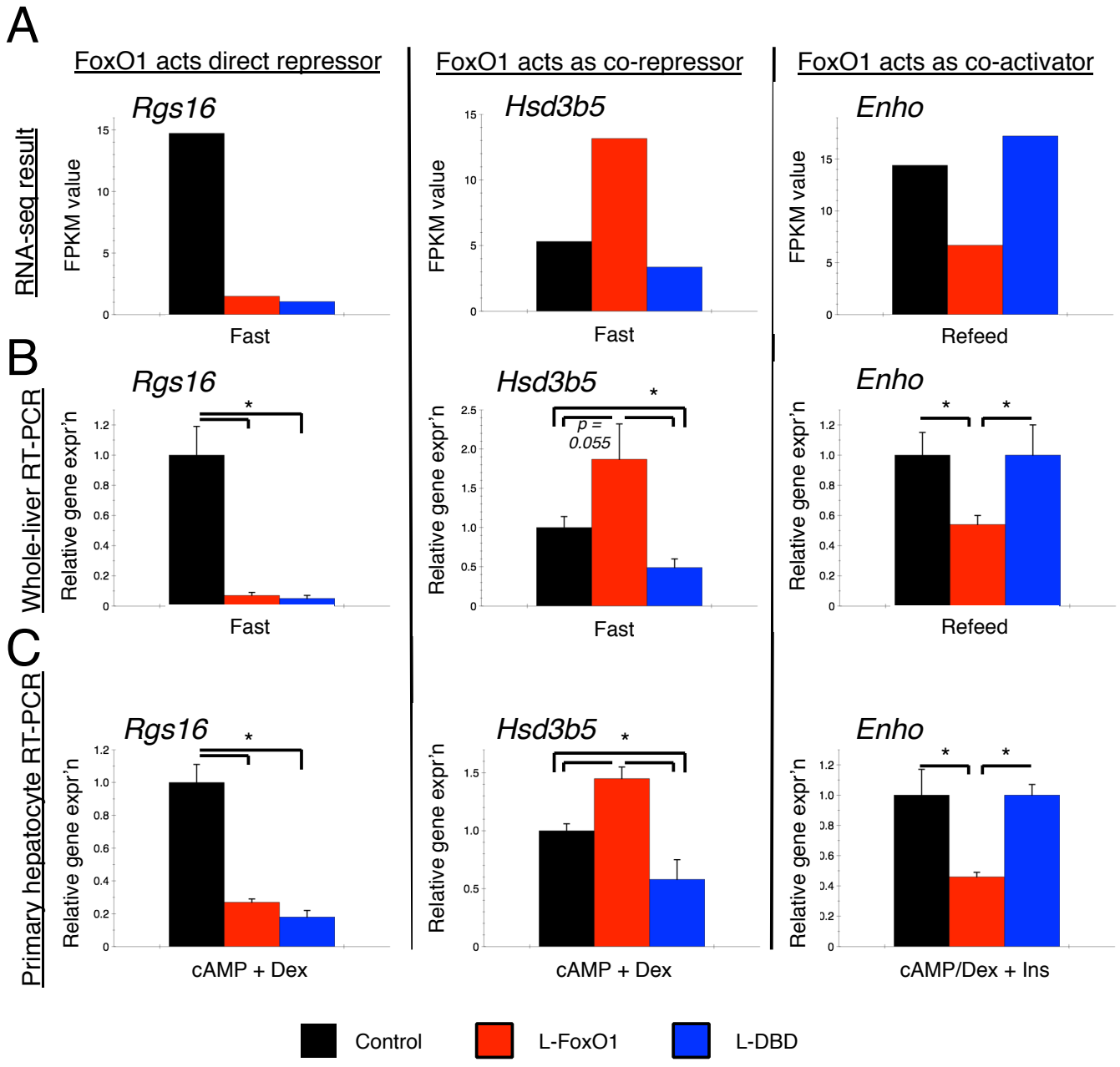


Figure 4.15

Figure 4.15. Validation of Selected RNA-seq Candidate Genes

(A) FPKM values of genes selected from RNA-seq to illustrate FoxO1's roles as direct transcriptional repressor (*Rgs16*, left column), co-repressor (*Hsd3b5*, middle column), and co-activator (*Enho*, right column).

(B-C) qPCR validation of the above gene targets in whole liver ($N \geq 7$ for each genotype) (B) and primary hepatocytes (c).

Data represent means ± SEM. * $p < 0.05$ by Tukey's post-hoc analysis following one-way ANOVA.

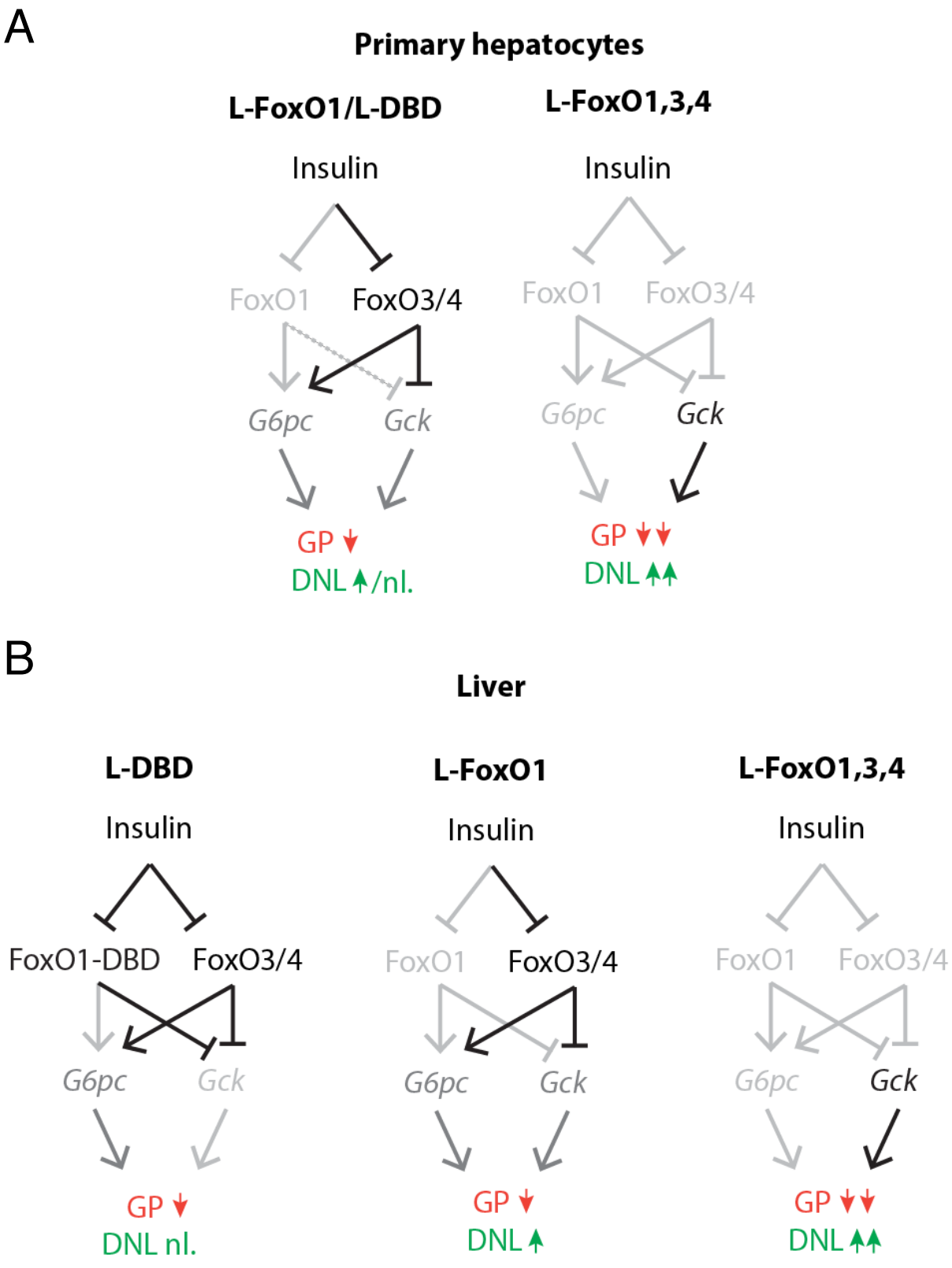

A
Primary hepatocytes
L-FoxO1/L-DBD
L-FoxO1,3,4
Insulin
Insulin
FoxO1
FoxO3/4
FoxO1
FoxO3/4
G6pc
Gck
G6pc
Gck
GP
DNL /nl.
GP
DNL
B
Liver
L-DBD
L-FoxO1
L-FoxO1,3,4
Insulin
Insulin
Insulin
FoxO1-DBD
FoxO3/4
FoxO1
FoxO3/4
FoxO1
FoxO3/4
G6pc
Gck
G6pc
Gck
G6pc
Gck
GP
DNL nl.
GP
DNL
GP
DNL

Figure 4.16

Figure 4.16. Model of FoxO Action in GP *vs.* DNL

Schematic diagram of insulin action on FoxO1 and FoxO3/4 with respect to *G6pc* expression → GP and *Gck* expression → DNL in (A) primary hepatocytes and (B) whole liver. Black type and strokes indicates that the indicated gene/protein or pathway is intact, while dark gray indicates partially intact and light gray indicates the absence of the indicated gene/protein or pathway. GP = glucose production, DNL = *de novo* lipogenesis, nl. = normal.

