HOW MUSCLE COMMUNICATES NUTRIENT STATUS TO REGULATE PHYSIOLOGIC HOMEOSTASIS

By

Marcus Seldin

A dissertation submitted to the Johns Hopkins University School of Medicine in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

May, 2014

©2014 Marcus Seldin

All Rights Reserved
Abstract

The integrated control of animal and human physiology requires intimate tissue crosstalk, a vital task mediated by circulating humoral factors. The recent realization that skeletal muscle, the largest organ in the human body, secretes a variety of biologically and metabolically active polypeptide factors (collectively called myokines) has provided a new conceptual framework to understand the coordination of whole-body physiology and energy balance. Here we report the identification and functional characterization of myonectin, the first skeletal muscle-specific secreted protein which links this critical organ to postprandial metabolic homeostasis. In mouse models we show myonectin mRNA and protein is suppressed with fasting, but dramatically induced upon nutrient availability. Further, myonectin exerts significant control over regulating whole-body glucose and fat metabolism through novel endocrine circuitry. Myonectin acts in a postprandial mechanism to reduce circulating lipid levels. This reduction is facilitated by enhanced expression of fatty-acid uptake genes in both adipose tissue and liver. Additionally, myonectin significantly reduces blood glucose levels by inhibiting glucose output, while simultaneously promoting uptake and deposition into liver. Given the dependence of myonectin expression on nutrient homeostasis, we further explored how the protein regulates hepatic autophagy, an important process in regulating metabolic fuels. Myonectin significantly reduced both acute and chronic liver autophagic functions by activating the well-described PI3Kinase/Akt/mTOR pathway. Our data suggests myonectin functions as a skeletal muscle nutrient sensor, which communicates the status of muscle energy reserves to other metabolically relevant organs. Given the important contribution of skeletal muscle (comprising ~40% of total body mass) to whole-body metabolism, these studies yield novel and fundamental insights into how myonectin links skeletal muscle to energy regulation in muscle and non-muscle tissues.
This basic knowledge can be utilized in a therapeutic setting to approach and treat Type 2 diabetes, obesity and other metabolic perturbations.

Thesis Advisor: Dr. Guang William Wong

Thesis Reader: Dr. Jennifer Pluznick
ACKNOWLEDGEMENTS

My mentor and advisor, Dr. Guang William Wong proved to be the most valuable and influential individual throughout my thesis career. He possesses an unmatched enthusiasm for science and facilitated a thoughtful and independent learning environment. Dr. Wong was not only the most productive, passionate and kind mentor on our entire campus, but I consider him one of my closest friends. I am also grateful to be in close communication with Dr. Wong’s best friend, Dr. Michael J. Wolfgang. Dr. Wolfgang spent countless hours with me reviewing experimental approaches, discussing current literature and instilled in me one of the most valuable tools for a scientist, critical thinking. Dr. Wolfgang taught me never to “believe the hype,” something which I will remember throughout my career. Dr. Wong and Wolfgang are also two of the most hilarious people I have ever met, a trait I consider of the utmost value.

I would also like to thank my lab colleagues for teaching me valuable scientific approaches, discussing various phenomena, troubleshooting problems and putting up with my gregarious nature. I was fortunate to receive guidance and input from Dr. Zhikui Wei and Dr. Jonathan Peterson during the beginning of my doctoral career. Dr. Wei showed me the value of focus in a scientific setting and helped guide me through how to approach scientific problems. Dr. Peterson taught me many experimental techniques and instructed me on areas of endocrine metabolism, muscle physiology and conservative politics. After Dr. Wei and Dr. Peterson moved on to their respective positions, the Wong lab underwent a dramatic shift in gender, aside from me. I am extremely fortunate to have been surrounded by some of the most intelligent, thoughtful and dedicated women. Dr. Xia (Shelley) Lei has proven to be the most efficient and talented mouse physiologist I have encountered. Importantly, Dr. Lei was always willing to go out of her way to help other members of the lab, enabling us to be so productive. I would also like to especially acknowledge my other colleague and one of my closest friends, Stefanie Tan.

~ iv ~
Stefanie possesses unrivaled dedication, intelligence and positive demeanor, which are quite helpful when working so closely during many late night hours and weekends.

Beyond the scope of those with whom I worked directly, there have been many other individuals who fostered my successful thesis career, especially Dr. Jessica Ellis and Dr. Susana Rodriguez. I hold Dr. Ellis in the highest of regards, as she guided me not only through thinking about science, but also the necessary steps to achieve a successful career. Dr. Rodriguez and her husband Colin Smith are dear friends who have helped me through difficult times and gone above-and-beyond to help me throughout my stay in Baltimore. I would also like to thank other colleagues Dr. Jing Zhu, Weibo Zho, Wan Fang Han, Dr. Santosh Ramamurthy, Hannah Little, Dr. Risa Wolf, Caitlyn Bowman and Jieun Lee.

I would like to thank my thesis committee Dr. Daniel Raben, Dr. Sejun Lee and especially, Dr. Paul Watkins for their guidance. Dr. Watkins has helped my through every stage of my graduate career, from serving on my orals committee to troubleshooting fatty-acid uptake assays; he is a very kind and supportive individual. Also, my graduate department has helped me through all the steps needed to become a successful Ph.D., as well as provide answers to my incessant questioning throughout the years.

On a personal note, a few individuals have served as a strong basis for support during my 5 years as a graduate student. I am lucky to have met one of my best friends in Baltimore, Basil Hussain. Basil is the among the most kind and considerate people I know, he is a true friend and also an incredible magician. I would also like to acknowledge two other individuals, Elizabeth Kolar and Michael Multhaup who have been great friends and shared many incredible memories throughout my doctoral career.
I want to thank my family for their love, support and kindness, despite being distanced from me. My mother, Lila Seldin, has instilled in me the enthusiasm, confidence, love and dedication needed to succeed. My father, Calvin Seldin, has given me the creativity and intellectual capacity to confront every problem with which I have been presented. My sister, Rebecca Seldin, has always shown me unconditional love and support and has shown me the importance of personal independence and strength. My Aunt, Robin Seldin, has given me a unique perspective on family and always been a strong foundation for support and role model. My grandmother, Leatrice Seldin, has taught both myself and my sister vital life lessons and always provided a foundation for love. My grandfather, Abe Seldin and Marion McManus, have always been kind, thoughtful and shared enthusiasm for my life and work. My Uncle, Aunt and Cousins, Frank, Gio, Emily and Elyse Seldin have provided me with a warm and welcoming family, as well as a new-found knowledge in Disney princesses.

Lastly, I would like to thank the love of my life, Phuong Nhat Duong for her support over the years. Phuong has always stood by my side with a loving demeanor and been the cornerstone for my continued success. Phuong has taught me the importance of strength, perseverance, not fearing confrontation and communication. Phuong has also shown me the value of questioning life at every turn and never backing down. Phuong is my personal hero and continues to serve as my foundation for love and support.
# TABLE OF CONTENTS

ABSTRACT ............................................................................................................................... i

ACKNOWLEDGEMENTS.......................................................................................................... iv

LIST OF FIGURES .................................................................................................................... ix

CHAPTER 1: Background and Overview ............................................................................... 10

Myokines: Skeletal muscle as an endocrine organ ............................................................... 11

C1q/TNF-related Proteins (CTRPs) ....................................................................................... 13

CHAPTER 2: Identification and characterization of myonectin regulation and its role in lipid metabolism .............................................................................................................. 29

Introduction ............................................................................................................................ 30

Experimental procedures ........................................................................................................ 31

Results .................................................................................................................................... 37

Identification of myonectin ................................................................................................. 37

Expression of myonectin in myotubes and skeletal muscle .................................................. 39

Myonectin forms disulfide-linked oligomers and heteromeric complexes with other CTRPs ................................................................................................................................. 39

Myonectin circulates in blood .............................................................................................. 41

Regulation of myonectin expression in myotubes ................................................................. 42

Exercise increases myonectin mRNA expression and circulating levels .............................. 44

Metabolic state regulates myonectin expression and circulating levels ................................. 45

Myonectin expression and circulating levels are reduced in the obese state ......................... 47

Recombinant myonectin administration lowers circulating free fatty acids levels ............... 48

Recombinant myonectin promotes fatty acid uptake but not adipose tissue lipolysis ........... 50

Discussion ............................................................................................................................... 53

CHAPTER 3: Myonectin Links Skeletal Muscle to Carbohydrate Metabolism in Liver ............ 56

Introduction ............................................................................................................................ 57

Experimental procedures ........................................................................................................ 58

Results .................................................................................................................................... 61

Myonectin lowers blood glucose in fasted mice ................................................................. 61

Myonectin suppresses hepatic glucose .............................................................................. 63

Myonectin enhances hepatic glucose uptake ...................................................................... 65

Myonectin activates glycogen synthase to promote glycogenesis ....................................... 66
Myonectin and insulin act independently to inhibit gluconeogenesis and promote glucose uptake .................................................................67
Discussion .................................................................................................................................69
CHAPTER 4: Myonectin activates the mTOR pathway to suppress autophagy in liver ..........71
Introduction .................................................................................................................................72
Experimental procedures ...........................................................................................................73
Results .....................................................................................................................................77

Myonectin expression is repressed by starvation and induced by nutrient supplementation .................................................................77

Myonectin suppresses the expression of autophagy genes in H4IIE hepatocytes and mouse liver ............................................................................79
Myonectin inhibits LC3 lipidation and autophagosome-dependent p62 degradation .....81
Myonectin activates the Akt/mTOR pathway to suppress autophagy ........................................84
Inhibition of mTOR signaling abrogates myonectin suppression of autophagy ....86

Discussion ................................................................................................................................ 89
Summary of Major Findings .........................................................................................................92
REFERENCES ........................................................................................................................ 96
CURRICULUM VITAE ........................................................................................................ 120
LIST OF FIGURES

FIGURE 1-1. Schematic of CTRPs ................................................................. 28

FIGURE 2-1. The deduced myonectin protein and its expression in skeletal muscle and cultured myotubes ................................................................. 38

FIGURE 2-2. Myonectin is secreted as a multimeric protein that can form heteromeric complexes with other CTRP family members ......................................... 40

FIGURE 2-3. Myonectin is produced by skeletal muscle and circulates in plasma ................. 42

FIGURE 2-4. Myonectin expression in myotubes is upregulated by an increase in cellular cAMP or calcium levels ................................................................. 43

FIGURE 2-5. Exercise increases myonectin expression in skeletal muscle as well as circulating levels ................................................................. 44

FIGURE 2-6. Nutritional state regulates the expression and circulating levels of myonectin ................................................................. 46

FIGURE 2-7. High-fat diet reduces myonectin expression and its circulating levels .......... 47

FIGURE 2-8. Recombinant myonectin administration reduces serum non-esterified free fatty acid levels in mice ................................................................. 49

FIGURE 2-9. Recombinant myonectin has no effect on adipocytes or adipose tissue lipolysis. ............................................................................................................ 50

FIGURE 2-10. Myonectin enhances fatty acid uptake in 3T3-L1 adipocytes and H4IIE hepatocytes via transcriptional mechanism ........................................... 52

FIGURE 3-1. Myonectin reduces fasting blood glucose levels in mice ................................ 62

FIGURE 3-2. Myonectin inhibits hepatic gluconeogenesis .............................................. 64

FIGURE 3-3. Myonectin promotes glucose uptake in hepatocytes .................................... 65

FIGURE 3-4. Myonectin enhances glycogenesis ............................................................. 66

FIGURE 3-5. Additive effects of myonectin and insulin on hepatocytes ............................. 68

FIGURE 4-1. Myonectin expression and circulating levels are reduced by starvation and increased by nutrient availability .................................................. 78

FIGURE 4-2. Myonectin suppresses the expression of starvation-induced autophagy genes ... 80

FIGURE 4-3. Myonectin reduces autophagosome formation and autophagy-mediated p62 degradation ................................................................. 83

FIGURE 4-4. Myonectin activates Akt/mTOR pathway in H4IIE hepatocytes and mouse liver ........................................................................................................... 85

~ ix ~
FIGURE 4-5. Inhibition of PI3K-AKT-mTOR signaling prevents myonectin from suppressing autophagy-mediated LC3 lipidation, $Atg$ expression and p62 degradation .......... 87

FIGURE 4-6. Inhibition of Akt-mTOR signaling abolishes the ability of myonectin to suppress autophagy in hepatocytes .......................................................... 88
HOW MUSCLE COMMUNICATES NUTRIENT STATUS TO REGULATE PHYSIOLOGIC HOMEOSTASIS

CHAPTER 1: Overview*

*Most text and figures in this chapter were adopted from (ref 270, Seldin MM and Wong GW, 2013 and ref 271, Seldin MM et al., 2013)
Myokines: Skeletal muscle as an endocrine organ

Skeletal muscle, the largest organ in the human body, plays a vital role in maintaining whole-body metabolic homeostasis. A major proportion of the circulating postprandial glucose is taken up by skeletal muscle in response to insulin via the GLUT4 glucose transporter, and metabolized or stored in the form of glycogen (1). Impaired insulin responsiveness in muscle is a hallmark of type 2 diabetes (2). The recent discovery that skeletal muscle in fact secretes a variety of biologically active polypeptide factors (collectively termed myokines) that can act in an autocrine, a paracrine, and/or an endocrine fashion to regulate metabolic and inflammatory processes gives a new dimension to the role of muscle in coordinating integrated physiology (3). Proteomics approaches to cataloging the secretome of cultured mouse and human myotubes reveal hundreds of secreted proteins (4, 5), many of which likely play a role in diverse cellular processes. The inter-tissue crosstalk mediated by myokines undoubtedly provides a greater sense of appreciation for the complexity of metabolic circuits governing systemic energy balance.

Myostatin was the first described myokine, a secreted protein belonging to the TGF-β superfamily and a negative regulator of muscle growth (6). A loss-of-function mutation of myostatin in human, or knockout mice lacking myostatin, results in striking doubling of muscle mass (6, 7). More recently, the functions of other myokines such as IL-6 (3), FGF-21 (8, 9), Insulin-like 6 (Insl6) (10), follistatin-like 1 (Fstl-1; also known as TSC-36) (11), LIF (12), IL-7 (13), IL-15 (14), musclin (15), and irisin (16) have been described. These myokines either act locally within skeletal muscle, serving as autocrine/paracrine factors, or circulating in blood as endocrine factors linking skeletal muscle to regulation of physiological processes in other tissues. In the context of metabolism, IL-6 is the most extensively characterized myokine (17). Secreted by skeletal muscle fiber in response to exercise, IL-6 has been shown to improve
whole-body insulin sensitivity and dampen inflammation, providing a link between exercise and the improvement in systemic metabolic parameters (17, 18). However, the contrasting role of IL-6 as a pro-inflammatory cytokine that induces hepatic insulin resistance has yet to be fully reconciled (19, 20). In mice, Fstl-1 links skeletal muscle to the vasculature, promoting endothelial cell function and revascularization in ischemic tissue (11). A gain-of-function mouse model demonstrates a role for muscle-derived IL-15 in regulating fat mass in response to metabolic insults resulting from high fat-feeding (14), highlighting a muscle-adipose axis controlling energy balance.

Much excitement and discussion surrounds the identification of Fndc5/Irisin, a gene whose expression is regulated by the transcriptional co-activator, PGC1-α (16). It was discovered as a gene up-regulated in skeletal muscle of mice that over-expressed a PGC1-α transgene. Fndc5 is synthesized as a type I transmembrane protein; proteolytic processing generates a soluble form (designated as irisin) that circulates in blood. Interestingly, exercise increases circulating levels of irisin in humans and mice. Remarkably, adenovirus-mediated over-expression of irisin turns on the thermogenic program in subcutaneous fat depot by inducing the “browning” of white adipose tissue. The increased number of brown adipocyte-like cells with UCP-1 expression within the white adipose tissue enhances fat oxidation, in so doing promotes energy expenditure and improves systemic insulin sensitivity. Thus, the metabolic action of muscle-derived irisin on fat depot provides one molecular mechanism accounting for the benefit of exercise. However, despite a major resurgence in the study of brown fat (21), its purported role in maintaining energy balance by burning off excess calories remains a hotly debated issue (22).
Analogous to the importance of fat tissue-derived adipokines in regulating systemic insulin sensitivity and glucose and lipid metabolism in multiple tissue compartments (33), skeletal muscle-derived myokines are poised to play an equally important role in mediating inter-tissue crosstalk to control integrated physiology. In a broader context, elucidating the myokine-mediated metabolic circuits will provide valuable insights into complex networks governing energy homeostasis, the disruption of which likely contributing to metabolic diseases.

**C1q/TNF-Related Proteins (CTRPs)**

Mammals use complex central and peripheral mechanisms to maintain proper energy balance (34-35). Among peripheral mechanisms, secreted hormones play a particularly important role (35-38). They mediate inter-organ communication and tissue crosstalk to coordinate the integrated control of whole-body metabolism. Circulating levels of many secreted hormones, such as insulin, leptin, adiponectin, and FGF21, are dynamically regulated in response to short-term changes in nutritional status or long-term changes in metabolic state (35-39). Sustained metabolic perturbations due to excess caloric intake, as in obesity, frequently disrupt the signaling pathways regulated by these circulating hormones. Consequently, hormonal axis deregulation is mechanistically linked to common metabolic disorders such as obesity, insulin resistance, and type 2 diabetes (39).