Section 4.5

CHAPTER 4 TABLES

		Control (n ≥ 8)	FoxO1-het (n ≥ 7)	DBD-het (n ≥ 9)
Body weight (g)	Fed	25.1 ± 0.7	25.2 ± 0.4	24.6 ± 1.1
Lean mass %	Fed	77.4 ± 0.6	78.8 ± 1.2	78.7 ± 0.7
Fat mass %	Fed	14.7 ± 0.5	13.2 ± 0.3	13.3 ± 0.6
Fluid mass %	Fed	7.9 ± 0.2	8.0 ± 0.1	8.0 ± 0.2
Glucose (mg/dL)	Fasted	72 ± 2	74 ± 4	73 ± 3
	Re-fed	200 ± 13	204 ± 17	177 ± 6
Insulin (ng/mL)	Fasted	0.44 ± 0.08	0.33 ± 0.04	0.42 ± 0.08
	Re-fed	1.06 ± 0.17	0.96 ± 0.14	1.03 ± 0.16
Free fatty acids (mEq/L)	Fasted	1.66 ± 0.16	1.65 ± 0.25	1.75 ± 0.15
	Re-fed	0.69 ± 0.07	0.76 ± 0.13	0.70 ± 0.08
Triglycerides (mg/dL)	Fasted	70 ± 5	57 ± 4	60 ± 4
	Re-fed	63 ± 5	55 ± 5	63 ± 6
Cholesterol (mg/dL)	Fasted	97 ± 5	83 ± 20	93 ± 12
	Re-fed	89 ± 4	91 ± 1	93 ± 3

Table 4.1. Metabolic Features of Heterozygous Mice

Data are means ± SEM. Animals were analyzed as described in Experimental Procedures.

Table 4.1

		Control (n ≥ 10)	L-FoxO1 (n ≥ 10)	L-DBD (n ≥ 7)
Free fatty acids (mEq/L)	Fasted	1.09 ± 0.19	1.23 ± 0.09	1.14 ± 0.13
	Re-fed	0.19 ± 0.02	0.20 ± 0.03	0.21 ± 0.03
Triglycerides (mg/dL)	Fasted	76 ± 6	84 ± 4	72 ± 12
	Re-fed	102 ± 11	92 ± 7	121 ± 9
Cholesterol (mg/dL)	Fasted	92 ± 4	102 ± 5	96 ± 6
	Re-fed	92 ± 3	85 ± 4	90 ± 3
Liver cholesterol (mg/g)	Fasted	1.66 ± 0.16	1.56 ± 0.13	1.75 ± 0.11
	Re-fed	1.17 ± 0.10	1.10 ± 0.07	1.12 ± 0.13

Table 4.2. Metabolic Features of L-FoxO1 and L-DBD Mice

Data are means ± SEM. Animals were analyzed as described in Experimental Procedures.

Table 4.2

		Control (n ≥ 9)	DBD-het (n ≥ 11)	L-FoxO1 (n ≥ 11)	L-DBD (n ≥ 9)
Body weight (g)	Fed	33.2 ± 1.2	34.9 ± 1.8	38.0 ± 1.0	36.0 ± 2.8
	Fasted	33.7 ± 0.3	33.3 ± 1.6	34.3 ± 1.0	33.3 ± 1.1
Glucose (mg/dL)	Fed	218 ± 3	223 ± 2	190 ± 4	203 ± 7
	Fasted	241 ± 14	242 ± 10	214 ± 7	220 ± 9
Insulin (ng/mL)	Fed	2.87 ± 0.22	2.43 ± 0.55	4.22 ± 0.95	6.66 ± 3.15
	Fasted	2.58 ± 0.30	2.11 ± 0.27	2.68 ± 0.32	2.25 ± 0.42
Free fatty acids (mEq/L)	Fed	0.76 ± 0.04	0.72 ± 0.08	0.71 ± 0.05	0.79 ± 0.06
	Fasted	0.71 ± 0.06	0.70 ± 0.05	0.74 ± 0.05	0.71 ± 0.07
TG (mg/dL)	Fed	113 ± 11	118 ± 12	104 ± 7	118 ± 7
	Fasted	59 ± 6	67 ± 6	53 ± 3	63 ± 9
Cholesterol (mg/dL)	Fed	296 ± 21	344 ± 27	364 ± 28	428 ± 42
	Fasted	337 ± 34	342 ± 27	398 ± 31	376 ± 55

Table 4.3. Metabolic Features of WTD-reared L-FoxO1 and L-DBD Mice

Data are means ± SEM. Animals were analyzed as described in Experimental Procedures.

Table 4.3

	Gene Ontology Category	*P* value	No. of genes
Fasted (287 genes)	Lipid metabolic process	2.1×10^{-7}	32
	Steroid metabolic process	3.0×10^{-7}	15
	Oxidation-reduction	1.0×10^{-6}	30
	Alcohol metabolic process	1.3×10^{-6}	21
	Monocarboxylic acid metabolic process	6.4×10^{-6}	17
	Oxo/carboxylic acid metabolic process	8.6×10^{-5}	21
	Cellular ketone metabolic process	1.2×10^{-4}	21
	Regulation of catalytic activity	4.5×10^{-4}	19
	Sterol homeostasis	7.2×10^{-4}	5
Re-fed (262 genes)	Lipid metabolic process	1.3×10^{-5}	26
	Oxidation-reduction	6.2×10^{-5}	24
	Steroid metabolic process	8.6×10^{-5}	11
	Monocarboxylic acid metabolic process	3.8×10^{-4}	13
	Cellular lipid metabolic process	4.2×10^{-4}	18
	Cholesterol metabolic process	4.2×10^{-4}	7
	Brown fat cell differentiation	6.6×10^{-4}	5
	Sterol metabolic process	7.1×10^{-4}	7
	Acute-phase response	7.5×10^{-4}	5

Table 4.4. Gene Ontology Analysis: Control *vs.* L-FoxO1

Analysis was performed using DAVID Bioinformatics Database.

Table 4.4

	Gene Ontology Category	*P* value	No. of genes
Fasted (298 genes)	Steroid metabolic process	8.3×10^{-9}	17
	Alcohol metabolic process	2.4×10^{-8}	24
	Sterol metabolic process	4.6×10^{-7}	11
	Cholesterol metabolic process	1.9×10^{6}	10
	Lipid metabolic process	3.3×10^{-6}	30
	Oxidation-reduction	5.0×10^{-6}	29
	Oxo/carboxylic acid metabolic process	1.2×10^{-4}	21
	Monocarboxylic acid metabolic process	1.3×10^{-4}	15
	Steroid biosynthetic process	1.5×10^{-4}	8
Re-fed (240 genes)	Oxidation-reduction	1.3×10^{-7}	28
	Lipid metabolic process	3.2×10^{-6}	26
	Steroid metabolic process	7.4×10^{-6}	12
	Monocarboxylic acid metabolic process	9.7×10^{-6}	15
	Cholesterol metabolic process	3.2×10^{-5}	8
	Antigen processing/present. via MHC-II	4.3×10^{-5}	5
	Oxo/carboxylic acid metabolic process	4.7×10^{-5}	19
	Sterol metabolic process	5.9×10^{-5}	8
	Cellular ketone metabolic process	6.4×10^{-5}	19

Table 4.5. Gene Ontology Analysis: Control *vs.* L-DBD

Analysis was performed using DAVID Bioinformatics Database.

Table 4.5

	Gene Ontology Category	*P* value	No. of genes
Fasted (121 genes)	Steroid metabolic process	7.6×10^{-9}	12
	Steroid biosynthetic process	1.9×10^{-8}	9
	Sterol metabolic process	3.6×10^{-8}	8
	Lipid metabolic process	2.3×10^{-6}	18
	Alcohol metabolic process	3.8×10^{-6}	13
	Oxidation-reduction	2.8×10^{-5}	13
	Acute-phase response	4.1×10^{-5}	5
	Sterol biosynthetic process	4.1×10^{-5}	5
	Monocarboxylic acid metabolic process	3.7×10^{-4}	9
Re-fed (131 genes)	Acute-phase response	2.2×10^{-6}	6
	Immune response	7.3×10^{-6}	15
	Response to stimulus	2.5×10^{-5}	36
	Immune system process	5.0×10^{-5}	18
	Antigen processing/presentation	1.9×10^{-4}	4
	Regulation of developmental process	2.3×10^{-4}	14
	Negative regulation of signal transduction	2.4×10^{-4}	8
	Defense response	3.9×10^{-4}	12
	Regulation of cell differentiation	4.8×10^{-4}	11

Table 4.6. Gene Ontology Analysis: L-FoxO1 *vs.* L-DBD

Analysis was performed using DAVID Bioinformatics Database.

	Gene Ontology Category	*P* value	No. of genes
Fasted (159 genes)	Homeostatic process	7.2×10^{-4}	15
	Regulation of biological quality	9.3×10^{-4}	22
	Alcohol metabolic process	1.4×10^{-3}	11
	Cellular amino acid derivative metabolism	1.7×10^{-3}	7
	Regulation of catalytic activity	2.8×10^{-3}	12
	Steroid metabolic process	3.2×10^{-3}	7
	Lipid metabolic process	3.7×10^{-3}	15
	Second messenger signaling	4.1×10^{-3}	6
	Glycerolipid metabolic process	6.0×10^{-3}	6
Re-fed (107 genes)	Cholesterol metabolic process	2.9×10^{-5}	6
	Sterol metabolic process	4.6×10^{-5}	6
	Lipid metabolic process	4.7×10^{-5}	14
	Monocarboxylic acid metabolic process	6.7×10^{-5}	9
	Oxidation-reduction	1.5×10^{-4}	13
	Cellular lipid metabolic process	1.7×10^{-4}	11
	Steroid metabolic process	1.7×10^{-4}	7
	Oxo/carboxylic acid metabolic process	7.4×10^{-4}	10
	Cellular ketone metabolic process	8.8×10^{-4}	10

Table 4.7. Gene Ontology Analysis: Transcription Factor Mode

Analysis was performed using DAVID Bioinformatics Database using genes significantly altered in both L-FoxO1 and L-DBD relative to control but not to each other.

Table 4.7

	Gene Ontology Category	*P* value	No. of genes
Fasted (31 genes)	Oxidation-reduction	4.2×10^{-4}	7
	Oxo/carboxylic acid metabolic process	5.7×10^{-3}	5
	Cellular ketone metabolic process	6.3×10^{-3}	5
	Monocarboxylic acid metabolic process	7.8×10^{-3}	4
	Alcohol metabolic process	1.7×10^{-2}	4
	Lipid metabolic process	2.1×10^{-2}	5
	Immune system process	3.1×10^{-2}	5
Re-fed (44 genes)	Negative regulation of signal transduction	5.0×10^{-5}	6
	Negative regulation of cell communication	7.4×10^{-5}	6
	Negative regulation of biological process	7.8×10^{-4}	11
	Immune response	7.8×10^{-4}	7
	Negative regulation of cellular process	1.5×10^{-3}	10
	Immune system process	2.3×10^{-3}	8
	Neg. regulation of response to stimulus	1.1×10^{-2}	3
	Regulation of developmental process	1.1×10^{-2}	6
	Positive regulation of cellular process	1.1×10^{-2}	9

Table 4.8. Gene Ontology Analysis: Co-Regulatory Mode

Analysis was performed using DAVID Bioinformatics Database using genes significantly altered in both control and L-DBD relative to L-FoxO1 but not to each other.

Table 4.8

Chapter 5

IMPRESSIONS AND CONJECTURES

5.1. General Summary of Findings

Through the work described in this thesis I have undertaken to enrich the current understanding of how the liver responds to insulin in both normal and pathophysiologic states. Specifically, my goal was to understand the etiology of selective insulin resistance – that is, the dissociation of insulin's effects on hepatic glucose and lipid metabolism. I approached this question through the use of two distinct but related hypotheses.

First, in Chapter 3, I surmised that selective IR represents not a pathogenic paradox, but rather a manifestation of the inherent differences in the insulin responsiveness of these processes. In order to test this hypothesis, I employed two models of insulin resistance in primary hepatocytes: chronic hyperinsulinemia (CHI) and acute competitive antagonism. CHI treatment *in vitro* showed that it is possible to recreate the uncoupling of insulin's effects on glucose production and *de novo* lipogenesis within the context of the clinically relevant *in vivo* CHI that marks the run-up to type 2 diabetes. Next, treatment of hepatocytes with variable doses of S961, a peptide competitive antagonist of InsR, demonstrated that insulin's suppression of GP and its stimulation of *de novo* lipogenesis (DNL) follow distinct dose-response patterns. The half-maximal dose of S961 to inhibit insulin's effect on GP was about four times greater than its ability to halfway inhibit insulin's effect on DNL. Taken together, these data suggest that hepatic IR *in vivo* may not only cause but also result from CHI, and that the causal relationship between CHI and IR occurs at least in part at the level of InsR itself. These findings contravene much of the conventional wisdom that intricate and arcane alterations in cellular metabolites or distantly related signaling pathways are required for the pathogenesis even of garden-variety IR [2, 51, 65, 90, 362, 418, 419].

Second, in Chapter 4, I consider another possible culprit behind selective IR, FoxO1, the most distal insulin-regulated metabolic effector that is agreed to impact on both glucose and lipid metabolism. Despite much phenotypic evidence linking FoxO1 to insulin's control of these processes, surprisingly little is known about the actual mechanisms FoxO1 uses to accomplish them. We therefore took an unbiased, genetic approach to the question by taking advantage of the fact that FoxO1 has been shown to act both as a transcription factor and as a transcriptional co-regulator. We therefore have developed mice (L-DBD)

that possess only DNA binding-defective FoxO1 (FoxO1-DBD) in its liver and compared their phenotype and gene expression to wild-type controls and to L-FoxO1 mice that completely lack the protein in hepatocytes. We found that FoxO1's well-established role as an activator of hepatic glucose production requires its ability to bind to DNA (*i.e.*, act as a transcription factor). On the other hand, while L-FoxO1 mice exhibited increased liver TG levels, L-DBD mice were equivalent to controls in this regard. Thus, on the whole, FoxO1 regulates hepatic TG metabolism in co-regulatory mode. Interestingly, in spite of this *in vivo* conclusion, primary hepatocytes from both L-DBD and L-FoxO1 mice showed higher rates of DNL and lower rates of fatty acid oxidation. Thus, the ability of FoxO1-DBD to properly control liver TG metabolism *in vivo* either does not depend strictly upon regulation of hepatocyte-autonomous DNL or FAO or requires input from extrahepatic factors.

Taken together, these results suggest that selective IR can be a phenomenon inherent to the hepatocyte and that FoxO1 may play a dual role in its development. This conclusion is further explored in the discussion that follows.

5.2. Primary Hepatocytes: Limitations and Lessons

5.2.1. Rationale for Using Primary Hepatocytes

The liver exists at the center of a staggeringly complex web of metabolic regulation; extensive mouse genetic work emphasizes the particular importance of liver InsR action in the preservation of whole-body metabolic health. As discussed extensively in previous chapters, the liver *Insr* knockout (LIRKO) mouse develops diabetes due to unrestrained glucose production [273, 274]. Mice completely lacking *Insr* die in the perinatal period of extreme hyperglycemia and ketoacidosis. However, restoration of *Insr* expression only in liver and, to a lesser extent, in pancreatic β cells and certain brain regions (*Ttr-Insr* ; $Insr^{-/-}$ "L1" mice) largely reverses these effects [165]. Only about 30% of L1 mice go on to develop diabetes later in life *versus* 100% of neonatal mice with complete *Insr* knockout [164, 420, 421]. On the other hand, reconstitution of *Insr* expression in Glut4-expressing tissues including skeletal muscle and WAT (GIRKI mice) did not result in a notable prolongation of lifespan or reprieve from diabetes [422]. Moreover, rescue of *Insr* expression in neurons alone (NIRKI mice) also did not extend lifespan to adulthood or prevent

diabetes [422]. This finding further emphasizes the importance of *Insr* restoration in liver *per se* rather than in brain in the rescue of the L1 mouse [165, 420, 422].