*Adiponectin and the C1q family*

Among peripheral tissues, adipose fulfills two critical roles—as the major storage depot for triglycerides and as an endocrine organ that secretes active polypeptide hormones into circulation (40). Adipose-derived secretory proteins are collectively called adipokines and act in an
autocrine, paracrine, and/or endocrine manner to modulate insulin sensitivity and glucose and fatty acid metabolism (39). Adipokines may also influence whole-body metabolism by indirectly modulating inflammatory processes in adipose and other metabolic tissues (41). Adiponectin is a pleotropic insulin-sensitizing adipokine that has undergone extensive genetic and correlative studies in humans and mechanistic studies in transgenic and knockout (KO) mice (42-43).

Despite the widely described anti-diabetic, anti-atherogenic, and anti-inflammatory properties of adiponectin (42-44), adiponectin KO mice exhibit highly variable phenotypes (45-49). Three independent adiponectin KO mouse lines develop some degree of insulin resistance when fed a high-fat diet, while another line exhibits enhanced fat oxidation; all four KO mouse lines have mild or undetectable metabolic abnormalities when fed a standard chow diet (45-47). The generally mild metabolic phenotypes of adiponectin KO mice have been partially attributable to enhanced leptin sensitivity (50). However, despite attempts to provide a unifying mechanism of action for adiponectin (51), a clear understanding of the pleiotropic functions of adiponectin has been confounded by conflicting studies concerning its role in glucose metabolism (52), atherosclerosis (49), food intake (53, 54), inflammation (55, 57), and tumor angiogenesis (58, 59). Additional mechanisms may partially account for the variable and mild metabolic alterations of adiponectin KO mice (60-61).

Adiponectin belongs to the larger C1q protein family, defined by the presence of a C-terminal globular domain with sequence homology to the immune complement protein C1q (62). Human and mouse genomes encode >30 C1q domain-containing proteins (62-65). These include the founding immune complement C1q (A-, B-, and C-chain) (66), as well as multimerins (7), emilins (64), C1q/TNF-related proteins (CTRPCs) (56), cerebellins (36), adiponectin (67), otolin (68), C1q-related factor (CRF) (69), C1qDC1/caprin-2 (70), and non-fibrillar collagen VIII (71,
72) and collagen X (74). With the exception of cytosolic C1qDC1 (72), all C1q family members are secreted proteins that likely evolved from a common C1q domain-containing ancestral protein. C1q proteins play diverse roles in mammalian physiology, including immunity, development, and metabolism (7, 62, 66, 74, 75). Of the C1q family members, CTRP1-15 share strikingly similar structural organization and biochemical properties with adiponectin (24-25, 28-30, 60, 70-78). Here, we highlight the unique and shared characteristics between CTRPs and adiponectin; overlapping metabolic functions with CTRPs could account for the mild phenotypes of adiponectin KO mice (61).

CTRPs were identified by sequence homology with the globular domain of adiponectin (56). Further, the overall CTRP domain organization is similar to adiponectin; every CTRP contains a signal peptide to direct protein secretion, an N-terminal domain with one or more conserved Cys residues, a collagen-like domain with variable numbers of Gly-X-Y repeats, and a C-terminal globular C1q domain (Fig. 1). All CTRPs are highly conserved throughout vertebrate evolution, and clear orthologs can be identified from zebrafish, frog, mouse, and human genomes. Of the CTRPs, CTRP9 shares the highest degree of sequence identity (54%) with adiponectin at the presumed functional globular domain (25). Unlike adiponectin, whose transcript is expressed almost exclusively by adipocytes (67), CTRPs are widely expressed in human and mouse tissues (24-25, 28, 30, 60, 73-78). Although adipose predominantly expresses CTRP1, CTRP2, CTRP3, CTRP5, CTRP7, CTRP9, CTRP12, and CTRP13, each CTRP has a unique tissue expression profile that may reflect unique functions (25, 27-28, 30).

CTRП structural organization

~ 6 ~
All characterized C1q members, including CTRPs, form trimers (79-84). Adiponectin trimers can be further assembled into higher-order hexameric and octadecameric complexes (85-90). Assembly of these structures requires the conserved N-terminal Cys-39 residue and is achieved with the help of endoplasmic reticulum proteins such as ERp44 and DsbA-L (91, 92). CTRP3, CTRP5, CTRP6, CTRP9, CTRP10, CTRP12, CTRP13, and CTRP15/myonectin also form multimeric complexes that are assembled through the conserved N-terminal Cys residues (25, 30, 55, 60, 75, 76). CTRP11 forms multimeric complexes through an interaction with the oxidoreductase ERp44, but conserved N-terminal Cys-28 and Cys-32 residues are not required for assembly (51). While different adiponectin oligomers may have distinct signaling properties (85, 93, 94) that correlate with insulin sensitivity in humans (89, 95-97), the functional significance of CTRP oligomers remains largely undefined, with the exception of CTRP12 (96).

CTRPs posttranslational modifications

When secreted in mammalian cells, CTRP1, CTRP2, CTRP6, CTRP12, and CTRP15/myonectin contain N-linked glycans, but CTRP3, CTRP5, CTRP9, CTRP10, CTRP11, and CTRP13 contain other carbohydrate moieties (25, 30, 55, 60, 75, 76). Mass spectrometry revealed that proline residues within the Gly-X-P repeats and lysine residues within the consensus GXKG(E/D) motif in the N-terminal collagen domain of CTRP9 are hydroxylated and glycosylated, respectively (25). Proline hydroxylation and lysine glycosylation within the collagen domain affect the stability, function and biological potency of adiponectin (99-101). One or more GXKG(E/D) motifs are present in the collagen domain of all CTRPs except CTRP4, CTRP12, and CTRP15/myonectin, suggesting these modifications may occur in other CTRPs to affect protein stability and/or function. Adiponectin also contains sialic acids important for stability (102-103), although it is presently unknown whether any CTRPs are modified with sialic acid. The potential
influence of posttranslational modifications on assembly of higher-order CTRP structures underscores the importance of using recombinant CTRPs produced in mammalian expression systems to conduct functional studies. Recombinant proteins produced in mammalian cells, rather than bacteria, are likely to possess native posttranslational modifications and higher-order structures to confer biological activity.

CTRPs in circulation

When specific antibodies are available, endogenous CTRPs can be detected circulating in the blood (25, 30, 55, 60, 75, 76). Although adiponectin circulates in human plasma at high concentrations (10-30 μg/mL), CTRPs circulate at 1-2 orders of magnitude less than adiponectin (80-83). In both humans and mice, sex and genetic background influence metabolic hormone levels and signaling pathways, and thus variably influence the development of obesity, insulin resistance, and type 2 diabetes (108-111). Females have significantly higher circulating adiponectin levels (86, 112-113), which may be due to the ability of testosterone to suppress adiponectin (112-114). Likewise, the expression and circulating levels of some CTRPs also exhibit sexually dimorphic patterns, with female mice expressing higher levels of CTRP5, CTRP9, CTRP11, and CTRP13 relative to males (25, 27, 60, 75). However, it is unclear whether sex hormones directly modulate CTRP expression levels. Alterations in metabolic state also affect circulating CTRP levels consistent with metabolic function (25, 27-30, 55). Adiponectin expression levels are consistently reduced in obesity and type 2 diabetes (115-120); similarly, circulating levels of CTRP1, CTRP3, CTRP9, CTRP12, and CTRP15/myonectin are also reduced in diet-induced obese (DIO) mouse models (28-30, 55).

CTRP metabolic functions
The metabolic function and regulation of adiponectin has been extensively studied in the past decade (42-43); however, much less is known about the regulation and function of CTRPs. Here, we summarize current understanding of CTRP metabolic functions, as non-metabolic roles have been reviewed elsewhere (74).

**CTRP1**

CTRP1 is predominantly expressed by adipose tissue (60). Circulating CTRP1 levels decrease in DIO (28) and increase in adiponectin KO mice or mice given daily injections of the anti-diabetic drug rosiglitazone (60), suggesting that increased circulating CTRP1 levels may have a positive metabolic effect. Concordantly, administration of physiological amounts of recombinant CTRP1 to wild-type mice acutely and substantially lowers blood glucose (60). Further, a two-fold increase in circulating CTRP1 levels modestly improves insulin sensitivity and decreases high-fat diet-induced weight gain in transgenic mouse models (28). Reduced weight gain in response to high-fat feeding is primarily due to increased energy expenditure from enhanced fat oxidation in skeletal muscle (28). These *in vivo* effects are mediated by the highly conserved AMP-activated protein kinase (AMPK). In skeletal muscles of transgenic mice, AMPKα and its downstream target acetyl-CoA carboxylase (ACC) are hyperphosphorylated (28).

Phosphorylation of AMPKα at Thr-172 activates the kinase, whereas AMPK phosphorylation of ACC at Ser-79 inactivates the carboxylase (121). Inactivation of ACC reduces malonyl CoA levels to promote fatty acyl-CoA import into mitochondria for β-oxidation (121), reflecting a direct action of CTRP1 on AMPK signaling in muscle cells. In addition, recombinant CTRP1 can recapitulate increases in skeletal muscle AMPKα (Thr-172) and ACC (Ser-79) phosphorylation in wild-type mice (28). These studies collectively indicate that CTRP1 is a novel secreted regulator of skeletal muscle fat oxidation.
CTRP3

CTRP3, also known as CORS26/cartducin (122), is expressed by adipocytes (124-126) and stromal cells (60) in adipose tissue and in other tissues (60, 123, 126-128). Overnight fasting increases circulating CTRP3 levels relative to mice fed *ad libitum*, while high-fat feeding reduces CTRP3 levels by ~50% (29). In humans, circulating CTRP3 levels positively correlate with adiponectin and negatively correlate with waist circumference, blood pressure, fasting glucose, triglycerides, and cholesterol (105). Additionally, CTRP3 regulates glucose metabolism. Increased circulating CTRP3 levels substantially lower blood glucose in wild-type and insulin-resistant, leptin-deficient obese *ob/ob* mice (29). These results suggest CTRP3 influences metabolism independently of insulin. CTRP3 targets liver hepatocytes to suppress gluconeogenic gene expression (*G6Pase* and *PEPCK*) and gluconeogenesis via the activation of protein kinase B/Akt signaling (29). This differs from the function of adiponectin in liver to activate AMPK to suppress hepatic glucose output (129, 130), although the role of AMPK in this process has been recently challenged (131).

We generated transgenic mice with elevated circulating CTRP3 levels (132). When challenged with a high-fat diet, CTRP3 transgenic mice exhibit remarkable resistance to the development of hepatic steatosis despite similarities in body weight, food intake, and energy expenditure (132). Further, DIO mice administered daily physiological amounts of recombinant CTRP3 for five consecutive days exhibit reduced hepatic triglyceride content, confirming the direct action of CTRP3 in the liver (132). Mechanistically, CTRP3 reduces hepatic triglyceride content by inhibiting the expression of enzymes involved in triglyceride synthesis (GPATs, AGPATs, and DGATs) (132); it has no appreciable effect on hepatic fat oxidation, unlike adiponectin (133).
Adiponectin also improves alcoholic and non-alcoholic fatty liver phenotypes, although triglyceride synthesis enzymes were not examined (133).

Given the importance of chronic inflammation in obesity (134), it is interesting to note that CTRP3 may also modulate metabolism indirectly through anti-inflammatory processes (74). Recombinant CTRP3 reduces lipopolysaccharide-induced inflammation in monocytes (135), adipocytes (136) and colonic fibroblasts (137); however, the physiological relevance of these in vitro studies awaits in vivo confirmation.

CTRP3 also has non-metabolic functions in the vasculature and heart. Recombinant CTRP3 induces endothelial cell proliferation and migration in vitro by activating ERK1/2 and p38 MAPK signaling (138), implying a potential role of CTRP3 in regulating angiogenesis. Myocardial infarction induced by coronary artery occlusion substantially reduces CTRP3 expression in adipose tissue and in circulation in mice, although reconstitution of CTRP3 expression significantly restores cardiac function and survival rates (139). CTRP3 activation of Akt, but not AMPK, signaling attenuates cardiomyocyte apoptosis, suppresses interstitial fibrosis, and increases revascularization following myocardial infarction (139). Together, these studies highlight a novel and important role for CTRP3 in modulating metabolic, immune, and cardiovascular functions.

CTRP5 is widely expressed, with highest levels detected in the eye and adipose tissue (60). Prolonged mitochondrial depletion induces CTRP5 expression in rat L6 myocytes (140). Further, expression of recombinant GST-tagged CTRP5 or an un-tagged C-terminal globular head stimulates AMPK signaling to translocate GLUT4 and enhances fat oxidation via the AMPK-ACC pathway in vitro (140). These studies imply an autocrine function for CTRP5 in
muscle in response to reduced mitochondrial content. CTRP5 is also expressed by cultured mouse and human adipocytes and circulates in human serum (107). Saturated fatty acids upregulate CTRP5 expression in adipocytes, where CTRP5 acts in an autocrine fashion to reduce adiponectin and resistin secretion (107). While the physiological relevance of CTRP5 in metabolism remains uncertain, a point mutation (S163R) impairs folding and secretion of human CTRP5 to cause an autosomal dominant form of late-onset retinal macular degeneration (L-ORD) (141-143). S163R knock-in mouse models of L-ORD have yielded conflicting results—one model successfully recapitulates the retinal degeneration phenotype (145), while another does not (146).

**CTRP9**

CTRP9 is the closest paralog of adiponectin and shares the highest degree of amino acid identity (54%) at the globular domain. Adipose predominantly expresses CTRP9 (25). In leptin-deficient obese *ob/ob* mice, CTRP9 expression increases in adipose tissue and in circulation at 8 weeks of age relative to lean controls, but subsequently normalizes by 12 weeks of age (25). This observation suggests a possible compensatory response in young *ob/ob* mice prior to developing severe obesity and insulin resistance. Concordantly, circulating CTRP9 levels are significantly reduced in DIO mice, a model more closely resembling the common form of human obesity induced by excess caloric intake (Peterson and Wong, unpublished data). A modest (~40%) increase in circulating CTRP9 levels by adenoviral-mediated expression acutely reduces serum glucose levels in *ob/ob* mice without altering body weight and plasma insulin levels (25). In transgenic mouse models where circulating CTRP9 levels are chronically elevated, animals have reduced caloric intake and increased metabolic rate in response to high-fat feeding. Consequently, CTRP9 transgenic mice are strikingly resistant to weight gain when challenged
with a high-fat diet for 12 weeks (216). Reduced adiposity is due to chronic activation of AMPK in the skeletal muscle, increasing mitochondrial content and up-regulating expression of genes that mediate fat oxidation. As expected, lean CTRP9 transgenic mice fed a high-fat diet exhibit improved systemic insulin sensitivity and fail to develop hepatic steatosis (216). These observations suggest CTRP9 enhances fat oxidation in an AMPK-dependent manner.

The metabolic roles of CTRP9 functionally overlap with those of adiponectin. Adiponectin, in particular its globular domain, enhances skeletal muscle fat oxidation by activating the AMPK signaling pathway (130, 147, 148). Over-expression of adiponectin also improves insulin function in rat skeletal muscle (148); however, other studies concluded that liver, not skeletal muscle, is the predominant adiponectin target in vivo (120, 150). It remains to be confirmed, using skeletal muscle-specific adiponectin receptor KO mice, whether adiponectin acts directly on skeletal muscle to exert some of its whole-body metabolic effects (151).

CTRP9 also controls cardiac and endothelial cell functions. In human umbilical vein endothelial cells and in freshly isolated vascular rings, recombinant CTRP9 increases nitric oxide production to induce vasodilatation through the AMPK/eNOS pathway (152). CTRP9 also benefits cardiac function in response to stress. Overexpression of CTRP9 reduces myocardial infarct size and hypoxia-induced apoptosis of cardiac myocytes following myocardial ischemia/reperfusion through AMPK signaling (153). Expression of only the C-terminal globular domain of CTRP9 also attenuates oxidative stress and myocardial infarct size induced by ischemia/reperfusion injury and enhances cardiac output in DIO mice (154). In another model of femoral artery injury, adenoviral CTRP9 overexpression substantially reduces neointimal formation by suppressing vascular smooth muscle cell proliferation and migration through the cAMP/PKA/ERK pathway (155). Since obesity and insulin resistance are risk factors for vascular and cardiac dysfunction...
(156-158), these studies highlight the potential protective roles of CTRP9 in the heart in response to stress.

**CTRP11**

CTRP11, predominantly expressed by white and brown adipose tissues (76). Sequence alignment indicates striking degree of amino acid identity between CTRP11 of different vertebrate species (76). Within white adipose tissue, CTRP11 is primarily produced by stromal vascular cells (76). CTRP11 expression is acutely regulated by changes in metabolic state, as overnight fasting followed by re-feeding upregulates expression (76). The lack of a CTRP11-specific antibody precludes analysis of the endogenous protein; hence, it remains uncertain whether CTRP11 also circulates in blood. Functional studies suggest a role for CTRP11 in regulating adipogenesis (76). CTRP11 suppresses 3T3-L1 mouse cell differentiation into mature adipocytes by inhibiting the expression of PPAR-γ and C/EBP-α, two major transcriptional regulators that drive adipogenesis (159-161). Further, CTRP11 inhibits mitotic clonal expansion of 3T3-L1 pre-adipocytes (98), a process essential for adipocyte differentiation in culture (162). Suppression of adipogenesis is specific, as expression of other structurally-related proteins such as CTRP1, CTRP10, and adiponectin do not influence 3T3-L1 differentiation. These *in vitro* studies indicate that CTRP11 likely acts in a paracrine manner to mediate crosstalk between adipocytes and cells of the stromal vascular compartment to maintain adipose tissue homeostasis (76).

**CTRP12**

Sequence alignments between CTRP12 and other members of the C1q family indicate only modest sequence identity at the globular C1q domain (30, 77). Human adipose tissue predominantly expresses CTRP12, although expression is more widespread in mice (30). Unlike other CTRPs, endogenous CTRP12 exists in two differently sized isoforms—a full-length and a
cleaved globular isoform. Furin/PCSK3, a member of the proprotein convertase family, cleaves CTRP12 at Lys-91 within the N-terminal KKXR motif (98). The two CTRP12 isoforms differ in oligomeric structure and signaling specificity; full-length protein preferentially activates Akt signaling, whereas the globular gCTRP12 isoform preferentially activates p44/42-MAPK and p38-MAPK signaling (98). While CTRP12 expression in the adipose tissue of DIO mice is significantly reduced (30, 77), its expression in cultured adipocytes is acutely induced by insulin or rosiglitazone treatment (30, 98). Cleavage of CTRP12 appears to be enhanced in DIO mice (163).

A modest increase in circulating CTRP12 levels by adenoviral expression or recombinant protein administration is sufficient to lower blood glucose and improve insulin sensitivity in three different mouse models—lean wild-type, DIO, and leptin-deficient ob/ob mice (30). These metabolic improvements are mediated by enhanced insulin signaling in the liver and adipose tissue, but not in skeletal muscle (30). Independent of insulin, recombinant CTRP12 suppresses gluconeogenesis in cultured hepatocytes and promotes glucose uptake in adipocytes. Thus, CTRP12 improves metabolic function through both insulin-dependent and -independent mechanisms. Adipose tissue inflammation is dampened in obese mice overexpressing CTRP12, suggesting the anti-inflammatory action of CTRP12 is responsible for whole-body insulin sensitivity improvements (76). In contrast, adenoviral overexpression of CTRP12 does not alter adipose tissue histomorphology or inflammatory gene expression (e.g., IL-1β, IL-6, TNF-α) despite remarkable improvements in systemic insulin sensitivity and glucose homeostasis (30). CTRP12 is thus a novel adipokine with beneficial anti-diabetic properties, although future studies using loss-of-function mouse models will help clarify its mechanism of action.

\textit{CTRP13}
CTRP13 is preferentially expressed in adipose and brain tissues of mice and in adipose tissue of humans (27). Within mouse adipose tissue, CTRP13 is also mainly produced by cells of the stromal vascular compartment. In cultured adipocytes, myotubes, and hepatocytes, recombinant CTRP13 activates AMPK signaling to promote glucose uptake. CTRP13 also partially reverses lipid-induced insulin resistance in hepatocytes by suppressing SAPK/JNK stress signaling, which impairs insulin signaling (27). Further, CTRP13 activates AMPK signaling to reduce gluconeogenesis in H4IIE hepatocytes by inhibiting gluconeogenic enzymes G6Pase and PEPCK. However, the physiological relevance of CTRP13 in peripheral tissues remains to be determined in vivo.

In the brain, CTRP13 modulates food intake as an anorexigenic factor (164). Wild-type mice given restricted access to food downregulate hypothalamic CTRP13 expression, whereas DIO mice upregulate expression. Further, recombinant CTRP13 administered via intracerebroventricular cannulae suppresses food intake and reduces body weight in mice (164). Interestingly, CTRP13 and the orexigenic neuropeptide AgRP reciprocally regulate each other’s expression in the hypothalamus; central CTRP13 delivery suppresses Agrp expression, while AgRP delivery increases Ctrp13 expression. Food-restricted mice have reduced CTRP13 and increased orexigenic neuropeptide (NPY and AgRP) expression in the hypothalamus. In contrast, when food restriction is coupled to enhanced physical activity in an activity-based anorexia mouse model, hypothalamic expression of both CTRP13 and AgRP increases (164). These results suggest that CTRP13 and AgRP form a hypothalamic feedback loop to modulate food intake and that this neural circuit may be disrupted in anorexia.

CTRP15/myonectin
Unlike other CTRPs, CTRP15 is a myokine expressed predominantly by mouse and human skeletal muscle (55). The term myonectin was used for CTRP5 in a recent study (165). To prevent confusion in nomenclature, CTRP5 retains its original designation (24, 140, 141), while CTRP15 is now referred to as myonectin (55). Expression and circulating levels of CTRP15/myonectin are upregulated upon re-feeding after fasting and significantly downregulated in DIO mice (55). Interestingly, CTRP15/myonectin expression is differentially regulated between different muscle fiber types. At basal levels, CTRP15/myonectin is expressed more in oxidative slow-twitch fibers compared to fast-twitch glycolytic fibers. Re-feeding after fasting increases CTRP15/myonectin expression ~80-fold in slow-twitch fibers but only ~4-fold in fast-twitch fibers (55). Administration of glucose or lipid after overnight fasting increased serum CTRP15/myonectin levels four-fold; likewise, nutrient-starved myotubes upregulated CTRP15/myonectin expression similarly to glucose or fatty acid supplementation. These observations suggest that CTRP15/myonectin is a postprandial hormone produced and secreted by skeletal muscle in response to nutrient flux. Consistent with this notion, administration of recombinant CTRP15/myonectin to mice significantly lowered circulating free fatty acids by promoting hepatocyte uptake through upregulation of CD36, fatty acid binding proteins, and fatty acid transport proteins (55).

**CTRP receptors**

Three adiponectin receptors—adipoR1, adipoR2, and T-cadherin—have been identified by expression cloning strategies (166, 167). An additional distinct macrophage receptor for adiponectin is thought to exist, although this receptor remains elusive (168). In addition, the calreticulin/CD91 complex may bind adiponectin on the plasma membrane of macrophages to facilitate the removal of apoptotic cells (169). Since T-cadherin is a GPI-anchored plasma
membrane protein (170), it is unclear how T-cadherin transduces a signal in response to adiponectin binding. Nonetheless, KO mice demonstrate that T-cadherin largely mediates the cardioprotective effects of adiponectin (171). AdipoR1 and adipoR2 contain seven transmembrane domains with inverse GPCR topologies. APPL1 is an intracellular adaptor protein that couples adiponectin binding to adipoR1/2 to intracellular signal transduction (172-176). Mice lacking adipoR1 or adipoR2 show variable and opposing phenotypes (177-179). In general, the metabolic phenotypes of adipoR1/2 KO mice are more striking than the relatively mild metabolic phenotypes of adiponectin KO mice. This complex story is ongoing (51, 180).

No CTRP receptors have been definitively identified. AdipoR1 may partially mediate the effects of CTRP9 on vascular endothelial cells (152) and cardiomyocytes (153), although these studies relied entirely on RNA interference approaches. No evidence demonstrates that CTRP9 interacts with adipoR1 on the plasma membrane of intact cells. FACS analysis can monitor receptor-ligand interactions on live cells, suggesting these experiments are feasible. Since adipoR1 and adipoR2 belong to the 11-member PAQR family of transmembrane proteins (181), it is tempting to speculate that other PAQR family members may be CTRP receptors.

Concluding remarks on CTRP Family

Recent studies have provided insight into the metabolic roles of CTRP proteins. Although much has been learned since CTRPs were initially described, many more questions remain to be addressed. CTRPs possess unique and shared functions supported by a high degree of vertebrate conservation. Future studies with gain-of-function and loss-of-function mouse models will reveal novel insights into the physiological function, mechanism of action, and possible redundancy of each CTRP in normal and disease states. Going forward, identifying CTRP receptors will remain
a major challenge but will provide enormous insights into the signaling pathways controlled by each CTRP to mediate its unique biological function.

FIGURE 1-1. Schematic of CTRPs. With the exception of CTRP4, every CTRP consists of four domains: a signal peptide for secretion (dark grey), an N-terminal domain with one or more conserved Cys residues (white), a collagen domain with varying numbers of Gly-X-Y repeats (light gray), and a C-terminal globular domain homologous to the immune complement C1q (blue). The numbers on the right refer to the percent amino acid identity between human and mouse orthologs when comparing the full-length protein (first column) or the C-terminal globular domain (second column). CTRP4 and CTRP8 are omitted—the former consists of only two tandem C1q domains, while the latter is absent in the mouse genome.
HOW MUSCLE COMMUNICATES NUTRIENT STATUS TO REGULATE PHYSIOLOGIC HOMEOSTASIS

CHAPTER 2: Identification and characterization of myonectin regulation and its role in lipid metabolism*

*Most text and figures in this chapter were adopted from (ref 23, Seldin MM et al., 2012)
Introduction

A large proportion of diet-derived glucose is taken up by skeletal muscle in response to insulin, and excess glucose is stored in muscle as glycogen until mobilized (182). In addition, muscle burns large amounts of fat via mitochondrial β-oxidation in response to energy demands (183). Insulin resistance in skeletal muscle has long been recognized to be an important underlying mechanism of type 2 diabetes (2). While the importance of skeletal muscle in controlling whole-body glucose and lipid metabolism is well established, its role as an endocrine tissue that secretes biologically active polypeptide hormones and cytokines (collectively termed myokines) involved in modulating metabolic, inflammatory, and other physiological processes in non-muscle tissues has only recently been appreciated (3).

Recent proteomics studies focusing on the secretome (the entire complement of secreted proteins) of cultured mouse or human myotubes have revealed a large number (~250 in human and ~600 in mouse) of muscle cell-derived secretory proteins with potential autocrine, paracrine, and/or endocrine functions (4, 5). IL-6 (3), FGF-21 (6,9), Insulin-like 6 (Insl6) (10), follistatin-like 1 (Fstl-1; also known as TSC-36) (11), LIF (12), IL-7 (13), IL-15 (14), and musclin (15) are the currently characterized myokines. These myokines act locally in an autocrine/paracrine manner and/or as endocrine factors linking skeletal muscle to regulation of physiological processes in other tissues. IL-6 is the first myokine described and remains the best studied (17). Secreted by skeletal muscle fiber in response to exercise, IL-6 has been shown to improve whole-body insulin sensitivity and dampen inflammation, providing a link between exercise and
the improvement in systemic metabolic parameters (17, 18). In mice, skeletal muscle-derived Fstl-1 promotes endothelial cell function and revascularization in ischemic tissue (11). Elevated serum levels of IL-15 via transgenic expression in mouse skeletal muscle reduce fat mass and decrease adiposity in response to high fat-feeding (14). These studies highlight the potential roles of myokines in mediating tissue crosstalk to control integrated physiology.

However, the expression of all myokines described to date is not restricted to skeletal muscle—they are generally expressed by a variety of cell types, and most are, in fact, expressed at much higher levels by non-muscle tissues (184-186). Prior to this study, no myokine has been discovered to be preferentially expressed by skeletal muscle.

While characterizing the metabolic function of the CTRP family of proteins we recently uncovered (24-29), we identified myonectin (CTRP15) as a novel member of the family on the basis of sequence homology in the shared C1q domain—the signature that defines this protein family.

Here, we provide the first molecular, biochemical, and functional characterization of myonectin and show that it is a novel nutrient-responsive myokine secreted by skeletal muscle to regulate whole-body fatty acid metabolism.

**Experimental procedures**

*Antibodies and chemicals*—Mouse monoclonal anti-FLAG M2 antibody was obtained from Sigma, and rat monoclonal anti-HA (clone 3F10) antibody was obtained from Roche Applied Science. AICAR (an AMPK activator) (187) was obtained from Calbiochem; insulin, isoproterenol, ionomycin, and epinephrine from Sigma; forskolin from Cell Signaling Technology.
*Animals*—Eight-week-old male or female wild-type C57BL/6J mice were purchased from the Jackson Laboratory and were housed in polycarbonate cages on a 12-hr light-dark photocycle with free access to water and standard laboratory chow diet (no. 5001, Lab Diet, PMI Nutrition International, St. Louis, MO) throughout the study period. Separate cohorts of mice were fed a high-fat diet (60% kcal derived from fat; D12492) or an isocaloric-matched low-fat diet (10% kcal derived from fat; D12450B) purchased from Research Diets Inc. (New Brunswick, NJ). HF diet was provided for a period of 12-14 weeks, starting at 4-5 weeks of age, to make mice diet-induced obese (DIO). Blood samples were collected for serum analysis. Tissues were collected, snap-frozen in liquid nitrogen, and kept at -80°C. All animal protocols were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University School of Medicine.

*Cloning of mouse myonectin*—A search for CTRP-like proteins in the NCBI GenBank databases identified a novel cDNA encoding a previously undescribed member of the CTRP family, which we designated as CTRP15/myonectin. Based on genomic DNA and EST sequences, a PCR approach was used to clone the entire coding region of mouse myonectin from a skeletal muscle cDNA library (Clontech). For mouse myonectin, the primer pair used was 5’-CAGCATGGCCCTCGACCCGCCGCCCCGTCGGAG-3’ and 5’-CAGCTGCTGCAGGCTCTTACCCTTA-3’. The PCR product was agarose gel-purified and cloned into the pCR2.1 TOPO vector (Invitrogen). The entire cDNA insert was sequenced. The nucleotide sequence of mouse myonectin reported in this study has been deposited in GenBank database under the accession number HQ285249.

*cDNA constructs*—C-terminal FLAG epitope (DYKDDDDK)-tagged mouse myonectin cDNA was generated by PCR, cloned into the mammalian expression vector (pCDNA3.1; Invitrogen),
and verified by DNA sequencing. C-terminal HA epitope (YPYDVPDYA)-tagged mouse adiponectin and all CTRPs used in this study were generated as previously described (20-23).

**Generation of myonectin-specific antibody**—Rabbit polyclonal anti-peptide antibody that can specifically recognize mouse myonectin (epitope 77-KQSDKGI NSKRRSKARR-93) was generated (YenZym Antibodies, LLC) and tested against conditioned medium harvested from myonectin-transfected HEK 293T cells.

**Protein purification**—Recombinant full-length mouse myonectin, containing a C-terminal FLAG-tagged epitope, was produced in mammalian cells as described previously (60). The presence of carbohydrate moieties and the formation of higher order oligomers necessitate that recombinant myonectin be produced in mammalian cells to ensure biologically active protein. Briefly, HEK 293 cells (Grip-Tite™ cells, Invitrogen) were cultured in DMEM containing 10% (v/v) bovine calf serum supplemented with antibiotics. Transfections were performed in HEK 293 cells using the calcium phosphate method (188). At 48 hr post-transfection, medium was replaced with serum-free Opti-MEM (Invitrogen) supplemented with vitamin C (0.1 mg/mL). Supernatants were collected three times, every 48 hr, pooled, and purified using an anti-FLAG affinity gel according to the manufacturer's protocol (Sigma), then eluted with 150 μg/mL FLAG peptide (Sigma). Purified proteins were dialyzed against 20 mM Hepes buffer (pH 8.0) containing 135 mM NaCl in a 10 kDa cut-off Slide-A-Lyzer dialysis cassette (Pierce). Protein concentration was determined using a Coomassie Plus protein assay reagent (Thermo Scientific), and samples were stored at -80°C.

**Recombinant protein injection**—Experiments were carried out as previously described (60, 29). Briefly, food was removed in the morning (around 8-9 am), 2 hr prior to recombinant protein injection; drinking water was supplied for the duration of the experiment. Recombinant
myonectin (5 μg/g body weight) or the equivalent volume of vehicle buffer [20 mM Hepes (pH 8.0) containing 135 mM NaCl] was injected intraperitoneally into 10-week-old C57BL/6 mice (n=6). Serum samples were harvested by tail bleeding at baseline (time 0) and every hour for 5 hr post injection and separated using Microvette® CB 300 (Sarstedt). Glucose concentrations were also measured using a glucometer (BD Pharmingen) when tail blood was collected at the indicated time points.

*Isolation of skeletal muscle*—Mice were sacrificed and soleus and plantaris muscles were immediately isolated and snap-frozen in liquid nitrogen. Homogenized muscle cell lysates were prepared in lysis buffer (TPER, Thermo Scientific) containing protease and phosphatase inhibitor cocktails (Sigma). Protein content was quantified using Coomasie Plus protein reagent (Thermo Scientific).

*Cell culture*—Mouse C2C12 myocytes and mouse 3T3-L1 pre-adipocytes were cultured and differentiated into myotubes and adipocytes, respectively, as previously described (27,29). Differentiated cells were stimulated with insulin (100 nM), AICAR (1 mM), epinephrine (1 μM), ionomycin (1 μM) or forskolin (1 μM) for the indicated time and total RNAs were isolated and subjected to quantitative real-time PCR analysis for myonectin expression.

*Fatty Acid uptake assay*—Cells were washed twice in PBS and placed in stimulation media (0.5% BSA for 3T3-L1 adipocytes and 0.1% BSA for H4IIE hepatocytes in high glucose, fatty acid-free DMEM) at 37°C and 5% CO₂ in an incubator for 2 hr. Next, media was changed to the same DMEM (with 0.5% and 0.1%, respectively, fatty acid-free BSA) containing vehicle control, recombinant myonectin (5 ug/mL), or insulin (50 nM) overnight. Cells were transferred to a 37°C water bath where 1 μCi/well (in a 24-well format) of [H]3-labelled palmitate (dissolved previously for 1 hr in the fatty-acid-free BSA DMEM) was added for either 0, 30, or
60 seconds. Media was then aspirated out and cells were washed twice in cold PBS. Cells were lysed in 10% SDS and transferred to a scintillation vial. Radioactive counts were measured and normalized to protein concentration of final cell lysate.