Despite the indubitable importance of liver insulin action in regulating global metabolism and preventing diabetes, studying the mechanisms of liver insulin action and resistance *in vivo* is fraught with complications. For example, L1 mice remain markedly hyperinsulinemic and are unable to normally suppress HGP during a hyperinsulinemic-euglycemic clamp in spite of normal Akt phosphorylation and *G6pc*/*Pck1* expression [420]. It is therefore possible that this apparent defect in liver metabolism is due to defects in other, *Insr*-deficient tissues (*e.g.*, unchecked WAT lipolysis, severely decreased skeletal muscle glucose uptake) rather than to primary defects in the liver itself [420]. We therefore undertook to address the liver-autonomous aspects of insulin action and resistance. We made extensive use of isolated primary hepatocytes to measure the production of glucose and lipids in response to insulin as well as associated changes in gene expression. Although we have made significant progress using this model, it is necessarily imperfect. In this spirit, our data indicate that the utilization of primary hepatocytes represents a double-edged sword; this system does not faithfully recapitulate many important facets of *in vivo* metabolism, but in certain cases this apparent disadvantage has actually proved educational.

5.2.2. Primary Hepatocyte Studies of Glucose Metabolism

The downsides of using primary hepatocytes, especially from mice, are many and varied, even aside from the obvious differences between the signaling and nutrient inputs available to hepatocytes *in situ* as opposed to in culture. If we first consider glucose production, for example, we mentioned in Chapter 1 that about 75% of glucose production over the first 16 h or so of fasting results from glycogenolysis and the rest from gluconeogenesis. In fact, the most acute effects of glucagon (analogous to cAMP treatment in our *ex vivo* experiments) are entirely attributable to enhanced glycogenolysis [423]. On the other hand, work form our laboratory using this glucose-production system has indicated that only about 10% of glucose production from primary hepatocytes results from glycogenolysis [370], similar to findings by other groups [424]. Whether or not this speaks to an inherent difference in the source of glucose for release between liver and primary hepatocyte or merely to the obvious differences in ambient conditions,

the conclusions we draw from these experiments are likely more germane to gluconeogenesis (*i.e.*, the minor source of HGP in physiologic fasting) than to glycogenolysis.

There are, however, several upsides to this complication. Although in human liver gluconeogenesis plays a minor role in HGP under normal conditions, both the relative and absolute rates of gluconeogenesis are significantly increased in patients with diabetes [103, 104, 106, 425]. Similar to the case of increased HGP in the L1 mouse, it has been unclear whether the increased gluconeogenesis of diabetes is attributable principally to mass action by increased influx of gluconeogenic precursors from other insulin-resistant tissues (*i.e.*, a more passive role for the hepatocyte) or if there is some primary derangement in the liver's handling of these substrates [126, 426, 427]. As our studies were performed within a milieu of constant glucose, lactate, and pyruvate levels, we can state that the derangement in gluconeogenesis in our cell-culture models of hepatic IR is inherent to the hepatocyte. Both CHI and acute InsR antagonism hinder the ability of insulin to blunt cAMP-induced glucose production. Indeed, these experiments support an important role of insulin in the regulation of gluconeogenesis in hepatocytes even if not so readily *in vivo.* In fact, although several previous studies have found a similar effect as ours of glucagon mimetics on GP in primary hepatocytes, most of these do not remark on its suppression by insulin [145, 171, 424, 428, 429]. Several *in vivo* studies, mostly in dogs undergoing hyperinsulinemic-euglycemic clamp, have cast doubt on the regulation of gluconeogenesis by insulin [94, 114, 399]. These reports have generally concluded that an effect of insulin on gluconeogenesis is transient and requires very high concentrations of insulin. Although these studies were performed with great rigor, they do not speak directly to processes in the hepatocyte itself. Even studies in whole liver in mice do not consistently reveal insulin suppression of gluconeogenesis [112]. The apparent inconsistency with our data may also stem from physiologic differences between rodents and large mammals [423, 430].

Although our data support a dynamic regulation of gluconeogenesis by cAMP (*i.e.*, glucagon) *vs.* insulin that can be perturbed by IR, the most important mechanisms remain unclear. The expression of *G6pc* and *Pck1*, the two rate-limiting enzymes of gluconeogenesis, are often used as a surrogate marker of HGP, but we found a poor correlation between transcript levels and observed GP. Although treatment of hepatocytes with InsR antagonist revealed a dose-dependent change in *G6pc* and *Pck1* expression

that generally paralleled GP, the IC_{50} values for gene expression *versus* GP differed by an order of magnitude (Fig. 3.6). Despite this finding, one may still argue that the discrepancy between gene expression and GP is merely a quantitative matter. However, several other experiments revealed qualitative differences between the regulation of *G6pc*/*Pck1* expression and GP, strongly suggesting that the dynamics of these processes are not synchronized within the temporal context of the experiment. In all of our GP experiments in both Chapters 3 and 4, cAMP treatment increases *G6pc* expression by at least 30-fold even as GP increases only by 50-100%. In our model of CHI, there was no alteration in basal or cAMP-induced GP even as the effect of insulin was nearly lost (Fig. 3.2A). On the other hand, both basal and especially cAMP-induced *G6pc* and *Pck1* expression were markedly decreased, the latter by up to two thirds (Fig. 3.2B). Perhaps even more strikingly, primary hepatocytes from L-FoxO1 and L-DBD mice exhibited a tenfold decrease in cAMP/dex-stimulated *G6pc* expression despite only a roughly 25% decrement in GP (Fig. 4.8A-C). Interestingly, *in vivo G6pc* expression was decreased by about 25% in L-FoxO1 mice, commensurate with the decreased AUC of the glucose and pyruvate tolerance tests (Fig. 4.5). Further indicative of a dissociation between FoxO1-mediated GP and *G6pc* expression, we found *G6pc* expression to be decreased by about two thirds even in cells from *Foxo1*-heterozygous mice in spite of no defect in GP (Fig. 4.3A-C). As discussed earlier, FoxO1-associated GP has customarily been ascribed to its incontrovertible regulation of *G6pc*; that we have managed to quantitatively and qualitatively uncouple these events lends further credence to the hypothesis that FoxO1 affects gluconeogenic/glycolytic flux by as yet uncharacterized means.

To reemphasize, we can be sure that FoxO1 is a key regulator of HGP, but we are skeptical of the idea that this occurs primarily through its regulation of *G6pc*. Finally, both our *in vivo* and primary hepatocyte data cast doubt on the importance of *Ppargc1a* expression for glucose production despite early enthusiasm in its favor [173, 372, 431]. Not only is expression of the gene in hepatocytes unaffected by insulin treatment – quite unlike its marked repression by re-feeding, and therefore also representing a dual drawback/benefit of primary hepatocytes – but its expression is not decreased in L-DBD mice even as GP is.

Overall, these conclusions regarding transcriptional regulation of gluconeogenesis are nicely concordant even with several *in vivo* models. As mentioned earlier, even liver-specific deletion of *G6pc* or *Pck1* expression do not cause a physiologic impairment in glucose production due to compensation by other tissues [379, 380]. Moreover, although the expression of *G6pc* and *Pck1* are clearly regulated by insulin, the activity levels of the enzymes are not well correlated with insulin action; flux through gluconeogenesis to G6P occurs even in the fed state [94, 114]. Even in the context of diabetes, when gluconeogenesis *per se* is elevated, studies of human liver samples did not reveal differences in *G6pc* or *Pck1* expression [381]. It therefore appears that although G6Pase and PEPCK are essential to the biochemical process of glucose production, physiologic fluctuations in their mRNA levels may not be as important as once thought to the acute and subacute regulation of HGP.

5.2.3. Primary Hepatocyte Studies of Lipid Biosynthesis

The importance of differences between whole liver and primary hepatocytes with respect to *de novo* lipogenesis has already been extensively discussed in Chapter 4 and is revisited in Section 5.3.1 below. Aside from these phenomenological considerations, however, it is important to understand why these *in* and *ex vivo* differences occur. There are several different causes that are relevant to these studies.