**Palmitate and glucose treatment**—Differentiated mouse C2C12 myotubes were washed twice with PBS, followed by addition of 0.1% fatty acid-free BSA (Sigma) in serum- and glucose-free DMEM for 2 h. Next, the same solution was added with or without 25 mM glucose or 1 μM palmitic acid. The palmitic acid and fatty acid-free BSA mixture was made 1 hr prior to addition to cells and kept at 37°C to completely dissolve into solution. Total RNA was harvested from cells treated for 18 hr.

**Running wheel-induced exercise**—C57BL/6 male mice were placed in a cage with or without a running wheel for a period of 2 weeks. They were given *ad libitum* food access. At the end of the two-week period, mice were fasted overnight (12 hr) and serum and skeletal muscle were harvested for analysis.

**Intra-gastric gavage**—Mice were fasted for 12 hr and gavaged with 10% glucose solution (10 μl/g body weight) or 20% emulsified intralipid (soybean oil; Sigma; 10 μl/g body weight). Sera were collected before and after gavage for blood chemistry and Western blot analysis.

**Serum and blood chemistry analysis**—Mouse serum samples were harvested by tail-bleed and separated using microvette® CB 300 (Sarstedt). Glucose concentration was determined at time of collection with a glucometer (BD biosciences). Serum triglycerides (Thermofisher), NEFA (Wako), and insulin (Millipore) were determined using commercially available kits.

**Quantitative real time PCR analysis**—The tissue expression profile of myonectin was determined using mouse tissue cDNA panels (Clontech). Otherwise, total RNAs were isolated from tissues or cell lines using Trizol® and reverse transcribed using Superscript II RNase H-
reverse Transcriptase (Invitrogen). Myonectin-specific primers used were 5’-TGCTTGATGGCTGGTGCAAA-3’ and 5’-CAGATGGGATAAAGGCGCTG-3’. Quantitative real-time PCR analyses were performed on an Applied Biosystems Prism 7500 Sequence Detection System. Samples were analyzed in 25-µL reactions according to the standard protocol provided in the SyBR® Green PCR Master Mix (Applied Biosystems). Myonectin expression was normalized to 18 S rRNA in each sample.

*Immunoblot Analysis*—Serum samples were diluted 1:20 in SDS loading buffer [50 mM Tris-HCl, pH 7.4, 2% SDS w/v, 6% glycerol w/v, 1% 2-mercaptoethanol v/v, and 0.01% bromophenol blue w/v] and were separated on 10% Bis-Tris NuPAGE gel (Invitrogen). Each well was loaded with an equivalent of 1 µl serum. For skeletal muscle lysates, 10 µg of protein were loaded and separated on a 10% Bis-Tris NuPAGE gel. Fractionated proteins were then transferred to Protran BA8 nitrocellulose membranes (Whatman), blocked in 2% non-fat milk for 1 h, and probed with primary antibodies in the presence of 2% non-fat milk overnight. Immunoblots were washed 3x (10 min each) in PBS containing 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) (1:2000) for 1 hr. Blots were washed 3x (10 min each) in PBS containing 0.1% Tween 20, developed in ECL reagent (Millipore) for 2-5 min, and visualized with MultiImage III FluorChem ® Q (Alpha Innotech). Quantifications of signal intensity were performed using Alphaview Software (Alpha Innotech).

*Native gel electrophoresis*—Non-denaturing, non-reducing gel electrophoresis was carried out as previously described (68).

*Adipose tissue and 3T3-L1 adipocytes lipolysis*—Experiments were performed as previously described (189). Food was removed from mice 2 hr prior to the isolation of epididymal fat pads.
Fat pads were cut into 20 mg sections and placed in 500 µL (per piece) of DMEM (high glucose) containing 0.5% fatty acid-free BSA, with or without 1 µM isoproterenol or purified myonectin (5 µg/mL). Tissue samples in media were kept in a 24-well cell culture dish at 37ºC and 5% CO₂. Media were collected at various time points and non-esterified free fatty acid (NEFA) content was measured using an absorption-based HR series NEFA kit (Wako Diagnostics). In the case of differentiated 3T3-L1 Adipocytes, cells were plated in a 24-well culture dish. Prior to treatment, cells were washed once with PBS and placed in high glucose DMEM containing 0.5% fatty acid-free BSA, with or without 1 µM isoproterenol or purified myonectin (5ug/mL). Media were collected prior to- and one hour following treatment and NEFA content was measured.

Statistical analysis—Comparisons were performed using two-tailed Student’s t-tests. Values were considered to be significant at \( p < 0.05 \). All data are presented as mean ± standard error (SE).

Results

Identification of myonectin

We cloned a previously undescribed member of the CTRP family from mouse skeletal muscle on the basis of sequence homology shared between their C1q domains (Fig. 1A). We designated this novel cDNA and its encoded protein CTRP15/myonectin. While current work was ongoing, Lim et al. reported on a distantly related but distinct C1q family member, CTRP5 (C1qTNF5), in human and had inadvertently coined the term “myonectin” for CTRP5 (165) although that term was not used in a previous CTRP5 study by the same authors (140). Given that mouse “myonectin” cDNA and protein sequences (HQ285249 and ADP00570, respectively) were deposited in the GenBank database and released to the public prior to the report by Lim et
al. (165), we propose that CTRP5 retains its original designation (24,141) and CTRP15 retains its current designation as “myonectin” to prevent confusion in nomenclature. The deduced mouse myonectin protein consists of five domains: a signal peptide for secretion, an N-terminal domain-1 (NTD1), a short collagen domain with 6 Gly-X-Y repeats, an N-terminal domain-2 (NTD2), and a C-terminal C1q/TNF-like domain. This protein consists of 340 amino acids and contains four Cys residues and four potential N-linked glycosylation sites that conform to the consensus sequence N-X-(Ser/Thr) (190). The 7.8 kb mouse myonectin gene is located on chromosome 1 (contig NC_000002.11) and contains 8 exons.

FIGURE 2-1. The deduced myonectin protein and its expression in skeletal muscle and cultured myotubes. A, The deduced domain structure of mouse myonectin. SP, signal peptide; NTD1, N-terminal domain-1; NTD2, N-terminal domain-2. B, Expression profile of myonectin in mouse tissues. C, Expression of myonectin transcript in isolated plantaris and soleus muscle fiber type (n=5 per group). D, Expression of myonectin transcript in un-differentiated mouse C2C12 myocytes and differentiated myotubes (n=8 per group). All quantitative real-time PCR data were normalized to 18S rRNA and expressed as mean fold change ± SE (***p<0.005)
Expression of myonectin in myotubes and skeletal muscle

In mice, myonectin transcript was predominantly expressed by skeletal muscle, with significantly lower expression in other tissues (Fig. 1B). Within skeletal muscle, soleus (a predominantly slow-twitch, oxidative muscle fiber type) tended to have higher expression of myonectin transcript compared to plantaris (a predominantly fast-twitch, glycolytic muscle fiber type) (Fig. 1C). Consistent with preferential skeletal muscle expression, myonectin was greatly induced in differentiated mouse C2C12 myotubes compared to undifferentiated myoblasts (Fig. 1D), suggesting that myonectin is produced by skeletal muscle fiber and not satellite cells. These data indicate that myonectin is a novel myokine.

Myonectin forms disulfide-linked oligomers and heteromeric complexes with other CTRPs

Consistent with the presence of a signal peptide, myonectin was robustly secreted into the conditioned medium when expressed in vitro (Fig. 2A). Secreted myonectin contains N-linked glycans; treatment with PNGase F (an N-glycosidase) reduced the apparent molecular weight of myonectin on immunoblot (Fig. 2B), confirming that one or more of the conserved Asn residues are glycosylated. In the absence of reducing agent (β-ME), myonectin migrated as a dimer on immunoblot (Fig. 2C), indicating the presence of intermolecular disulfide bond. On a non-reducing, non-denaturing, native gel immunoblot, myonectin existed as a multimeric complex, revealing its higher-order oligomeric structure (Fig. 2D). Formation of oligomers is a biochemical feature shared by all CTRPs and proteins of the C1q family (25, 60, 62, 90). Further, heteromeric complex formation between different CTRPs has been previously demonstrated (25, 27, 60, 191), suggesting a potential mechanism to generate an expanded repertoire of functionally distinct complexes. Similarly, when co-expressed in mammalian cells
(HEK 293T), myonectin formed heteromeric complexes with CTRP2 and CTRP12, and, to a lesser extent, with CTRP5 and CTRP10 (Fig. 2E).

**FIGURE 2-2.** Myonectin is secreted as a multimeric protein that can form heteromeric complexes with other CTRP family members. A, Immunoblot analysis of cell pellet (P) or supernatant (S) from transfected HEK 293T cells expressing pCDNA3.1 control vector or FLAG epitope-tagged myonectin. B-C, Immunoblot analysis of myonectin subjected to PNGaseF (B) or β-mercaptoethanol (C) treatment. D, Native gel immunoblot analysis of myonectin. E, Immunoprecipitation (IP) followed by immunoblot (IB) analysis of supernatants from HEK 293T cells expressing a combination of FLAG-tagged myonectin and HA-tagged adiponectin or CTRPs.
**Myonectin circulates in blood**

To examine the endogenous expression of myonectin and to address whether myonectin is a plasma protein and hence may function as an endocrine factor, we generated an anti-peptide antibody that can specifically recognize mouse myonectin secreted from the conditioned medium of transfected cells (Fig. 3A). In mouse skeletal muscle lysate, endogenous myonectin was detected in multiple isoforms, with apparent molecular weights between 40-50 kDa on immunoblot (Fig. 3B), likely reflecting different degrees of glycosylation (Fig. 2A,B) due to the presence of multiple conserved Asn residues (Fig. 1A). Western blot analysis of mouse serum revealed the presence of myonectin (Fig. 3C), confirming that it is a secreted protein that circulates in blood. Using purified recombinant myonectin as a standard, serum concentration of myonectin in *ad libitum* wild-type 12-week-old male mice was determined to be approximately 0.4 μg/mL (Fig. 3D).
FIGURE 2-3. Myonectin is produced by skeletal muscle and circulates in plasma. A, Immunoblot analysis of supernatant from HEK 293T cells expressing control vector or FLAG-tagged myonectin using a rabbit anti-myonectin antibody. B, Immunoblot detection of myonectin in mouse skeletal muscle lysate. Arrows indicate possible isoforms of myonectin resulting from differential glycosylation. C, Immunoblot detection of myonectin in mouse serum. D, Estimation of serum concentration of myonectin in wild-type 12-week-old C57BL/6J male mice. Purified recombinant myonectin was used to construct a standard curve.

Regulation of myonectin expression in myotubes

Expression of myonectin under different cellular conditions was examined to address whether factors that are known to regulate skeletal muscle physiology also affect myonectin expression.
Raising intracellular levels of cAMP (by forskolin and epinephrine) or calcium (by ionomycin) substantially induced expression of myonectin transcript in mouse C2C12 myotubes (Fig. 4A-C). In contrast, insulin and AICAR (an AMPK activator) had no effect on myonectin expression in myotubes (Fig. 4D,E).

**FIGURE 2-4.** Myonectin expression in myotubes is upregulated by an increase in cellular cAMP or calcium levels. A-E, Quantitative real-time PCR analysis of myonectin expression mouse C2C12 myotubes treated with vehicle control or 1 μM forskolin, Fsk (A); 1 μM epinephrine, Epi (B); 1 μM ionomycin, Iono (C); 1 mM AICAR (D); or 100 nM insulin, Ins (E). n= 8 for each experiment. All expression data were normalized to 18S rRNA and expressed as fold change (normalized to vehicle control). All data presented as mean ± SE (*p<0.05; **p<0.01; ***p<0.005).
Exercise increases myonectin mRNA expression and circulating levels

The in vitro results of myonectin expression in myotubes suggest that exercise-induced rises in intracellular calcium levels may also upregulate myonectin expression in intact skeletal muscle. To test this, we subjected a cohort of mice to voluntary exercise for 2 weeks. In contrast, control mice had access to a locked wheel. An average of 86 ± 29 revolution per day was recorded for mice with access to a running wheel. Exercise was confirmed by the expected reduction of fast-twitch muscle transcripts (troponin C2, MHCIIIB, and MyoH4) resulting from exercise-induced fiber type switch (Fig. S1) (192). In support of the in vitro data, myonectin expression was significantly induced in soleus and plantaris of mice given access to a running wheel for 2 weeks (Fig. 5A). Consistent with enhanced mRNA expression in skeletal muscle of mice subjected to voluntary exercise, circulating levels of myonectin also increased (Fig. 5B), suggesting a potential role of myonectin in exercise-induced physiology.

FIGURE 2-5. Exercise increases myonectin expression in skeletal muscle as well as circulating levels. A. Quantitative real-time PCR analysis of myonectin expression in plantaris and soleus muscle from mice given access to a running wheel (RW) for two weeks or matched controls with access to locked wheel. All expression data were normalized to 18S rRNA and expressed as fold change (normalized to control mice). B. Immunoblot analysis of serum myonectin from the same cohort of mice. All data are expressed as fold change (normalized to control mice) and shown as mean ± SE (n=6 mice/group; *p<0.05; **p<0.01).
Metabolic state regulates myonectin expression and circulating levels

Given that exercise induces myonectin expression in skeletal muscle, we next addressed whether short- and long-term changes in nutritional/metabolic state also regulate myonectin expression and circulating levels. Surprisingly, an overnight fast greatly suppressed myonectin expression, but a 2-hr refeeding period (following an overnight fast) dramatically upregulated its mRNA expression in skeletal muscle (Fig. 6A,B). Intriguingly, refeeding induced myonectin mRNA expression to a much greater extent in soleus than in plantaris muscle fiber of both male and female mice (data not shown), suggesting that myonectin expression may be regulated differentially depending on muscle fiber type. Consistent with the mRNA data, fasting reduced, but refeeding substantially increased, circulating levels of myonectin (Fig. 6C). Interestingly, fasted females also tended to have higher circulating levels of myonectin compared to male mice (Fig. 6D), a trend which persisted upon refeeding (data not shown). In a separate cohort of mice, we determined whether the carbohydrate or lipid component within the diet was responsible for the induction of myonectin expression and secretion in the fasted/re-fed state. Overnight fasted male mice were gavaged with a bolus of glucose or emulsified intralipid. Sera were collected from these animals 2 hr post gavage and subjected to blood chemistry and Western blot analysis. As expected, blood glucose and serum TG rose significantly in response to glucose and intralipid gavage, respectively (data not shown). It appeared that both glucose and lipid were equally potent at increasing circulating levels of myonectin in the re-fed state (Fig. 6E,F). These data raised the possibility that the presence of nutrient (carbohydrate or lipid) in the gastrointestinal tract induced the secretion of a gut-derived hormone (e.g., incretins such as GLP-1 and GIP) that, in turn, upregulated myonectin expression and secretion from the skeletal muscle. To rule out this possibility, differentiated C2C12 myotubes cultured in serum-free media were starved of
glucose and lipid, and then stimulated with glucose or free fatty acid (palmitate). In the absence of any potential gut-derived hormones, both glucose and free fatty acid were able to acutely upregulate myonectin expression in vitro (Fig. 6G). These data suggest that nutrient flux through muscle cells may directly regulate myonectin expression.

FIGURE 2-6. Nutritional state regulates the expression and circulating levels of myonectin. A-C, Quantitative PCR analysis of myonectin expression in soleus (A) and plantaris muscle (B), as well as immunoblot quantification of serum levels (C) after a 12-hr fast (Fasted) or fasted followed by 2 hr of unrestricted food access (Re-fed) (n=10 mice/group). All PCR data were normalized to 18S rRNA and expressed as fold change (normalized to fasted control mice). D, Immunoblot quantification of serum myonectin levels in male and female mice subjected to a 12-hr fast (n=10 mice/group). E-F, Immunoblot quantification of serum myonectin levels in mice subjected to a 12-hr fast (Fasted) or fasted and gavaged with glucose (E) or emulsified intralipid (F) (n=10 mice/group). All data are expressed as fold change (normalized to fasted control mice). G, Quantitative PCR analysis of myonectin expression in C2C12 myotubes cultured in serum-free media containing no glucose/lipids (control) or treated with 25 mM glucose or 1 μM palmitate for 18 hr (n=8/group). All data are expressed as fold change (normalized to control) and shown as mean ± SE (*p<0.05; **p<0.01; ***p<0.005)
Myonectin expression and circulating levels are reduced in the obese state

To test if myonectin expression and circulating levels are responsive to long-term chronic alteration in whole-body energy balance, we examined its mRNA and serum levels in DIO mice. As compared to mice fed an isocaloric-matched low-fat diet, mice fed a high-fat diet had lower myonectin mRNA and serum levels (Fig. 7A,B), suggesting that obesity-induced alteration in energy balance may be linked to dysregulation of myonectin-mediated processes in the obese state.

FIGURE 2-7. High-fat diet reduces myonectin expression and its circulating levels. A, Immunoblot quantification of serum myonectin levels in mice fed a high-fat diet (HFD) or an isocaloric-matched low-fat diet (LFD) for 12 weeks. B, Quantitative PCR analysis of myonectin expression in calf muscle isolated from LFD- or HFD-fed male mice. All data are expressed as fold change (normalized to control mice fed a LFD) and shown as mean ± SE (n=8 mice/group; **p<0.01)
Recombinant myonectin administration lowers circulating free fatty acids levels

To address the possible metabolic function of myonectin in vivo, we administered purified recombinant myonectin to wild-type male mice. Due to multiple posttranslational modifications (glycosylation and oligomerization), recombinant myonectin was produced in mammalian cells to ensure production of biologically active protein. When injected intraperitoneally into mice at a dose of 5 μg/g body weight, circulating levels of recombinant myonectin reached their maximum at 1 hr and gradually declined over time (Fig. 8A). At the injected dose we could raise serum levels of myonectin by 60-70% above endogenous steady-state levels (Fig. 8B). Elevating the circulating level of myonectin did not result in lowering of blood glucose over time when compared to mice injected with vehicle control (data not shown). In contrast, a relatively modest rise in serum myonectin levels was sufficient to lower (by ~30%) non-esterified free fatty acid (NEFA) levels over time relative to vehicle-injected controls (Fig. 8C). However, no significant difference was observed in serum triacylglycerol levels between the two groups of mice (Fig. 8D). These data suggest a potential role of myonectin in regulating systemic fatty acid metabolism.
FIGURE 2-8. **Recombinant myonectin administration reduces serum non-esterified free fatty acid levels in mice.** *A*, Immunoblot detection of FLAG epitope-tagged myonectin in mouse serum before and after recombinant protein injection. *B*, Immunoblot quantification revealed a ~60% elevation in serum myonectin levels above normal baseline levels after recombinant protein injection (n=5 per group). *C-D*, Male mice were injected i.p. with vehicle or myonectin (5 μg/g body weight) and sera were harvested every hour for 5 hr following recombinant protein administration. Food was removed 2 hr prior to protein injection. Serum non-esterified fatty acid (*C*) and triglyceride (*D*) levels were quantified (n=5 mice/group). All data are expressed as mean ± SE (*p<0.05)
**Recombinant myonectin promotes fatty acid uptake but not adipose tissue lipolysis**

Myonectin can lower circulating NEFA levels via two possible mechanisms—suppressing adipose tissue lipolysis or promoting fatty acid uptake into cells. Treatment of 3T3-L1 adipocytes *in vitro* or primary adipose tissue (epididymal fat pads) *ex vivo* with recombinant myonectin had no effect on lipolysis (Fig. 9). Myonectin also had no effect in suppressing isoproterenol-induced lipolysis in cultured 3T3-L1 adipocytes or primary adipose tissue explants (Fig. 9). Thus, the suppression of adipose tissue lipolysis is likely not the mechanism by which myonectin lowers serum NEFA levels in mice.

**FIGURE 2-9. Recombinant myonectin has no effect on adipocytes or adipose tissue lipolysis.**

*A*, NEFA concentration in media of 3T3-L1 adipocytes treated for 1 hr with vehicle, isoproterenol (1 μM), myonectin (5 μg/mL), or a combination of myonectin and isoproterenol (n=12 per group). B, Time course of NEFA release into conditioned media of adipose tissue (epididymal fat pad) explants treated with vehicle, isoproterenol (1 μM), myonectin (5 μg/mL), or a combination of myonectin and isoproterenol (n=6 per group). All data are expressed as mean ± SE (**p<0.005). Iso, isoproterenol; n.s., not significant.
Next, we investigated whether myonectin promotes fatty acid uptake in differentiated mouse 3T3-L1 adipocytes. As expected, treatment of adipocytes with a saturating dose of insulin (50 nM) led to a maximum 50% increase in fatty acid uptake (Fig. 10A). Similarly, treatment of adipocytes with recombinant myonectin (5 μg/mL) also enhanced fatty acid uptake to the same extent as insulin. A dose response curve indicated that maximum fatty acid uptake in adipocytes can be achieved with 5 µg/ml of myonectin (Fig. 10B). Several proteins, such as CD36, FATP1, Cav1, and Fabp4, are known to play important roles in fatty acid uptake into cells (193-195). Quantitative real-time PCR analysis showed that expression of these genes was upregulated in adipocytes by recombinant myonectin (Fig. 10C). To determine whether myonectin-mediated enhancement of lipid uptake is specific to adipocytes, we also tested the effect of myonectin on lipid uptake in rat H4IIE hepatocytes. We observed a modest (~25%) but consistent increase in fatty acid uptake into hepatocytes stimulated with myonectin (5 μg/mL), an effect similar to cells treated with a saturating dose of insulin (50 nM) (Fig. 10D). A dose response curve indicated that maximum fatty acid uptake in hepatocytes can also be achieved with 5 µg/ml of myonectin (Fig. 10E). Enhanced lipid uptake is coupled to increased expression of Cav1 and Fabp1 in H4IIE hepatocytes (Fig. 10F). Together, these results indicate that myonectin promotes lipid uptake into adipocytes and hepatocytes via transcriptional up-regulation of genes involved in fatty acid uptake.
FIGURE 2-10. Myonectin enhances fatty acid uptake in 3T3-L1 adipocytes and H4IIE hepatocytes via transcriptional mechanism. Mouse 3T3-L1 adipocytes (A) or rat H4IIE hepatocytes (D) were treated overnight with vehicle buffer, recombinant myonectin (5ug/mL), or insulin (50nM), and subjected to [3H]-palmitate uptake assay for 10, 30, or 60 seconds (n=8/group). Data represents cumulative uptake over 60 seconds. (B and E) Dose response curves of [3H]-palmitate uptake in 3T3-L1 adipocytes (B) and H4IIE hepatocytes (E) stimulated with various concentrations of myonectin. (C and F) Quantitative real-time PCR analysis of CD36, FATP1, Caveolin-1 (Cav1), and FABP4 or FABP1 expression in adipocytes (C) or hepatocytes (F) treated with vehicle buffer or myonectin (5 ug/mL) for 12 hr (n=8/group). All expression data were normalized to 18S rRNA. All data are expressed as fold change (normalized to vehicle control) and shown as mean ± SE (*p<0.05; **p<0.01).
Discussion

We provide the first characterization of myonectin, with in vitro and in vivo evidence that it is a novel myokine with important metabolic function. Unlike the other CTRPs characterized to date (25, 27, 60), myonectin (CTRP15) is expressed and secreted predominantly by skeletal muscle. While some CTRPs function as adipokines linking adipose tissue to regulation of systemic insulin sensitivity and energy metabolism (28, 29), myonectin functions as a myokine that mediates crosstalk between skeletal muscle and other metabolic compartments (e.g., adipose tissue and liver) to coordinate the integration of whole-body metabolism. Consistent with this notion, the expression and secretion of myonectin by skeletal muscle is highly responsive to acute nutritional and metabolic changes (e.g., fast/re-fed cycle and exercise), as well as chronic alteration in energy state of the animals (e.g., diet-induced obesity).

A rise in intracellular cAMP or calcium levels due to muscle contraction or pharmacologic agents (forskolin, epinephrine, or ionomycin) increases expression of myonectin. In contrast, insulin and AICAR (an AMPK activator) have no apparent effect on the transcription of myonectin in myotubes. Intriguingly, in starved myotubes cultured in serum-free medium, the presence of glucose or free fatty acid is sufficient to induce the expression of myonectin transcript (4-fold), analogous to overnight-fasted mice gavaged with a bolus of glucose or lipid (Fig. 6E,F). The observed in vitro results implicate a direct effect of glucose and/or fatty acids in inducing myonectin expression in myotubes, rather than through an indirect effect of incretin hormones (e.g., GLP-1 and GIP) secreted in response to the presence of nutrients in the gastrointestinal tract. These results suggest that myonectin is a nutrient-responsive metabolic regulator secreted by skeletal muscle in response to changes in cellular energy state resulting from glucose or fatty acid fluxes.
Many metabolically relevant secreted proteins (e.g., adiponectin, leptin, resistin, RBP4) and the signaling pathways they regulate in tissues are known to be dysregulated in the condition of obesity (32, 38, 42, 196, 197). The reduction in expression and circulating levels of myonectin in the obese state may represent yet another component of the complex metabolic circuitry dysregulated by excess caloric intake. Although exercise has long been known to have profound positive impacts on systemic insulin sensitivity and energy balance, the underlying mechanisms remain incompletely understood. That voluntary exercise dramatically increases the expression and circulating levels of myonectin to promote fatty acid uptake into cells may underlie one of the beneficial effects of physical exercise.

A modest rise in the circulating levels of myonectin resulting from recombinant protein administration is sufficient to lower serum NEFA without altering serum triglyceride levels. Unlike CTRP1 (60) and CTRP3 (29), injection of recombinant myonectin into mice appears to have no glucose-lowering effect. Reduction in circulating NEFA is not due to suppression of adipose tissue lipolysis; rather, it results from increased fatty acid uptake by adipocytes and hepatocytes. Although the myonectin-mediated enhancement of lipid uptake in vitro appears modest (25-50%)—in fact, the magnitude of this effect is comparable to cells stimulated with 50 nM insulin—a saturating dose that leads to maximum increase in fatty acid uptake. Even in primary muscle cells that over-express molecules that promote fatty acid uptake (e.g., CD36, FATP1, FATP4), only a 20-60% increase in lipid uptake is observed compared to control cells (32). Stimulation of adipocytes with a combination of insulin and myonectin does not result in further increases in fatty acid uptake (data not shown), suggesting that myonectin and insulin likely activate similar pathway(s) in cells to enhance lipid uptake. In support, recombinant myonectin treatment up-regulates the expression of CD36, Cav1, and Fabp1/Fapb4 in adipocytes.
and hepatocytes, similar to insulin (data not shown), to mediate its effect on fatty acid uptake. In accordance with myonectin mediating its metabolic effect through a transcriptional mechanism, a reduction in circulating NEFA in mice occurred only 2 hr after recombinant protein injection, a lag period presumably required for mRNA and protein synthesis.

In summary, myonectin is a novel multimeric protein of the C1q family that is synthesized and secreted by skeletal muscle, with its expression subjected to complex metabolic regulation. Circulating myonectin functions as a myokine linking skeletal muscle to lipid metabolism in liver and adipose tissue, providing insights into tissue crosstalk that underlies the integrated control of whole-body metabolism.
HOW MUSCLE COMMUNICATES NUTRIENT STATUS TO REGULATE PHYSIOLOGIC HOMEOSTASIS

CHAPTER 3: Myonectin links skeletal muscle to carbohydrate metabolism in liver
Introduction

Skeletal muscle plays a vital role in maintaining whole-body energy balance by taking up a majority of circulating postprandial glucose and storing it as glycogen (1,198). Importantly, impaired insulin responsiveness in skeletal muscle is mechanistically linked to type 2 diabetes (199-203). Given its integrative control of energy balance and function as a major metabolic tissue, skeletal muscle is assumed to communicate with other metabolic tissues via secreted hormones to influence whole-body metabolism. The discovery that skeletal muscle indeed dynamically secretes a variety of myokines—secreted proteins that can engage muscle and non-muscle tissues to regulate various biological processes—has provided a novel and critical conceptual framework to understand skeletal muscle’s role in coordinating integrated physiology (3, 8, 204-211).

Of the hundreds of proteins secreted by skeletal muscle (4, 212, 213), only some have been functionally characterized; these include myostatin (207, 214, 215), IL-6 (3), fibroblast growth factor 21 (FGF21) (8, 9), insulin-like 6 (INSL6) (10), follistatin-like 1 (FSTL1) (11), leukemia inhibitory factor (LIF) (12), IL-7 (13), IL-15 (14), musclin (15), and irisin (16). These myokines either act locally within skeletal muscle as autocrine/paracrine factors or circulate in blood as endocrine factors, linking skeletal muscle to the regulation of physiological processes in non-muscle tissues (3, 8, 204-209).

We recently described myonectin/CTRP15 as a novel myokine expressed predominantly by skeletal muscle (23). Myonectin is a secreted protein with a globular C1q domain, the signature feature shared by other C1q/TNF-related proteins (CTRPs) (23-27, 30, 60, 76). Several CTRPs have important metabolic and cardioprotective functions (28, 29, 30, 77, 132, 153-155, 164, 216, 217), but myonectin is the only one whose expression is primarily restricted to skeletal
muscle. Several lines of evidence suggest myonectin is a physiologically relevant metabolic regulator (23, 218). First, overnight fasting reduces and re-feeding increases myonectin mRNA and serum levels. Second, circulating myonectin levels increase similarly to re-feeding when overnight-fasted mice are given a bolus of glucose or emulsified lipid (23). Addition of glucose or free fatty acids to cultured myotubes also upregulates myonectin expression, suggesting that nutrient uptake and metabolism by muscle cells is sufficient to induce myonectin expression. Third, myonectin expression and circulating levels are significantly reduced in diet-induced obese and insulin-resistant mice (23). Fourth, infusion of recombinant myonectin into mice reduces circulating free fatty acid levels by promoting free fatty acid uptake (23). Finally, myonectin suppresses liver autophagy (218), an intracellular recycling pathway activated in the fasted state and inhibited in the fed state. Based on these observations, we hypothesized that myonectin likely regulates other physiological processes in the postprandial state.

Here we established the function of myonectin in regulating hepatic glucose metabolism and provided further evidence to support a hormonal role for myonectin in mediating skeletal muscle-liver crosstalk to maintain metabolic homeostasis.

**Experimental Procedures**

*Serum and blood chemistry analysis*—Mouse serum samples were harvested by tail-bleed and separated using Microvette® CB 300 (Sarstedt). Samples were centrifuged at 10,000 x g for 5 min and sera were collected and stored at -80°C.

*Cell culture*—Rat H4IIE hepatocytes (ATCC) were cultured and differentiated as previously described (98). In brief, cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen)
containing 5 mM glucose, 10% fetal bovine serum (Atlanta Biologicals), penicillin, and streptomycin (Life Technologies). In all cell culture treatments, 5 µg/mL of recombinant myonectin were used unless otherwise stated.

_Gluconeogenesis assay_—Glucose production assays were performed as previously described (219). In brief, H4IIE cells were plated 3 h prior to the assay at 100,000 cells in 100 µL of growth medium per well in 96-well tissue culture plates. Cells were washed with PBS and incubated overnight in glucose production buffer (DMEM lacking phenol red, serum, and glucose and supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate). The following day, cells were washed in PBS and fresh glucose production buffer with or without recombinant myonectin (5 µg/mL) was added for 8 h. Cells and supernatant were harvested and glucose concentration in the media was measured using an Amplex Red Glucose Kit (Invitrogen).

_Glucose uptake assay_—To evaluate glucose uptake in H4IIE hepatocytes, a previously published assay was adapted (220). H4IIE cells were plated in a 24-well format, washed with PBS, and incubated overnight with vehicle control or recombinant myonectin (0.5 or 5.0 µg/mL) in DMEM containing 5 mM glucose and 0.5 g/L BSA (Sigma). The following day, cells were washed in PBS and incubated with DMEM containing 0.5 g/L BSA and 0.1 µCi/mL [3H]-2-deoxy-D-glucose for 15 min. Cells were washed in cold PBS and lysed in 500 µL/well of lysis buffer. Ten microliters of cell lysate from each well were used for protein quantification. Three hundred microliters of each sample were placed in a vial with Ecoscint Ultra scintillation fluid (National Diagnostics) and radioactivity was quantified using a Beckman Coulter scintillation counter (model LS 6000SC). Values were normalized to protein content.
**Glycogen deposition assay**—An assay to measure total mouse liver glycogen deposition was adapted from previously published protocols (221, 223). Briefly, mice fed *ad libitum* were injected with vehicle saline or recombinant myonectin (1 µg/g body weight). Ninety minutes following injection, livers were harvested, separated into 20 mg sections, and snap-frozen in liquid nitrogen. Each 20 mg section was boiled in 500 µL of 30% KOH (w/v in water) at 95°C for 30 min. Samples were vortexed and glycogen was precipitated on ice in 1.5 mL of 95% ethanol for 60 min. Next, samples were centrifuged at 3000 rpm for 2 min and glycogen pellets were resuspended in 500 µL distilled water. Resuspended glycogen was added to 1.5 mL of 66% ethanol, precipitated on ice for 20 min, and centrifuged at 3000 rpm for 2 min. This series was repeated twice. Glycogen pellets were then dissolved in 1 M HCl and boiled at 95°C for 60 min. Samples were diluted 1:200 in water and total glucose was measured using an Amplex Red Glucose Kit (Invitrogen). To ensure proper glycogen extraction, samples were compared to livers from overnight-fasted (16 h) mice with depleted hepatic glycogen content.