First, as has been discussed, the major transcriptional control of lipogenesis by insulin occurs *in vivo* through the cleavage-induced activity of SREBP-1c [237, 276]. However, as discussed in Chapter 3, we found considerable variability in insulin's regulation of SREBP-1c cleavage. This is not surprising, however, as mouse primary hepatocytes are generally considered a poor venue for the study of SREBP-1c dynamics [218, 226]. Whereas SREBP-1c is the dominant SREBP-1 isoform in intact liver, in cultured cells including hepatocytes, SREBP-1a appears to predominate [226]. Even though SREBP-1a is far more powerful a driver of lipogenesis both in liver and primary hepatocytes than is the 1c isoform, its activity is not regulated by insulin [211, 226, 432]. Although we did not measure mRNA or protein levels of SREBP-1a in our studies, it is unlikely that 1a could account for the acute induction of DNL we observe following insulin treatment. Even the SREBP-1c that does remain in mouse hepatocytes loses its

responsiveness to insulin almost immediately after explantation, suggesting the requirement for extrahepatic inputs in regulating this system [218].

It is for these reasons that we proposed in Chapter 3 a modification to the Brown and Goldstein model of selective IR, in which the relative preservation of insulin-stimulated DNL proceeds via SREBP-1c activation [276]. That is, even though it may account for the slight increase in basal DNL in CHI-treated cells *vs.* control, it would not likely explain the retained acute increase in DNL, especially as we did not detect increases in expression of SREBP-1c target genes such as *Fasn or Srebf1c* itself over this time period [198]. We therefore hypothesize that insulin's acute regulation of DNL in mouse primary hepatocytes proceeds via posttranslational modifications (such as of ACC1, PFK-2, and ACL) that affect carbon flux within the hepatocyte [48, 94, 158, 258]. Interestingly, however, rat hepatocytes differ from mouse in retaining a considerable degree of insulin-sensitive SREBP-1c cleavage [218]. Although several studies have modeled CHI in rat hepatocytes, none to date have directly studied the effect on SREBP-1c cleavage [285, 325, 432]. We have performed a pilot experiment in rat primary hepatocytes that showed a preservation of SREBP-1c cleavage in response to acute insulin following CHI treatment, however we have not yet assessed DNL in this system.

Aside from SREBP-1c, there are numerous other potential confounders of insulin-stimulated DNL in mouse primary hepatocytes. As will be further discussed below, insulin induction of *Gck* expression via FoxO1 inhibition apparently requires some potentiating factor outside of the liver. One possibility in this vein is neuronal modulation, as acute infusion of insulin into the head arteries of dogs resulted in increased *Gck* expression in liver [433]. Furthermore, blockade of hypothalamic insulin signaling prevented insulin's induction of *Gck* expression in liver [399]. Interestingly, this effect may be mediated through a downregulation of SHP (*Nr0b2*), a negative regulator of *Gck* expression that was identified as a FoxO1 target by our RNA-seq analysis and previous studies [168, 423, 434]. Additionally, our laboratory has shown that targeted deletion of *Foxo1* in AgRP neurons decreases fasting *Gck* expression in liver [435]. Studies are currently underway in on mice lacking *Foxo1* both in liver and in AgRP neurons. Finally, we have demonstrated that FoxO1 (and therefore, insulin) regulates lipid metabolism in part through modulation of bile acid signaling [168], a regulatory circuit not present in isolated hepatocytes.

5.2.4. Primary Hepatocyte Studies of Fatty Acid Oxidation

Those considering the relationship between insulin action and hepatic lipid metabolism generally focus on lipid biosynthesis as the primary driver of liver lipid levels. However, as illustrated starkly by poorly controlled T1DM patients, insulin also plays an important role in the regulation of fatty acid oxidation. As mentioned in the introduction, however, it has been unclear whether the functional inhibition of FAO by insulin results primarily from blunted WAT lipolysis or due to direct effects on liver and/or skeletal muscle. By addressing this issue in primary hepatocytes, we have begun to parse out the relative contribution of liver itself in the absence of acute changes in FFA supply [436]. Specifically, we found that FoxO1 ablation reduces FAO by over half, suggesting that insulin may exert a primary inhibition of FAO through FoxO proteins in the liver in addition to its well-documented effects on WAT lipolysis. Indeed, *Insr*$^{-/-}$; l-*Foxo1* mice have normal levels of β-hydroxybutyrate despite increased circulating FFA [145]. We did not, however, directly assess the effect of acute insulin treatment on FAO in primary hepatocytes.

That insulin might suppress FAO directly in liver is also supported by tracer studies in humans with obesity and/or NAFLD that suggest increased rates of FAO [418, 437-440]. (Apparently, even though rates of FAO are increased in these patients, it is insufficient to lower liver TG levels to normal.) Although this may merely be due to increased delivery of FFA from WAT lipolysis, garden-variety IR is not strictly associated with tonically elevated fasting circulating FFA [441]. Indeed, adipocyte lipolysis is exquisitely sensitive to insulin – in fact, more so than any other major physiologic process [426, 442]. It is for this reason that even many patients with severe IR due to mutations in *Insr* do not develop DKA [65]. In a striking example of selective IR on a global scale, even moderate residual InsR action in these patients can evidently suppress lipolysis enough to keep FFA levels from creeping up into the danger zone [65, 270]. Only in the most severe cases of absolute (*e.g.*, untreated type 1 diabetes) or relative (*e.g.*, Rabson-Mendenhall syndrome) insulin deficiency does DKA arise, presumably due principally to unchecked lipolysis rather than to a primary defect leading to increased hepatic FAO [183, 443]. Going forward, it would be of interest to test the effects of CHI or S961 treatment on insulin inhibition of FAO in primary hepatocytes. CHI treatment especially may prove a useful model for teasing out the acute *vs.* chronic

effects of insulin on FAO as it would be expected to lead to derepression of FoxO1, thereby helping to elucidate its contribution to the process.

5.3. Toward a Mechanistic Understanding…

5.3.1. Identifying the True "Branch Point" in Selective IR

Whether qualitative or quantitative, the defect in CHI-induced IR is at the level of InsR itself. Selective IR, on the other hand, has generally been attributed to postreceptor defects [65, 444]. Many different potential explanations have been invoked, generally focusing on identifying a discrete branch point beyond which the common upstream mediators of insulin signaling diverge to regulate disparate downstream metabolic processes [65, 90, 213, 218, 276, 295, 306]. These models generally posit that one branch (*i.e.*, controlling hepatic glucose metabolism) becomes resistant to the effects of insulin while the other (*i.e.*, controlling lipid metabolism) remains relatively sensitive, potentiated by hyperinsulinemia. Thus, in these cases, hyperinsulinemia is largely the result of altered glucose metabolism rather than a cause. These models generally ignore the precise mechanism whereby the sensitive *vs.* resistant pathways are differentially activated, although the implication is that this regulatory bifurcation requires signaling input from nebulous other pathways. Gonzalez, *et al.* [305] took a more sophisticated mechanistic approach by studying a model of CHI in cultured adipocytes, demonstrating that activation of downstream branches are selectively regulated by the different isoforms of Akt. Even this study, however, evaded the question of how exactly CHI induces IR in Akt1/2 activation and did not address the possibility of differential regulation of IRS1/2 or PI3K [305]. Another valid view that has been articulated posits that the concept of "selective IR" is somewhat misguided. That is, although insulin is certainly a key regulator of glucose homeostasis, its regulation of lipid metabolism requires a complex crosstalk between different tissues that is in some ways independent of insulin and therefore difficult to classify as "sensitive" or "resistant" [362].

Our studies, on the other hand, have indicated that selective IR can be both hepatocyte-autonomous and related directly to alterations in InsR number and/or function. Thus, selective IR does not require input from other signaling molecules or pathways; it also rebuts the implication that InsR plays a somewhat passive role in the development of selective IR [276]. Thus, rather than dividing IR along

receptor-level *versus* postreceptor lines, it behooves us to better understand how common mediators in both pathways may differentially regulate glucose *vs.* lipid metabolism. In this thesis we have employed three different models of hepatic insulin resistance; the data offer novel insights into the mechanism by which this altered InsR action translates into disparate effects on glucose *vs.* lipid metabolism. We therefore revisit our discussion of the proximal insulin-signaling pathway from Section 1.6.5 in order to integrate what we have learned from the experiments in this work.