**Quantitative real-time PCR analysis**—Total RNA was isolated from tissues or cells using Trizol® (Life Technologies) and reverse-transcribed using Superscript II RNase H-reverse transcriptase (Invitrogen). Primers included: mouse glucose-6-phosphatase (*G6pc*) forward 5’-CGACTCGCTAT CTCCAAGTGA-3’ and reverse 5’-GTTGAACCA GTCTCCGACCA-3’; mouse phosphoenolpyruvate carboxykinase (*Pck1*) forward 5’-CTGCATAACGGTCTGGACTTC-3’ and reverse 5’-CAGCAACTGCCCGTACTCC-3’; mouse glucose transporter (*Glut2*) forward 5’-TCAGAAGACAAGATCACCGGA-3’ and reverse 5’-GCTGGTGTGACTGTAAGTG-3’; rat *G6pc* forward 5’-CTACCTTGCGCCTCACTTTC-3’ and reverse 5’-ATCCAAGTGCGAAACC AC-3’; rat *Pck1* forward 5’-GAGTGCCCATCGAGTGCAACAT-3’ and reverse 5’-CCAGTGCCCGTACTTG-3’; and rat *Glut2* forward 5’-

~ 51 ~
CAAGATCACCGGAACCTTGG-3’ and reverse 5’-ATTCCGCCTACTGCAAAGCT-3’.

Quantitative real-time PCR analyses were performed on an Applied Biosystems Prism 7500 Sequence Detection System. Samples were analyzed in 25 µL reactions per SyBR® Green PCR Master Mix (Applied Biosystems) standard manufacturer directions. Expression values were normalized to β-actin levels using the C_{t} method (223).

**Immunoblot analysis**—Serum samples were diluted 1:20 in SDS loading buffer [50 mM Tris-HCl (pH 7.4), 2% SDS (w/v), 6% glycerol (w/v), 1% 2-mercaptoethanol (v/v), and 0.01% bromophenol blue (w/v)] and separated on 10% Bis-Tris NuPAGE gels (Invitrogen). Each well was loaded with an equivalent of 1 µL serum. For H4IIE hepatocytes and mouse liver lysates, 10 µg of protein was loaded per lane and proteins were separated on 12% Bis-Tris NuPAGE gels. Western blots were carried out as previously described (23). Immunoblot results were visualized with MultiImage III FluorChem® Q (Alpha Innotech). Signal intensity quantifications were performed using Alphaview Software (Alpha Innotech).

**Statistical analysis**—Comparisons were performed using two-tailed Student’s t-tests with 95% confidence intervals. Values were considered significant at \( p<0.05 \). All data is presented as mean ± S.E. and *=\( p<0.05 \); **=\( p<0.01 \); and ***=\( p<0.001 \).

**Results**

**Myonectin lowers blood glucose in fasted mice**

During fasting when plasma insulin levels are low, the liver buffers blood glucose to prevent hypoglycemia by initiating gluconeogenesis (224). Given that myonectin expression and circulating levels are reduced in the fasted state and increased in the fed state (23), myonectin may serve as a postprandial hormone to modulate hepatic gluconeogenesis. To test this
hypothesis, we injected recombinant myonectin into overnight-fasted mice at a dosage that mimicked the physiologic induction of myonectin that occurs with re-feeding after a fast (23). Although overnight-fasted mice had depleted hepatic glycogen content and had engaged gluconeogenesis, infusion of myonectin significantly reduced blood glucose over time in fasted mice to <50 mg/dL (Fig. 1).

FIGURE 3-1. Myonectin reduces fasting blood glucose levels in mice. Blood glucose levels were measured with a glucometer at different time points in 16 h-fasted mice intraperitoneally injected with 1 μg/g body weight of recombinant myonectin or volume-matched saline control (n=5). ***p<0.001
Myonectin suppresses hepatic glucose production by inhibiting gluconeogenic gene expression

The failure of fasted mice to maintain euglycemia following recombinant protein administration suggested that myonectin inhibited hepatic gluconeogenesis. Indeed, *in vitro* assays showed that myonectin reduced glucose production in H4IIE hepatocytes in a dose-dependent manner (Fig. 2A). The significant drop in blood glucose levels 2 h after myonectin administration suggested transcriptional changes were required, so we measured gene expression via real-time PCR. Expression of hepatic G6pc and Pck1, two important gluconeogenic genes, was significantly suppressed in myonectin-injected mice (Fig. 2B). Similarly, recombinant myonectin suppressed G6pc and Pck1 expression in rat H4IIE hepatocytes (Fig. 2C,D). These results demonstrate that myonectin acts directly on hepatocytes to regulate gluconeogenesis.
FIGURE 3-2. **Myonectin inhibits hepatic gluconeogenesis.** *A*, Total glucose production was measured with gluconeogenesis assays in rat H4IIE hepatocytes treated with recombinant myonectin (0.5 or 5.0 µg/mL) or volume-matched vehicle control (n=12). *B*, Overnight-fasted (16 h) mice were injected with 1 µg/g body weight of recombinant myonectin or volume-matched saline control (n=5). Four hours post-injection, liver RNA was analyzed for expression of gluconeogenic genes *G6Pc* or *Pck1* with quantitative real-time PCR. *C-D*, Expression of *G6Pc* (C) and *Pck1* (D) was also measured in H4IIE hepatocytes treated with recombinant myonectin (5 µg/mL) or vehicle control (n = 6) for 0, 3, 6, 12, and 24 h. All real-time PCR data were normalized to β-actin levels and expressed as mean ± S.E. *p < 0.05; **p < 0.01; ***p < 0.001.
Myonectin enhances hepatic glucose uptake

In the fed state, hepatic gluconeogenesis is suppressed while glucose uptake and utilization is increased (224). To test if myonectin is a postprandial hormone that regulates glucose homeostasis in the fed state, we treated H4IIE hepatocytes with vehicle control or recombinant myonectin and measured $[^3]$H-2-deoxyglucose uptake. Myonectin enhanced glucose uptake in a dose-dependent manner (Fig. 3A) and increased hepatic mRNA and protein levels of Glut2 (Fig. 3B,C), the predominant glucose transporter (225, 226).

FIGURE 3-3. *Myonectin promotes glucose uptake in hepatocytes.* A, Glucose uptake was measured with $[^3]$H-2-deoxyglucose in rat H4IIE hepatocytes treated for 12 h with recombinant myonectin (0.5 or 5.0 µg/mL) or vehicle control (n=6). B, Expression of Glut2 mRNA in rat H4IIE hepatocytes treated with recombinant myonectin (5.0 µg/mL) or vehicle control was measured by real-time PCR. C, Expression of GLUT2 protein in rat H4IIE hepatocytes treated with recombinant myonectin (5.0 µg/mL) or vehicle control was measured by Western blot. All data are normalized to β-actin (mRNA) or tubulin (protein) expression levels. *p<0.05; **p<0.01
Myonectin activates glycogen synthase to promote glycogenesis

The protein kinase B/Akt signaling pathway regulates glycogenesis in the fed state (227). In cultured rat H4IIE hepatocytes and mouse liver, recombinant myonectin activates Akt (218). Downstream of Akt, myonectin also induced inactivation of GSK3β (Fig. 4A, B) through phosphorylation at the residue phosphorylated by Akt, Ser-9 (228). Inactivated GSK3β also reduced inhibitory phosphorylation on GS at Ser-641 (Fig. 4A, B), leading to net activation of GS and increased glycogenesis (198). To functionally verify activation of glycogenesis, mice fed ad libitum were i.p. injected with recombinant myonectin or control saline and total liver glycogen was quantified. As a positive control, a separate cohort of fed mice was injected with recombinant insulin (1 U/kg). As expected, insulin injection increased liver glycogen content by ~26% (Fig. 4C), while myonectin injection increased liver glycogen content by ~16% (Fig. 4D).

FIGURE 3-4. Myonectin enhances glycogenesis. A-B, Expression of GSK3β, phospho-GSK3β (Ser-9), glycogen synthase (GS), and phospho-GS (Ser-641) was measured by Western blot following stimulation with vehicle control or myonectin (5.0 µg/mL) in H4IIE hepatocytes (A) or mouse liver (B) (n=3). C-D, Mouse liver glycogen content was measured and quantified following injection with saline control (v/v; n=10), insulin (1 U/kg; n=10) (C), or myonectin (1 µg/g body weight; n=10) (D). Liver glycogen content was normalized to tissue weight and expressed as mean ± S.E. *p<0.05
Myonectin and insulin act independently to inhibit gluconeogenesis and promote glucose uptake

Insulin levels rise rapidly following a meal to control hepatic glucose output (224). During re-feeding following an overnight fast, circulating myonectin levels also increase substantially (23). To test whether myonectin and insulin act together, we performed functional assays for gluconeogenesis and glucose uptake in the presence of varying doses of recombinant insulin and myonectin. Maximum inhibition of gluconeogenesis and increase in glucose uptake were observed at a saturating dose of 10 pM and 100 nM insulin, respectively (Fig. 5A,C). Because hepatocytes do not express the insulin-responsive glucose transporter Glut4 and instead rely on Glut2 for glucose uptake, a much higher dose (10-100 nM) of insulin is required to observe increase in glucose uptake. For both gluconeogenesis and glucose uptake assays, we observed the maximum effect at 5 µg/mL myonectin (Fig. 5A,C). When combined at suboptimal doses, we did not observe synergistic effects of insulin and myonectin on gluconeogenesis or glucose uptake beyond which could be achieved by either alone (Fig. 5A-D). These results suggest that myonectin and insulin likely act independently in hepatocytes.
FIGURE 3-5. **Additive effects of myonectin and insulin on hepatocytes.** A-B, Glucose production was measured in H4IIE hepatocytes at varying doses of recombinant myonectin (0, 0.5, and 5.0 µg/mL) and insulin (0, 1, 2, 5, and 10 pM). Data are expressed as glucose output (A) and cumulative area-under-curve (AUC) insulin-inhibited production at three myonectin concentrations (B). C-D, Glucose uptake was measured in H4IIE hepatocytes at varying doses of recombinant myonectin (0, 0.5, and 5.0 µg/mL) and insulin (0, 10, and 100 nM). Data are expressed as glucose uptake (C) and cumulative area-under-curve (AUC) insulin-stimulated uptake at three myonectin concentrations (D). All data are expressed as mean ± S.E. NS = not significant; *p<0.05; **p<0.01; ***p<0.001
Discussion

We provide evidence here that skeletal muscle-derived myonectin regulates carbohydrate metabolism in liver. Our previous studies (23, 218) and current findings support a postprandial hormonal role for myonectin in mediating inter-tissue crosstalk to maintain metabolic homeostasis.

Although both insulin and myonectin are postprandial hormones secreted in response to food intake, their actions differ temporally in the fed state to regulate glucose and lipid metabolism. Secretion of insulin is rapid (within 15 min) following meal consumption. Insulin acutely orchestrates postprandial metabolism within the first hour (229, 230), but plasma insulin largely returns to baseline physiological levels at 60-90 min following a meal. Myonectin secretion, in contrast, is maximally induced 2-3 h following food intake (23). This suggests that nutrient uptake and metabolism in skeletal muscle is coupled to myonectin induction and secretion. Myonectin, in turn, acts on adipose tissue and liver to regulate metabolism in the postprandial state after plasma insulin levels return to baseline, but well before the next meal.

Aside from differences in the kinetics of secretion following a meal, our functional data also suggest that myonectin and insulin act independently. In gluconeogenic conditions, such as overnight-fasted mice, physiologically relevant myonectin levels suppressed gluconeogenic gene expression and glucose production. Our in vitro studies indicate that myonectin acts independently of insulin to modulate \textit{G6pc} and \textit{Pck1} expression and glucose production in cultured hepatocytes. These results are supported by \textit{in vivo} studies that show myonectin infusion in fasted mice does not alter plasma glucagon levels (218). Treatment of H4IIE hepatocytes with a combination of varying doses of insulin and myonectin suggests additive rather than synergistic effects on gluconeogenesis and glucose uptake. These results are consistent with the
independent action of insulin and myonectin in regulating hepatic glucose metabolism at different postprandial phases (early and late).

Of the myokines described thus far, only IL-6 and myonectin have been functionally shown to regulate hepatic glucose metabolism. IL-6 is a cytokine secreted by contracting skeletal muscle during exercise (231, 232); it has been shown to promote hepatic gluconeogenesis in cultured hepatocytes (233) and in mice (234). While glucagon accounts for a significant part of exercise-induced increases in hepatic glucose output (235), studies on IL-6 also suggest that this cytokine carries a signal from the skeletal muscle to liver to increase hepatic glucose output during intense and sustained exercise. Presumably this is due to a greater demand on liver to maintain euglycemia as a consequence of increased glucose flux into skeletal muscle (236). Thus, the metabolic effect of IL-6 on hepatic gluconeogenesis appears to be opposite of myonectin. We previously reported that circulating levels of myonectin are increased in mice that have access to a running wheel for 2 weeks (23). Since re-feeding increases myonectin expression (23), it remains unclear whether exercise or skeletal muscle contraction per se enhances myonectin expression and secretion or is secondary to increased food intake following exercise (237).

In summary, we provide further evidence for endocrine communication between skeletal muscle and liver and highlight the important and novel role of myonectin in coupling the metabolic states of skeletal muscle to the integrated control of hepatic glucose metabolism in the postprandial state. Aside from adipose tissue and liver, the potential autocrine, paracrine, and endocrine actions of myonectin on skeletal muscle and other non-muscle tissues remain to be uncovered. Future studies will elucidate whether the myonectin-mediated skeletal muscle-liver axis is dysregulated in pathophysiological conditions of obesity and type 2 diabetes.
HOW MUSCLE COMMUNICATES NUTRIENT STATUS TO
REGULATE PHYSIOLOGIC HOMEOSTASIS

CHAPTER 4: Myonectin activates the mTOR pathway to suppress autophagy in liver*

*Most text and figures in this chapter were adopted from (ref 218 Seldin MM et al., 2013)
Introduction

Autophagy helps recycle intracellular organelles and proteins into their constituent building blocks, serving as potential fuel sources for maintaining cellular homeostasis in times of nutrient deprivation (237, 238). In fact, exercise—a form of nutrient deprivation—triggers autophagic turnover of aged organelles, such as mitochondria, to accommodate renewal of cellular content; this is thought to be the origin of some exercise-related health benefits (239).

In contrast, dysregulation of autophagy is linked to diseases like obesity and atherosclerosis (240-242), and an absence of autophagy prohibits survival. Global deletion of autophagy genes (e.g., \textit{Atg5} and \textit{Atg7}) in mice results in perinatal lethality (243, 244), which likely results from a failure in catabolic breakdown of liver protein and glycogen (245, 246). This process is needed to maintain energy homeostasis within the first few hours after birth, before neonates have acquired a full capacity for gluconeogenesis (247, 248). Thus, autophagy is essential to normal cellular and organismal functions.

One organ in which autophagy has particular importance is the liver. The liver responds dramatically to starvation; up to 40% of its cellular protein content can be degraded via autophagy during prolonged periods of nutrient deprivation (249). Autophagy in the liver is remarkably sensitive to hormonal regulation. Elevated levels of glucagon, a hormone produced in the pancreas, in the fasted state induce autophagy in liver (250). Conversely, the anabolic hormone insulin, secreted in response to food intake, and diet-derived amino acids suppress hepatocyte autophagy by activating the nutrient-sensing mTOR signaling pathway (251). In diet-induced obese mice that are insulin-resistant, liver autophagy is dysregulated (252). Suppression of autophagy by insulin also extends to skeletal and cardiac muscle (253). While insulin and
glucagon play an important role in regulating cellular autophagy in the fed and fasted state, respectively, other hormonal signals may also affect this recycling process.

We recently described myonectin (also designated as CTRP15) as a nutritionally-regulated hormone secreted by skeletal muscle (23). It belongs to the C1q/TNF-related protein (CTRP) family of secreted proteins known to regulate glucose and fatty acid metabolism (24, 25, 27-30, 60, 132, 164). However, unlike many CTRPs that are predominantly expressed by adipose tissue, myonectin is primarily derived from skeletal muscle. Myonectin expression and circulating levels in mice are highly induced by re-feeding following an overnight (12-h) fast. Infusion of recombinant myonectin into mice lowers circulating levels of free fatty acids, in part by promoting cellular lipid uptake and upregulating the expression of genes (Cd36, Fabps, Fatps) involved in lipid uptake (23). Here, we establish the functional significance of myonectin as a novel regulator of autophagy in liver, highlighting its role in mediating skeletal muscle-liver crosstalk to modulate metabolic processes critical to maintaining tissue homeostasis.

**Experimental Procedures**

*Antibodies and chemicals*—Mouse monoclonal anti-FLAG M2 antibody was obtained from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-peptide antibody, which can specifically recognize mouse myonectin, has been previously described (18). Rabbit polyclonal antibodies recognizing p62, UNC-51-like kinase 1 (ULK1), phospho-ULK1 (Ser-757), Akt, phospho-Akt (Thr-308), mTOR, phospho-mTOR (Ser2448), insulin receptor substrate 1 (IRS-1), phospho-IRS (Ser-612), Forkhead box transcription factor 3 (FOXO3), phospho-FOXO3 (Thr24) and β-tubulin were obtained from Cell Signaling Technology (Danvers, MA). A rabbit polyclonal antibody recognizing LC3B was obtained from Sigma-Aldrich. Inhibitor, Akt IV, was obtained
from Millipore (Billerica, MA). mTOR inhibitor rapamycin and PI3K inhibitor LY294002 were obtained from Cell Signaling Technology.

Animals—Eight-week-old, male, wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in polycarbonate cages on a 12-h light-dark photocycle and had *ad libitum* access to water and standard laboratory chow diet (no. 5001 Lab Diet, PMI Nutrition International, St. Louis, MO) throughout the study period. Animals were fasted for 24 h and injected via tail vein with either 10 μL/g body weight of 20% (w/v) glucose solution, HEPES buffer (vehicle control), or 1 μg/g body weight recombinant myonectin. Serum and tissues were harvested in the *ad-libitum* state, 10 min or 4 h post-injection as indicated. All animal protocols were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University School of Medicine.

Protein purification—Recombinant full-length mouse myonectin, containing a C-terminal FLAG-tagged epitope, was produced in mammalian cells as previously described (23). Briefly, HEK 293 cells (Grip-Tite™, Invitrogen, Carlsbad, CA) were cultured in Dulbecco's Modified Eagle Medium (DMEM)(Invitrogen) containing 10% (v/v) fetal bovine serum (FBS) (Invitrogen) supplemented with antibiotics. Transfections were performed in HEK 293 cells using the calcium phosphate method (28). At 48 h post-transfection, medium was replaced with serum-free Opti-MEM (Invitrogen) supplemented with vitamin C (0.1 mg/mL). Supernatants were collected three times, every 48 h, pooled, and purified using an anti-FLAG affinity gel according to the manufacturer's protocol (Sigma-Aldrich). Proteins were eluted with 150 μg/mL FLAG peptide (Sigma-Aldrich) and dialyzed against 20 mM HEPES buffer (pH 8.0) containing 135 mM NaCl in a 10 kDa cut-off Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL). Protein concentration
was determined using a Coomassie Plus protein assay reagent (Thermo Scientific, Waltham, MA), and samples were aliquoted and stored at -80°C.

Isolation of skeletal muscle and liver—Liver and muscle samples were immediately harvested from euthanized mice and snap-frozen in liquid nitrogen. Homogenized cell lysates were prepared in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein content was quantified using Coomasie Plus protein reagent (Thermo Scientific).

Cell culture—Mouse C2C12 myocytes and rat H4IIE hepatocytes (ATCC) were cultured in 24-well plates and differentiated as previously described (23). To induce autophagy, cells were placed in autophagy media [DMEM containing 0.1% BSA (Sigma-Aldrich) but lacking glucose, L-glutamine, and sodium pyruvate] for 5 h or 24 h. Control cells were incubated with DMEM containing 10% FBS (Invitrogen). In all cell culture treatments, 5 μg/mL of recombinant myonectin was used.

Serum and blood chemistry analysis—Mouse serum was harvested by tail-bleed. Samples were separated using microvette® CB 300 (Sarstedt, Nümbrecht, Germany) and centrifuged at 10,000 x g for 5 min. Serum glucagon level was quantified using a glucagon ELISA kit (Millipore).

Quantitative real-time PCR analysis—Total RNAs were isolated from tissues or cell lines using Trizol® and reverse transcribed using Superscript II RNase H-reverse transcriptase (Invitrogen). Primer sequences are listed in Supplemental Table S1. Quantitative real-time PCR analyses were performed on an Applied Biosystems Prism 7500 sequence detection system (Invitrogen). Samples were analyzed in 25 μL reactions with SyBR® Green PCR Master Mix (Applied Biosystems, Invitrogen) per manufacturer’s directions. Data were normalized to β-actin and expressed as relative mRNA levels using the ΔΔCt method (223).
**Immunoblot analysis**—Serum samples were diluted 1:20 in sodium dodecyl sulfate (SDS) loading buffer [50 mM Tris-HCl pH 7.4, 2% SDS (w/v), 6% glycerol (w/v), 1% 2-mercaptoethanol (v/v), and 0.01% bromophenol blue (w/v)] and separated on 10% Bis-Tris NuPAGE gels (Invitrogen). Each well was loaded with an equivalent of 1 µL of serum. For H4IIE hepatocytes and mouse liver lysates, 10 µg of protein were loaded and separated on 12% Bis-Tris NuPAGE gels (Invitrogen). Fractionated proteins were then transferred to Protran BA8 nitrocellulose membranes (Whatman, GE Healthcare, Little Chalfont, UK), blocked in 2% non-fat milk for 1 h, and probed with primary antibodies overnight. Immunoblots were washed thrice (10 min each) in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and then incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) (1:5000) for 1 h. Blots were washed thrice (10 min each) in PBS containing 0.1% Tween-20, developed in enhance chemiluminescent reagent (Amersham Biosciences, GE Healthcare), and visualized with MultiImage III FluorChem® Q (Alpha Innotech, Santa Clara, CA). Signal intensity quantifications were performed using Alphaview Software (Alpha Innotech).

**Autophagy assay**—Autophagic degradation of long-lived proteins was assessed as previously described (255). Briefly, H4IIE hepatocytes (~80% confluence) were incubated for 18 h in complete media (DMEM containing 5 mM glucose, penicillin/streptomycin, and 10% FBS) supplemented with 0.2 µCi/mL of L-14C-Valine (Moravek Biochemicals, Brea, CA). Cells were washed thrice in PBS and placed in DMEM (lacking glucose, L-glutamine, and sodium pyruvate but containing 0.1% BSA and 0.5 mM cold valine) supplemented with either 10% FBS, vehicle (20 mM HEPES pH 8 containing 135 mM NaCl), 5 µg/mL myonectin, or myonectin plus LY294002 (50 µM), Akt IV (10 µM), or rapamycin (10 ng/mL). Short-lived proteins were degraded within 1 h of incubation, so media were changed at 1 h. Cells were then incubated for
an additional 4 h, after which media were collected. Cells were washed thrice with PBS, scraped, and collected. A matched volume containing 10% trichloroacetic acid and 1% phosphotungstic acid was added to cells, which were then centrifuged at 600 x g for 10 min at 4°C. Cell media and the acid-soluble cell lysate fraction were combined and placed in a scintillation vial containing 2 mL of Soluene 350 (National diagnostics). The acid-insoluble fraction was resuspended in PBS and placed in a separate scintillation vial containing 2 mL of Soluene 350. Percent protein degradation was calculated by dividing counts in the medium plus the acid-soluble fraction of cell lysate with counts in the acid-insoluble fraction of the cell lysate.

Statistical analysis—Comparisons were performed using two-tail Student’s t-tests with 95% confidence intervals. Values were considered significant at $p<0.05$. For all data *= $p<0.05$; **= $p<0.01$; and ***= $p<0.001$. All data are presented as mean ± standard error of the mean.

Results

**Myonectin expression is repressed by starvation and induced by nutrient supplementation**

We have previously shown that myonectin expression and circulating levels are highly upregulated in mice by re-feeding after a 12-h fast. However, a prolonged 24-h food deprivation (considered starvation in mice) is necessary to observe pronounced effects on liver autophagy (256). To evaluate changes in myonectin expression during starvation, mice were fed *ad-libitum* or fasted for 24 h and injected with a bolus of glucose or saline. A striking increase in myonectin mRNA (~10-fold) and circulating protein levels (~3-fold) was observed when starved mice were given a bolus of glucose compared to a bolus of saline or mice fed *ad-libitum* (Fig. 1A-B). In accordance, myonectin mRNA expression was markedly reduced in differentiated C2C12 myotubes when cells were subjected to serum deprivation (Fig. 1C), mimicking starvation-
induced autophagy (257). These results indicate that myonectin expression is repressed by starvation and robustly induced by nutrient availability in cultured skeletal muscle cells and in mice.

FIGURE 4-1. Myonectin expression and circulating levels are reduced by starvation and increased by nutrient availability. A-B, Animals fed ad-libitum (ad-lib) or fasted for 24 h were tail vein-injected with 10 µL/g body weight of 20% (w/v) glucose or a matched volume of saline (n=6). Skeletal muscle and sera were harvested 3 h later and subjected to quantitative real-time PCR analysis of myonectin expression (A) and immunoblot quantification of serum myonectin levels (B). C, Quantitative real-time PCR analysis of myonectin expression in differentiated mouse C2C12 myotubes (n=6) treated for 18 h in DMEM containing 5 mM glucose and 0.1% BSA, with 10% FBS (serum) or without (starved). All quantitative real-time PCR data were normalized to β-actin values. *p<0.05; **p<0.01
Myonectin suppresses the expression of autophagy genes in H4IIE hepatocytes and mouse liver

Expression of key autophagy genes in mouse liver is altered in response to nutrient deprivation. Autophagy-related protein (ATG) 7 is required for starvation-induced protein degradation in the liver (244), whereas ATG12 is critical for the formation of autophagosome precursors (258). Time-course treatments revealed a strong induction of Atg7 (Fig. 2A) and Atg12 (Fig. 2B) when H4IIE hepatocytes were serum-starved for 3 h and 6 h. Addition of recombinant myonectin was sufficient to suppress the induction of Atg genes in response to serum deprivation (Fig. 2A, B). Changes in mRNA levels for Atg7 and Atg12 in response to serum starvation and myonectin treatment were confirmed at the protein levels (Fig. 2C, D). To confirm our results in vivo, mice were fasted for 24 h and injected with a bolus of glucose, vehicle, or myonectin (1 μg/g body weight). Recombinant protein administration resulted in a four-fold increase in the circulating levels of myonectin 10 min after protein injection compared to saline injection (Supplemental Fig. S1). As expected, starved mice injected with control vehicle showed a significant upregulation of ATG7 and ATG12 levels in mouse liver, and this induction was suppressed when starved mice were given a bolus of glucose or injected with recombinant myonectin (Fig. 2E, F). These results suggest that myonectin regulates the expression of key autophagy genes in cultured hepatocytes and liver in response to nutrient availability. Further, recombinant myonectin administration did not alter circulating glucagon, a hormone known to activate liver autophagy in the fasted state, levels at 10 min or 4 h post injection (data not shown), suggesting that myonectin acts independently of glucagon (250).
FIGURE 4-2. Myonectin suppresses the expression of starvation-induced autophagy genes. A-B, Quantitative real-time PCR analysis of Atg7 (A), and Atg12 (B) mRNA in H4IIE hepatocytes (n=4) cultured for 3, 6, 12 or 24 h in medium containing 10% FBS (serum), HEPES buffer (starved), or 5 μg/mL recombinant myonectin (starved + myonectin). All quantitative PCR data were normalized to β-actin values. C-D, Representative immunoblots and corresponding protein quantifications for ATG7 (C) and ATG12 (D) in H4IIE hepatocytes cultured for 6 h in the same conditions (n=6). E-F, Mice (n=6) were fasted for 24 h and tail vein-infected with 10 μL/g body weight of 20% glucose (w/v), HEPES buffer (vehicle), or 1 μg/g body weight of recombinant myonectin. Four hours later, livers were harvested and ATG7 (E) and ATG12 (F) levels were quantified by Western blot analysis. Immunoblots show three representative liver samples. Tubulin was used as a loading control for immunoblot analyses. *p<0.05; **p<0.01; ***p<0.001
Myonectin inhibits LC3 lipidation and autophagosome-dependent p62 degradation

Lipidation of microtubule associated protein-1 light chain 3 (LC3) is a critical initial step in autophagy, leading to subsequent aggregation and autophagosome formation (256, 258). Autophagosomes contain p62/SQSTM1 protein complexes, which are eventually degraded by cellular autophagy (259, 260). Thus, the ratio of non-lipidated to lipidated LC3 and p62 degradation serve as markers for early and late stages of autophagy, respectively. H4IIE hepatocytes were deprived of serum for 2 h to activate autophagy, as indicated by a marked decrease in the proportion of non-lipidated (indicated by LC3-I) to lipidated (indicated by LC3-II) forms of LC3. Treatment with recombinant myonectin alone for 1 h post-induction was sufficient to reduce the lipidation of LC3 (Fig. 3A). Myonectin treatment increased the ratio of LC3-I/LC3-II by ~50%, an effect comparable to cells cultured in the presence of serum (Fig. 3B). To confirm the effects of myonectin on LC3 lipidation were indeed due to a shift in autophagic flux, the same experiments were repeated in the presence of chloroquine (261), a potent inhibitor of lysosomal degradation (Fig. 3A, C). Treatment with chloroquine enhanced the extent of starvation-induced LC3 lipidation, as well as the ability of myonectin to abolish these effects (Fig. 3C).

To assess late-stage autophagosome-dependent protein degradation, H4IIE hepatocytes were cultured for 24 h in the presence of serum, serum-starved, or serum-starved in the presence of recombinant myonectin and probed for p62 levels. Serum starvation significantly reduced p62 levels relative to cells cultured in the presence of serum (Fig. 3D). Recombinant myonectin treatment essentially reversed this process in starved cells, as indicated by the complete inhibition of starvation-induced p62 degradation (Fig. 3D). Cells starved for a shorter period of time (3, 6 or 12 h) elicited only a modest reduction of p62 when compared to serum- and
myonectin-treated samples (data not shown). To ensure that effects of myonectin on cellular autophagy are physiologically relevant in vivo, we subjected mice to a 24-h fast, then administered a bolus of glucose, vehicle, or recombinant myonectin. Consistent with our in vitro results, starved mice injected with vehicle control activated autophagy as indicated by significantly greater hepatic LC3 lipidation and p62 degradation compared to starved mice injected with a bolus of glucose (Fig. 3E, F). Importantly, myonectin administration largely suppressed starvation-induced LC3 lipidation and p62 degradation in the liver to a similar extent as glucose administration (Fig. 3E, F). These results indicate that myonectin inhibits early-stage autophagosome formation, as well as late-stage autophagy-dependent p62 degradation in vitro and in vivo.
FIGURE 4-3. **Myonectin reduces autophagosome formation and autophagy-mediated p62 degradation.**

_A_, Representative immunoblots and quantifications of autophagy marker LC3 in H4IIE hepatocytes cultured for 2 h in starvation medium in the presence or absence of 10 µM chloroquine (chloro) and then treated for 5, 15, 30 or 60 min with HEPES buffer (veh) or 5 µg/mL myonectin. LC3-II denotes the lipidated form, observed in a slight size shift from the non-lipidated band, LC3-I. 

_B-C_, Immunoblot and quantification (n=6) of LC3 in H4IIE hepatocytes cultured for 1 h in medium containing 10% FBS (serum), HEPES buffer (starved), or 5 µg/mL myonectin, in the absence (B) or presence (C) of 10 µM chloroquine. 

_D_, Immunoblot and quantification (n=6) of p62 in H4IIE hepatocytes cultured for 24 h in medium containing 10% FBS (serum), HEPES buffer (starved), or 5 µg/mL myonectin. 

_E-F_, Mice (n=6) were fasted for 24 h and tail vein-injected with 10 μL/g body weight of 20% glucose (w/v), HEPES buffer (vehicle), or 1 μg/g body weight of recombinant myonectin. Four hours later, livers were harvested and subjected to immunoblot quantification of LC3 (E) and p62 (F). All blots show tubulin as the loading control. Each lane represents an independent cell or liver sample. *p<0.05; **p<0.01
**Myonectin activates the Akt/mTOR pathway to suppress autophagy**

We next investigated what cellular signaling pathways are activated by myonectin to block autophagy. H4IIE hepatocytes were serum-starved for 1 h to reduce background signaling and then treated with vehicle control or recombinant myonectin for 5, 15, 30, or 60 min. Relative to vehicle-treated cells, myonectin dramatically induced phosphorylation of various components within the canonical Akt-mTOR pathway. Myonectin robustly induced the phosphorylation and activation of IRS-1 (Ser-612), Akt (Thr-308), and mTOR (Ser-2448) in cultured H4IIE hepatocytes (Fig. 4A) and in mouse liver (Fig 4B). Activation of Akt signaling elicits an mTOR-dependent suppression of cellular autophagy (263). Further, mTOR-dependent phosphorylation of ULK1 at Ser-757 disrupts its association with AMP-activated protein kinase (AMPK) and abolishes AMPK-mediated autophagosome formation (264, 265). Myonectin robustly induced ULK1 phosphorylation at Ser-757 in cultured hepatocytes and in mouse liver (Fig. 4). Activated Akt phosphorylates Forkhead box transcription factor 3 (FOXO3 at Thr-24, resulting in its nuclear exclusion (266). Under conditions when autophagic activity is high, FOXO3 binds to the promoters of relevant $Atg$ genes (e.g., $Atg12$) to activate gene transcription; this process is disrupted when FOXO3 is phosphorylated by Akt (267). Myonectin induced FOXO3 phosphorylation at Thr-24 in cultured H4IIE hepatocytes and in mouse liver (Fig. 4).
FIGURE 4-4. Myonectin activates Akt/mTOR pathway in H4IIE hepatocytes and mouse liver. A, H4IIE hepatocytes were serum-starved for 2 h, then placed in DMEM containing 5 mM glucose and 5 µg/mL myonectin or HEPES buffer (veh) for 5, 15, 30, or 60 min. Immunoblots were probed for phosphor-IRS (Ser612; P-IRS), IRS, phospho-AKT (Thr308; P-AKT), AKT, phospho-mTOR (Ser2448; P-mTOR), mTOR, phospho-ULK1 (Ser757; P-ULK1), ULK1, phospho-FOXO3 (Thr24; P-FOXO3), and FOXO3. B, Mice (n=6) were fasted for 24 h and tail vein-injected with HEPES buffer (vehicle) or 1 µg/g body weight of recombinant myonectin. Livers were harvested 10 min after injection and subject to immunoblot for the same signaling molecules. Each lane represents an independent cell or liver sample.
Inhibition of mTOR signaling abrogates myonectin suppression of autophagy

To confirm that myonectin indeed acts via the PI3K/Akt/mTOR pathway to inhibit autophagy, H4IIE hepatocytes were treated with recombinant myonectin in the presence or absence of LY294002, Akt IV, or rapamycin, potent pharmacological inhibitors of PI3K, Akt and mTOR function, respectively. Serum-starved cells treated with vehicle control or inhibitors activated autophagy, as indicated by increased LC3 lipidation, Atg7 and Atg12 expression, and p62 degradation. These processes were suppressed by myonectin treatment (Fig. 5). All three inhibitors completely abolished the ability of myonectin to inhibit starvation-induced LC3 lipidation, Atg gene expression, and greatly reduced its ability to prevent p62 degradation when simultaneously given to cells (Fig. 5).
Figure 4-5. Inhibition of PI3K-AKT-mTOR signaling prevents myonectin from suppressing autophagy-mediated LC3 lipidation, Atg expression and p62 degradation. Western blot analysis showing representative immunoblots (left column) and quantifications (right column; n=5) of H4IIE hepatocytes treated with or without 10% FBS (serum) and recombinant myonectin (5 ug/mL) in the presence or absence of signaling inhibitors LY294002 (50 µM), Akt IV (10 µM), or rapamycin (10 ng/mL) for 1, 5 or 24 h. *p<0.05; **p<0.01; ***p<0.001
As useful and informative markers, LC3 lipidation and p62 degradation represent the early and late stages of autophagy. However, we wanted to functionally verify our results using a biochemical assay to quantify the degradation of long-lived proteins in cells when autophagy is fully engaged (255). To do so, H4IIE hepatocytes were pulsed with $^{14}$C-labeled valine to radiolabel intracellular proteins before starving cells. As expected, serum-starved cells had about two-fold higher total protein degradation compared to cells cultured with serum (Fig. 6). Treatment with recombinant myonectin largely abolished starvation-induced protein degradation. LY294002, Akt IV, and rapamycin treatment, however, substantially inhibited the ability of myonectin to suppress starvation-induced cellular protein degradation (Fig. 6). Together, these results indicate that myonectin modulates cellular autophagy via the Akt/mTOR signaling pathway.