IRS1/2

First, we consider the role of the signaling mediators most proximal to InsR, IRS1/2. Although investigators have suspected that IRS1 and IRS2 exert differential control over glucose *vs.* lipid metabolism, much of the data have been conflicting (see Section 1.6.5). Our studies unfortunately do not provide much in the way of reconciliation. Previous modeling of CHI in rat primary hepatocytes revealed a selective downregulation of IRS2 at the mRNA and protein levels, suggesting that intact insulin signaling to SREBP-1c proceeds largely via IRS1 [295]. In our model, however, we found decreases in protein levels of both IRS1 and IRS2, similar to findings by another group [303] and consistent with observations in the livers of several hyperinsulinemic animal models [303, 445]. Insulin can downregulate IRS1 by inducing its proteasomal degradation via a PI3K- and mTORC1-dependent mechanism [303, 446-448], although similar studies on IRS2 have yielded conflicting data [303, 449]. Downregulation of IRS2 by insulin appears to occur primarily at the transcriptional level [295, 303], although we did not find any difference on average in *Irs2* mRNA levels in our CHI model. Overall, the similar decreases in both IRS1 and IRS2 do not allow us to discern any functional differences in their roles in GP *vs.* DNL in this system.

A further important aspect of IRS biology is its regulation by FoxO proteins. Our original study of the L-FoxO1 mouse demonstrated reductions in fasting-induced expression of *Irs2,* and FoxO1-deficient primary hepatocytes exhibited reductions in both *Irs1* and *Irs2* expression [145]. This regulatory scheme suggests a homeostatic loop whereby increased insulin signaling inhibits FoxO1, thereby decreasing IRS levels and preventing overstimulation of downstream pathway components; the subsequent decline in insulin signaling through InsR leads to the derepression of FoxO1 allowing it to restore IRS levels [145,

247, 304, 360]. This therefore represents an additional feedback layer in insulin signaling on top of InsR downregulation. Our studies confirm a key role for FoxO1 in the expression of *Irs2*, as fasted L-FoxO1 livers demonstrated a ~50% decrease in its mRNA. Moreover, *Irs2* represents a direct transcriptional target of FoxO1, as *Irs2* levels were equally decreased in fasted L-DBD livers. Interestingly, a study in MEFs deficient in either *Irs1* or *Irs2* indicates that the latter is required for the phosphorylation of Akt and FoxO1 while the former is dispensable, further substantiating an IRS2 → FoxO1 → IRS2 homeostatic loop [304, 360].

Akt

Our studies in primary hepatocytes verify that Akt signaling is required for mediating insulin's effects on both glucose and lipid metabolism (Fig. 3.8), consistent with *in vivo* data [112, 158, 167, 306]. While data such as these have been used in the past to support a neatly bifurcating model of insulin signaling (*i.e.*, glucose and lipid arms largely separate), our pharmacologic inhibition of Akt with Akti-1/2 results in a total abrogation of Akt1/2 signaling [327]. It therefore remains possible that Akt1 and Akt2 signal differentially to glucose *vs.* lipid metabolism, as proposed in Chapter 1 and buttressed by observations in an adipocyte model of CHI [305]. Nevertheless, the reliance of both glucose and lipid metabolism on the combined actions of Akt1 and Akt2 suggest that signaling pathways diverging upstream (*e.g.*, MAPK pathway) do not play a critical role. This result has been suggested by previous studies [213, 218] but these prior reports do not report directly on GP or DNL. Another potential interpretation is that Akti-1/2 does not inhibit both isoforms with equal potency.

Aside from potential isoform-specific actions of Akt, the kinase's functional outputs may be differentially regulated on the basis of the relative phosphorylation levels of Thr 308 and Ser 473 (see Section 1.6.5). Indeed, we have determined in Chapter 3 that the phosphorylation of Akt at these two sites does display some differential sensitivity to insulin (Fig. 3.5). The calculated IC_{50} for the inhibition of Thr 308 phosphorylation in response to 10 nM insulin by S961 treatment is 0.87 nM while that for Ser 473 is 2.69 nM. Thr 308 phosphorylation is therefore approximately three times less sensitive to this dose of insulin than is phosphorylation at Ser 473. Thus, we expect processes that can be regulated efficiently by

Akt phosphorylated at Ser 473 but not at Thr 308 to proceed more potently while those requiring full activation of Akt at both sites would be stunted, an example of selective IR.

Such a situation recalls the L-*Pdk1*KO mouse model, which interferes with Thr 308 but not Ser 473 phosphorylation and exhibits deficient phosphorylation of FoxO1 and S6K while that of GSK3β remains mostly intact [319]. Interestingly, however, we observed largely the opposite pattern in our experiments. That is, phosphorylation of FoxO1 qualitatively paralleled that of Ser 473, such that at 10^{-9} M S961, both were nearly maximal. On the other hand, at that concentration of inhibitor, both phosphorylation of Thr 308 and GSK3β were less than half maximal. That FoxO1 phosphorylation appears to be relatively more responsive to insulin than other targets is consistent with the CHI model of Gonzalez, *et al.* [305] and in keeping with our own observations regarding the exquisite sensitivity of FoxO1 localization even to glancing doses of insulin [143, 149, 150, 450]. Such a conclusion is surprising given that FoxO1 in primary hepatocytes appears to be relatively exclusively a mediator of glucose and not lipid synthesis, while we found the latter to be more sensitive to insulin than the former. Again, we found very limited responsiveness of SREBP-1c cleavage to S961 treatment. Together, these observations suggest that the most important mediators of insulin's acute effect on DNL in primary hepatocytes may be still more insulin-sensitive Akt target(s) that we have not assessed.

As discussed above, FoxO1 participates in a homoestatic loop regulating insulin action via IRS; an obligate intermediate in this pathway is Akt. Indeed, FoxO1 induction of IRS2 would be expected to increase Akt phosphorylation, as observed. A constitutively active mutant of FoxO1 has been shown to increase basal Akt phosphorylation independently of its effects on IRS2, as this effect proceeds via a co-regulatory mechanism [247] while we now know that its regulation of *Irs2* expression does not (Fig. 4.7G). Our previous work implicated FoxO1 inhibition of the pseudokinase tribble-3 (Trb3), a negative regulator of Akt phosphorylation, as the mechanism responsible [247, 451], although the regulation of *Trb3* expression by FoxO1-DBD was not directly tested. Whether through IRS and/or Trb3, we expect Akt phosphorylation to be dampened in the absence of FoxO1. Consistent with this expectation, primary hepatocytes lacking FoxO1 do display a notable decrease in insulin-stimulated Akt phosphorylation. Interestingly, L-DBD primary hepatocytes have reproducibly shown a defect in Akt phosphorylation even

relative to L-FoxO1, suggesting either that *Trb3* is not actually a FoxO1 co-regulatory target or that Trb3 is not the key mediator of the effect. That insulin's induction of DNL in L-DBD hepatocytes remained normal in spite of the stark decrease in Akt phosphorylation serves as further evidence of the sensitivity of this process to insulin.

The experiments contained in this work do not, however, speak to a functionally significant FoxO1 → Akt connection in the healthy liver. Although re-feeding-induced Akt phosphorylation (at both sites) was decreased in L-FoxO1 and L-DBD livers (Fig. 4.7A-C), this difference disappeared after matching samples for mean insulin levels (Fig. 4.7D-F). Intravenous insulin injection also did not reveal any difference in hepatic Akt phosphorylation (Fig. 4.6). On the other hand, in the fasted liver, basal phosphorylation of Akt at Thr 308 but not at Ser 473 was higher in L-FoxO1 than in control or L-DBD mice (Fig. 4.7A-C); this is the opposite of our expectations based on *Irs2* expression (Fig. 4.7G). It is tempting to speculate that the phenotypic correlation of this finding with differences in *Gck* expression and liver TG reflects differential Akt signaling on the basis of Thr 308 phosphorylation but we have no further data to imply causation. The potential importance of FoxO1 feedback may be magnified under conditions of IR, as Akt phosphorylation at Thr 308 and especially at Ser 473 were both decreased in the livers of L-FoxO1 and L-DBD WTD-fed mice *versus* control. It would be of interest to assess whether the relative worsening of Ser 473 *vs.* Thr 308 phosphorylation produces functional consequences such as altered HGP, although we did not detect any significant changes in gluconeogenic gene expression (data not shown).

Other Potential Mediators

Other signaling elements downstream of InsR have also been invoked to explain selective IR. Semple, *et al.* (2009) [65] proposed, for example, that selective IR results from a branching downstream of IRS1/2 wherein Akt signals to FoxO1 to regulate HGP while the path toward DNL proceeds via protein kinase C-λ (PKCλ) activation of SREBP-1c. This model is based on studies in demonstrating that overexpression of PKCλ increases SREBP-1c and Fasn levels in primary hepatocytes and liver [452, 453]. Liver-specific PKCλ-null mice, on the other hand, show decreased expression of SREBP-1c and its lipogenic targets as well as decreased TG content [419, 453]. The interpretation of these models, however, is complicated by

alterations in circulating insulin levels [90, 419, 453]. Moreover, we know both from experiments here and elsewhere using Akti-1/2 [213, 218] and from past studies in mice [112, 158, 167, 306] that Akt signaling is required for proper regulation of DNL in addition to HGP. The mechanism linking PKCλ to InsR also remains questionable [452, 454].

Another possible mediator of selective IR is GSK3β. Although this enzyme has classically been envisioned as a negative regulator of glycogen synthesis, recent studies have found that both liver-specific deletion of the protein [455] and expression of a constitutively active form [112] have no effect on hepatic glycogen metabolism *in vivo*, although in the former case this may be due to compensation by GSK3α [456]. (GSK3 activity appears to be more important for regulation of skeletal muscle glycogen metabolism [455, 457].) On the other hand, as described in Chapter 1, GSK3β may play a role in DNL via negative regulation of SREBP-1c stability [185, 215, 219, 220, 458]. Thus, a selective retention of insulin's inhibition of GSK3β would be expected to enhance SREBP-1c signaling and thereby increase DNL especially in the face of hyperinsulinemia [185]. Potentially arguing against this mechanism, treatment of rat primary hepatocytes with a GSK3β inhibitor had little to no effect on *Srebf1c* mRNA, the expression of which is an indicator of SREBP-1c activity [213, 459]. In fact, the inhibitor had a greater effect to decrease *Pck1* mRNA, suggesting more of an effect on the glucose than the lipid arm [213]. Furthermore, liver-specific deletion of GSK3β revealed no metabolic abnormalities in terms of glucose or lipids [455]. We have a conducted pilot experiments to test this hypothesis in CHI by infecting cells with an adenovirus encoding a constitutively active GSK3β mutant but the data were not consistent with the hypothesis.

Rather, the data reported in this thesis add considerably to the fund of knowledge regarding the dual regulation of hepatic glucose and lipid metabolism by FoxO1. We propose that FoxO1 (as well as other FoxOs) may itself be a key mechanistic link between the increased HGP and DNL of selective IR. In other words, again, rather than envisioning selective IR only as the product of a bifurcation in the InsR pathway, we see it potentially as arising parsimoniously *at least in part* from altered regulation of this single signaling node.

5.3.2. FoxO1: A Bridge Not Quite Far Enough

As discussed in Chapter 1, the prevailing model of insulin signaling anoints Akt as the most distal step in the InsR cascade at which insulin's control of glucose and lipid metabolism are unified [90]. This view, however, is inconsistent with repeated observations from our laboratory and others demonstrating FoxO1, a substrate of the Akt kinase, as playing a role in both metabolic arms [2, 90, 155]. Indeed, the L-FoxO1 mouse, completely lacking FoxO1 in hepatocytes, exhibits both decreased HGP and increased liver TG content, likely due to increased DNL and decreased FAO. Thus, in a reductionist sense, the phenotype of the L-FoxO1 mouse represents the inverse of the LIRKO mouse [202, 273, 274]; the pure IR of InsR deficiency could be wholly attributable to FoxO1 hyperactivity. Though oversimplified, the juxtaposition of L-FoxO1 and LIRKO models is salient in light of the demonstrated genetic epistasis between *Foxo1* and *Insr*, *Irs1/2*, and *Akt1/2* described in Chapter 1 [145, 166, 167]. Indeed, even in the model of acute, acquired IR via InsR antagonist treatment employed in this thesis reveals a close relationship between relative FoxO1 phosphorylation and the insulin responsiveness of GP and DNL (Fig. 3.2).

These observations might lead one to redraw the typical diagram of the proximal InsR signaling cascade to include FoxO1 as well. Such a remodeling, however, may be a bit premature, for although FoxO1 represents a downstream bridge between the glucose and lipid arms, several caveats highlighted by this thesis currently imperil the crossing. As discussed in Chapter 1, insulin signaling regulates DNL both at the transcriptional and posttranslational levels. The effect of insulin to stimulate DNL in our experimental setup likely proceeds largely due to posttranslational effects alone, as lipogenic gene expression in mouse primary hepatocytes, including *Srebf1c* and *Gck*, is fairly unresponsive to insulin (see Section 5.3; Fig. 3.1E) [218, 226]. This explains, at least in part, why we generally see increases only of up to 70% in DNL in response to insulin in primary cells compared to the roughly fivefold increase in DNL due to re-feeding over a similar time period *in vivo* [253]. An exception may lie in the increased basal expression of *Gck* and *Srebp1c* following CHI treatment, consistent with the augmented basal DNL in CHI-treated cells (Fig. 3.1D-E); the normal insulin treatment in our DNL assays is for a fraction of the time and at one tenth of the dose of CHI treatment. Nevertheless, WTD feeding of wild-type mice (*i.e.*,

driving CHI *in vivo*) results in constitutively increased *Gck* expression in liver with reduced fasting/feeding fluctuations.

Unlike tampering with InsR function, dysregulation of FoxO1 does not appear to greatly impact upon normal regulation of DNL in isolated hepatocytes. CHI treatment, which results in a loss of insulin-stimulated phosphorylation of FoxO1 and therefore unrestrained FoxO1 activity, was not associated with any defect in basal or insulin-stimulated DNL (Fig. 3.1A,D); in fact, both were higher following CHI treatment. Conversely, although *in vivo* studies indicate that loss of FoxO can enhance insulin-stimulated DNL [245], we find only a non-statistically significant ~35% increase in TG synthesis in L-FoxO1 primary hepatocytes compared to controls (Fig. 4.9G). Moreover, despite the higher baseline, L-FoxO1 (and L-DBD) cells exhibit the same proportional DNL response to insulin as control cells. This implies that the posttranslational modifications activated by insulin treatment – and that appear to be part of what is paradoxically "preserved" in selective IR – are still operational in the absence of FoxO1. Thus, it appears that FoxO1's major role in the regulation of DNL is at the transcriptional level, but that this comes into play mainly in the context of intact liver. The non-hepatocyte-autonomous nature of FoxO1's transcriptional control of DNL is echoed by our observation that basal *Gck* expression is increased following CHI treatment (Fig. 3.1E), a time when FoxO1 activity is expected to be unrestrained (Fig. 3.1A). The slight increase in DNL in the absence of FoxO1 therefore likely reflects alterations that are cell autonomous, such as *G6pc* expression and/or even GP as a whole. Indeed, as discussed extensively in Chapter 4, we hypothesize that the discrepancy between *in vivo* and *ex vivo* hepatocellular lipid handling in the L-FoxO1 mouse reflects on the nature of *Gck vs. G6pc*/GP regulation by insulin generally and FoxO1 in particular [253].

Our understanding does remain incomplete, however, as liver-specific deletion of *Akt2* decreases DNL and liver TG accumulation despite the expectation of increased FoxO1 activity [158]. Even simultaneous deletion of liver *Foxo1*, which on its own results in increased DNL and liver TG, does not overcome the decrease in DNL and liver TG content due to loss of *Akt2* [158]. The decrease in DNL in the absence of hepatic Akt2 was proposed to be due in part to decreased activation of the mTORC1 → SREBP-1c pathway, but that this alone was not sufficient to explain the mouse's phenotype [158]. Rather,

the authors of this study point to decreased Akt-mediated expression and phosphorylation of ATP citrate lyase (ACL) [158], an enzyme that participates in lipogenesis by facilitating the shuttling of acetyl-CoA from the mitochondrion to the cytosol [460].

The compendium of these data supports our hypothesis of a three-pronged control of hepatic lipogenesis by Akt (Fig. 5.1). First, as described in Chapter 1, insulin exerts posttranslational control over carbon trafficking through the hepatocyte, including via removal of phosphates placed by PKA and by Akt phosphorylation of ACL [158, 461, 462], among others. The inability to properly regulate these pathways in the absence of Akt2 may account for the aforementioned impairment in DNL [158]. We suspect that this pathway is largely responsible for insulin's acute induction of DNL in primary hepatocytes and may be preserved in selective IR. Second, representing the now-classic pathway of transcriptionally controlled lipogenesis is stimulation of SREBP-1c cleavage and expression via Akt's activation of mTORC1 and S6K [213, 215, 217, 218]. We believe that upregulation of this pathway underlies the increase in basal DNL we observe in CHI-treated primary hepatocytes and thus is also an important contributor to the phenotype of selective IR. Finally, owing in large part to the work of Rebecca Haeusler [253], we now understand that Akt's inhibition of FoxO facilitates DNL by reversing its dichotomous regulation of *Gck* and *G6pc*. This thesis extends our understanding of this process by indicating that the FoxO1 → ↓ *Gck* → ↓ DNL pathway is largely regulated by extrahepatic factors while the putative FoxO1 → ↑ *G6pc*/GP → ↑ DNL is intrinsic to the hepatocyte. Two of these mechanisms – posttranslational control of carbon flux and FoxO → *Gck*/*G6pc* – complement each other nicely in their ability to bridge the gap between insulin's control of glucose and lipid metabolism at both the transcriptional level and beyond.

5.4. One Wonders

Although the experiments presented in this thesis allow us new insights into hepatic insulin action and resistance, it brings up many more questions. Below are three of the more pressing issues and how we propose to test them.

5.4.1. Acute vs. Chronic Effects of Insulin on GP and DNL

We have proposed that selective IR operates both at the levels of acute and chronic insulin regulation of GP and DNL. Specifically, the former most likely proceeds via posttranslational modification of existing proteins while the latter hinges on *de novo* expression of new glucongeogenic/lipogenic enzymes. In order to test this hypothesis, we will treat hepatocytes with cycloheximide in order to inhibit new protein synthesis. We will then test the ability of insulin over the short term both to induce DNL and to inhibit GP. It is already known that the acute effect of glucagon on GP still proceeds even in the presence of cycloheximide [463], suggesting that the inhibition by insulin will be as well if it is merely a matter of reversing the acute effects of glucagon. We will also test the effect of cycloheximide on DNL in the setting of CHI treatment in order to assess whether the increase in basal *Srebp1c* and *Gck* expression are important in either increasing basal DNL or maintaining the acute insulin responsiveness of the process. Potential complication lies in our previous observation that cycloheximide treatment alone can increase basal Akt phosphorylation [247] and in an earlier report stating that cycloheximide treatment on its own can increase DNL in rats [464]. We will therefore be more concerned with the relative change (if any) with insulin compared to the cycloheximide/no insulin condition rather than compared to the vehicle/no insulin condition.

5.4.2. FoxO1 Regulation of Gck *Expression*

A key element of our proposed mechanism as to how FoxO1 mediates insulin's effects on both lipid and glucose metabolism is its coordinate regulation of *Gck* and *G6pc*. In the case of the former, our new *in vivo* data suggest that *Gck* is at least partially a co-regulatory target of FoxO1. This possibility is not without precedent in the literature, as FoxO1's regulation of *Gck* expression is mediated by interaction

with HNF-4α [179, 406]. We therefore are interested in directly testing whether FoxO1-DBD can indeed repress *Gck* expression. We have cloned the liver-specific *Gck* promoter into a luciferase vector and are currently performing reporter-gene experiments in isolated hepatocytes overexpressing FoxO1-WT and FoxO1-DBD. A determination that *Gck* is a *bona fide* co-regulatory target gene of FoxO1 would be a strong argument in favor of our model, as it would parallel the liver TG phenotype of L-DBD *vs.* L-FoxO1 mice. We are also interested in pursuing co-immunoprecipitation experiments in order to determine the regulatory factors most important for FoxO1 regulation of *Gck* and other co-regulatory targets (*e.g.*, *Hsd3b5*).

5.4.3. Hepatocyte-Autonomous Regulation of FAO

Our data provide evidence that insulin can regulate hepatocellular FAO at the transcriptional level through FoxO1 rather than only by affecting intracellular metabolite flux in the short term. Moreover, although FAO may be elevated in patients with hepatic IR or even NAFLD, the clinical approach to treatment of dyslipidemia includes treatment with fibrates, which act as PPARα agonists and thereby upregulate the FAO gene program to reduce steatosis and improve IR [465]. It is already well established that chronic treatment of primary hepatocytes with FFA induces IR [63, 466], but it is unclear whether the reverse holds. It would therefore be of interest to discern whether there is an alteration in FAO capacity in our hepatocyte model of CHI. We can test whether the selective IR of CHI affects FAO in the absence of confounding fluctuations in lipolysis, and then whether treatment of the cells with fenofibrate can reverse any adverse effects that arise. Studies have already demonstrated that treatment of primary hepatocytes with fibrates increases expression of genes not only involved in FAO *per se* but also those in glucose metabolism and various cell-stress responses [261, 467]. Indeed, the regulation of FAO in hepatocytes has been largely overlooked in favor of studying DNL, but it may well be an important contributor to the lipid phenotype of hepatic IR.

5.5. Conclusion

Type 2 diabetes and the metabolic syndrome are the great public-health crises of our day. Although we have learned much about their pathophysiology and treatment, even basic questions have remained. One of the most vexing of these has been the etiology of “selective” IR that contributes to the metabolic one-two punch of excessive glucose production *and* hepatic steatosis. In this work, however, we have taken a step forward in unraveling this apparent paradox. Although important questions remain – especially regarding the relative importance of insulin’s acute *vs.* chronic regulation of glucose and lipid homeostasis in the liver – we now understand that FoxO1 biology can at least partially to bridge the gap. Discovering what will get us all the way across to the other side, however, is our goal for the future.

Section 5.6:

CHAPTER 5 FIGURE

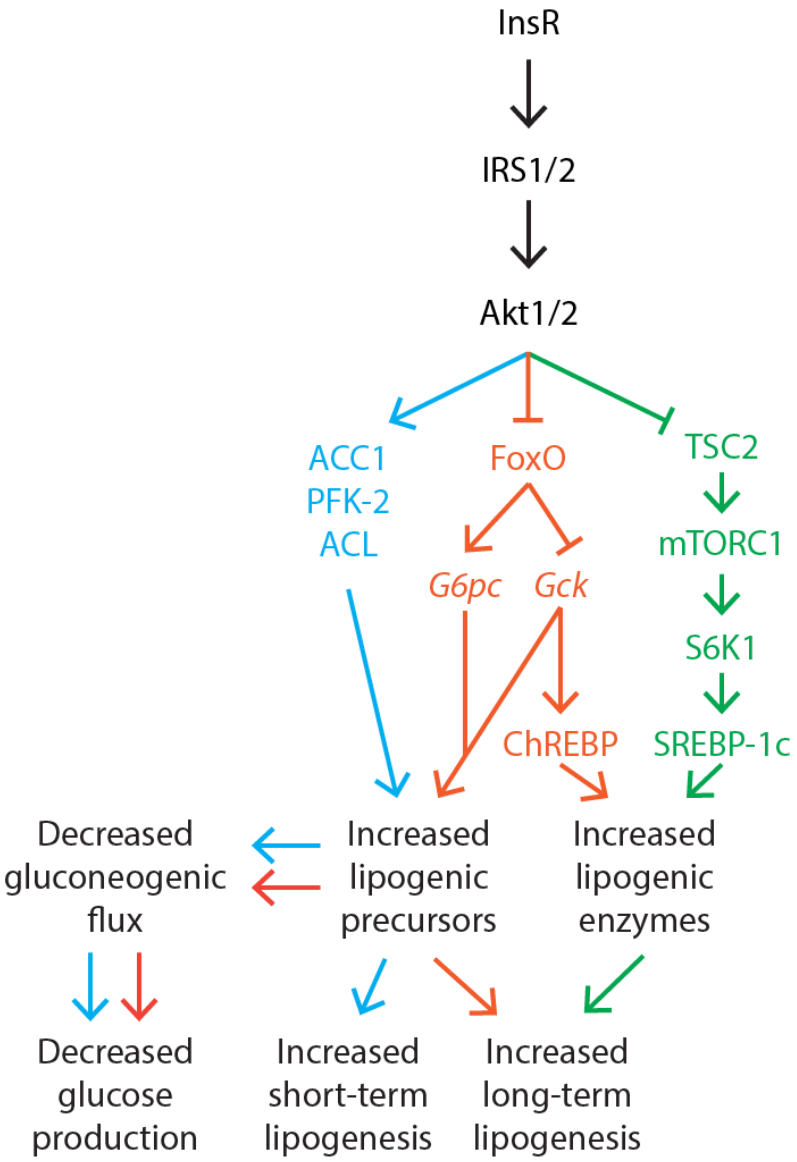


Figure 5.1. Three-Pronged Model of Insulin Action on DNL

Figure 5.1

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