FIGURE 4-6. Inhibition of Akt-mTOR signaling abolishes the ability of myonectin to suppress autophagy in hepatocytes. The rate of degradation of $^{14}$C-valine-labeled, long-lived proteins was measured in H4IIE hepatocytes (n=5) cultured in glucose-free medium (starved) or glucose-free medium containing 10% FBS (serum), myonectin (5 µg/mL), LY294002 (50 µM), Akt IV (10 µM), rapamycin (10 ng/mL), or myonectin plus inhibitors. a = $p<0.05$ compared to starved vehicle control; b = $p<0.05$ compared to starved myonectin sample.
Discussion

Here, we describe a novel function of myonectin as a nutrient-responsive regulator of liver autophagy. Prolonged periods of food deprivation turn on autophagy, the cell recycling pathway, by inducing the expression of autophagy-related genes and the formation of autophagosomes (237). This intracellular degradative pathway is highly regulated and sensitive to metabolic alterations. We provide evidence that skeletal muscle-derived myonectin, induced by food intake or the availability of nutrients (e.g., glucose and free fatty acids), conveys a hormonal signal to inhibit autophagy in hepatocytes; this is evidence of a novel skeletal muscle-liver axis in modulating tissue homeostasis.

The autophagy-related genes (Atg), first characterized in budding yeast, are required for autophagosome formation (268). The ATG-regulated cellular recycling pathway is phylogenetically conserved in metazoans (269), playing an essential role in maintaining cellular homeostasis in response to prolonged nutrient deprivation (237). As expected, we observed a pronounced induction of several key Atg genes in serum-starved H4IIE hepatocytes and in the liver of starved mice; remarkably, myonectin administration suppressed starvation-induced Atg expression in vitro and in vivo.

Degradation of the cytoplasmic component of liver cells is metabolically regulated and tightly coupled to prolonged fasting or starvation (237). After a 24-h fast, hepatic glycogen stores are mostly depleted to buffer blood glucose. Autophagosome-mediated recycling of cytoplasmic contents provides the liver, a major gluconeogenic tissue, an additional fuel source to power de novo glucose synthesis for maintaining physiological blood glucose levels during starvation. When nutrients become available, autophagy will be promptly turned off to prevent excessive catabolic breakdown of cytoplasmic components. We show that nutrient-induced myonectin
potently suppresses the LC3-dependent formation of autophagosomes and p62 degradation in cultured hepatocytes and in mouse liver. Importantly, myonectin inhibits autophagy to the same extent as conditions that maximally suppress autophagy (hepatocytes cultured in medium containing serum or starved mice given a bolus of glucose).

Activation of mTOR signaling, a nutrient-responsive anabolic pathway, potently inhibits autophagy (251). We show that myonectin activates the Akt/mTOR pathway to inhibit autophagy. Consistently, pharmacologic inhibition of PI3K, Akt, or mTOR function completely abolished myonectin’s ability to suppress autophagy in cultured hepatocytes. Because the receptor for myonectin is unknown, it is also unknown how skeletal muscle-derived myonectin conveys a hormonal signal to the liver to suppress autophagy through mTOR pathway activation. Identifying the receptor that mediates myonectin’s cellular effects will provide a better mechanistic understanding of its function and regulation.

The anabolic hormone insulin is a potent suppressor of autophagy in liver and skeletal muscle (253). The kinetics of insulin secretion is rapid; within 5-10 min after the ingestion of food or a bolus of glucose, insulin is secreted from pancreatic β-cells to promote glucose uptake in skeletal muscle and adipose tissue (1). In liver, insulin suppresses hepatic glucose output (224). Within one hour, circulating levels of insulin revert to baseline levels. In contrast, the kinetics of myonectin expression and secretion in response to food intake are slower than the time scale of insulin secretion and clearance. Peak induction of myonectin expression and secretion occurs 3 h after food intake or glucose gavage (23). Thus, insulin’s effect on liver autophagy is rapid and acute following food ingestion, while myonectin acts in the liver at a later postprandial time point to maintain liver autophagy in an “off” state.
Exercise induces autophagy in multiple tissues, leading to improved metabolic outcomes (239). We previously showed that myonectin mRNA and circulating levels are increased in mice that have access to a running wheel for two weeks (a model of chronic voluntary exercise) (23). The observed effect may result from a secondary response associated with increased bouts of feeding following voluntary exercise (237). Accordingly, the magnitude of myonectin induction resulting from re-feeding following a fast is much greater compared to that induced by chronic voluntary exercise. Further study is needed to determine whether acute muscle contraction or a single bout of exercise can regulate myonectin expression in skeletal muscle.

Collectively, this study provides evidence for direct endocrine communication between skeletal muscle and liver and highlights the complexity and importance of inter-tissue crosstalk in mediating integrated physiology. Elucidating hormone-mediated tissue crosstalk under different metabolic states, such as food deprivation and exercise, will undoubtedly provide insights into obesity and type 2 diabetes, two prevalent metabolic disorders characterized by failure to maintain systemic energy balance.
Summary of major findings

My doctoral thesis work was focused in the areas of integrative physiology, biochemistry and regulation of metabolic circuits. Here I acquired not only vital experimental laboratory techniques, but more importantly the approaches and critical thinking required to pursue a career in academic research. During my 5 years of doctoral research in Dr. Wong’s lab, I was able to identify and functionally characterize a novel protein we termed myonectin, a myokine belonging to the C1q/TNF-related protein (CTRP) family. Initially, we observed myonectin as a secreted polypeptide whose transcripts are almost exclusively expressed by skeletal muscle in both humans and mice. I next sought to elucidate the physiologically relevant conditions which regulate myonectin in vivo. We addressed whether changes in nutritional/metabolic state regulate myonectin expression and circulating levels. Surprisingly, an overnight fast greatly suppressed myonectin expression, but a 2-h refeeding period (following an overnight fast) dramatically up-regulated its mRNA expression in skeletal muscle. Interestingly, refeeding induced myonectin mRNA expression to a much greater extent in soleus (slow-twitch, oxidative) than in plantaris (fast-twitch, glycolytic) muscle fiber, suggesting that expression of the protein may be differentially regulated depending on muscle fiber type. Consistent with the mRNA data, fasting reduced, but refeeding substantially increased, circulating levels of myonectin. In a separate cohort of mice, we determined whether the carbohydrate or lipid component within the diet was responsible for the induction of myonectin expression and secretion in the fasted/re-fed state. Overnight fasted male mice were gavaged with a bolus of glucose or emulsified Intralipid. Both glucose and lipid were equally potent at increasing circulating levels of myonectin in the re-fed state. Intraperitoneal injection of the same dietary components enhanced myonectin serum protein levels to the same extent as gavage and feeding. Further, mice injected with
recombinant insulin, the well-described postprandial hormone did not display altered myonectin expression or circulation. Collectively, these data suggested that nutrient flux through muscle cells directly regulates myonectin expression and secretion.

To address the metabolic function of myonectin in vivo, we administered purified recombinant myonectin to wild-type male mice. When injected intraperitoneally into mice a physiologically relevant dose of recombinant myonectin which recapitulated the upregulation induced by nutrient availability. At this dosage, a relatively modest rise in serum myonectin levels was sufficient to lower (by \( \sim 30\% \)) non-esterified fatty acids (NEFA) levels over time relative to vehicle-injected controls. In the fasted state, myonectin can lower circulating NEFA levels via two possible mechanisms: suppression of adipose tissue lipolysis or promotion of fatty acid uptake into cells. Treatment of 3T3-L1 adipocytes in vitro or primary adipose tissue (epididymal fat pads) ex vivo with recombinant myonectin had no effect on lipolysis. Given that myonectin did not alter secretion of free fatty acids, we next hypothesized the protein exerts its NEFA-lowering effects by enhancing lipid uptake into adipose and liver tissue, the primary organs which take up fat to store as triglycerides. As expected, myonectin enhanced the ability of both hepatocytes and adipocytes to take up fatty acids in a dose-dependent fashion. This phenomenon was in part, mediated by enhanced expression of adipose and liver genes which facilitate fatty acid uptake into cells. Together, these results indicate that myonectin promotes lipid uptake into adipocytes and hepatocytes via transcriptional up-regulation of genes involved in fatty acid uptake, thus regulating whole-body lipid metabolism.

Next, we wanted to explore the role of myonectin in controlling glucose metabolism, especially given known tightly-regulated mechanisms of differentially shifting glucose homeostasis in the fasted and fed states. Fasted mice were injected with a similar bolus of
recombinant myonectin, resulting in a reduction in serum glucose levels over time. In the fasted state, peripheral tissue glycogen content is substantially depleted and several vital organs, such as the brain, rely on the capacity of the liver to produce glucose from metabolic substrates. Based on the dramatic induction of myonectin which accompanied refeeding, we hypothesized that myonectin would regulate postprandial glucose metabolism by inhibiting glucose production and enhancing uptake for storage in the form of glycogen. As expected, myonectin treatment was sufficient to inhibit glucose production in vitro, by suppressing mRNA expression of key gluconeogenic genes, Glucose 6-phosphatase (G6P) and Phosphoenolpyruvate carboxykinase (PEPCK). Additionally, myonectin enhanced hepatic glucose uptake through increased expression of the primary liver glucose transporter, GLUT2. To further refine the mechanism by which myonectin regulates these vital cellular functions, we showed recombinant myonectin protein alone activates the well-studied PI3-Kinase/Akt/mTOR canonical signaling cascade. Importantly, pharmacologic inhibition of this cascade abolished myonectin’s ability to regulate cellular functions. The protein kinase B/Akt signaling pathway also regulates glycogen synthesis in the fed state. In mouse liver, recombinant myonectin infusion induced the phosphorylation and inactivation of glycogen synthase kinase 3β (Gsk3β) at Ser-9, the residue phosphorylated by activated Akt. Inactivated Gsk3β exhibited reduced inhibitory phosphorylation on glycogen synthase (GS) at Ser-641, leading to net activation of glycogen synthase and increased glycogen synthesis. This data not only indicates myonectin exerts significant control over regulating physiologic glucose metabolism by activating postprandial functions in the liver, but also establishes the direct mechanism by which the protein acts.

The observations that myonectin expression and secretion is dependent on nutrient availability and its activation of the PI3-Kinase/Akt/mTOR canonical signaling cascade also lead
to the development of a different, but equally important project which explored how the protein acts on liver to specifically hinder autophagic function. This work showed myonectin exerts substantial control over hepatic autophagy by hindering expression of starvation-induced transcripts Atg7 and Atg12. Myonectin also inhibited autophagosome formation, as well as total cellular protein degradation. We concluded this study by showing that pharmacologic inhibition of the PI3K/Akt/mTOR pathway abolished the ability of myonectin to inhibit autophagic function.

Collectively, my thesis work demonstrated myonectin as a skeletal muscle protein which is secreted as a reflection of tissue-specific nutrient availability. Further, myonectin acts on other important metabolic organs to activate postprandial mechanisms, thereby regulating the physiologic flux of metabolic substrates and energy stores. Dr. Wong served as ideal mentor both in terms of productivity and oversight. My work resulted in 12 publications, 4 of which I served as first author. At the end of my graduate career, I was awarded the Johns Hopkins Paul Erlich Young Investigators Award for my research accomplishments. My time at Hopkins taught me how to approach scientific problems, ask the important questions, think in a critically and work in an ethical and collaborative fashion.
REFERENCES


~ 89 ~


~ 90 ~


~ 91 ~


~ 94 ~


~ 95 ~


tissue and exerts antiinflammatory and antifibrotic effects in primary human colonic fibroblasts. *Inflamm Bowel Dis* **17**, 2462-2471


~ 100 ~

~ 101 ~


~ 103 ~


H., Quaggin, S. E., Raben, N., Rabinowich, H., Rabkin, S. W., Raham, I., Rami, A.,
K., Reed, B. H., Reed, J. C., Reigjori, F., Regnier-Vigouroux, A., Reichert, A. S.,
Reiners, J. J., Jr., Reiter, R. J., Ren, J., Revuelta, J. L., Rhodes, C. J., Ritis, K., Rizzo, E.,
Robbins, J., Roberge, M., Rocca, H., Roccheri, M. C., Rocchi, S., Rodemann, H. P.,
Rodriguez de Cordoba, S., Rohrer, B., Roninson, I. B., Rosen, K., Rost-Roszkowska, M.
M., Rouis, M., Rouschop, K. M., Rovetta, F., Rubin, B. P., Rubinszttein, D. C.,
Ruckdeschel, K., Rucker, E. B., 3rd, Rudich, A., Rudolf, E., Ruiz-Opozo, N., Russo, R.,
Rusten, T. E., Ryan, K. M., Ryter, S. W., Sabatini, D. M., Sadoshima, J., Saha, T., Saitoh,
T., Sakagami, H., Sakai, Y., Salekdeh, G. H., Salomoni, P., Salvatera, P. M., Salvesen,
G., Salvioli, R., Sanchez, A. M., Sanchez-Alcazar, J. A., Sanchez-Prieto, R., Sandri, M.,
Sankar, U., Sansanwal, P., Santambrogio, L., Saran, S., Sarkar, S., Sarwal, M., Sasakawa,
C., Sasnauskiene, A., Sass, M., Sato, K., Sato, M., Schapira, A. H., Scharl, M., Schatzl,
H. M., Scheper, W., Schiaffino, S., Schneider, C., Schneider, M. E., Schneider-Stock, R.,
Schoenlein, P. V., Schorderet, D. F., Schuller, C., Schwartz, G. K., Scorrano, L., Sealy,
L., Seglen, P. O., Segura-Aguilar, J., Seiliez, I., Seleverstov, O., Sell, C., Seo, J. B.,
Separovic, D., Setaluri, V., Setoguchi, T., Settembre, C., Shacka, J. J., Shanmugam, M.,
Shapiro, I. M., Shaullian, E., Shaw, R. J., Shellhamer, J. H., Shen, H. M., Shen, W. C.,
Sheng, Z. H., Shi, Y., Shibuya, K., Shidoji, Y.,sie, J. J., Shih, C. M., Shimada, Y.,
Shimizu, S., Shintani, T., Shirihai, O. S., Shore, G. C., Sibirny, A. A., Sidhu, S. B.,
Sikorska, B., Silva-Zacarin, E. C., Simmons, A., Simon, A. K., Simon, H. U., Simone, C.,
M., Sivridis, E., Skop, V., Skulachev, V. P., Slack, R. S., SmaI, S. S., Smith, D. R.,
S., Spies, C. D., Springer, W., Srinivasula, S. M., Stefanis, L., Steffan, J. S., Stendel, R.,
Stenmark, H., Stephanou, A., Stern, S. T., Sternberg, C., Stork, B., Stralfors, P.,
Suzuki, T., Swanson, M. S., Swantox, C., Sweeney, S. T., Sy, L. K., Szabadkai, G.,
Tabas, I., Taegtmeyer, H., Tafani, M., Takacs-Vellai, K., Takano, Y., Takegawa, K.,
Takemura, G., Takeshita, F., Talbot, N. J., Tan, K. S., Tanaka, K., Tang, D., Tanida, I.,
S., Terman, A., Tettamanti, G., Thevissen, K., Thompson, C. B., Thorburn, A., Thumm,
M., Tian, F., Tian, Y., Tocchini-Valentini, G., Tolkovsky, A. M., Tomino, Y., Tonges, L.,
Tooze, S. A., Tournier, C., Tower, J., Towns, R., Tranjkovic, V., Travassos, L. H., Tsai, T.
Uchiyama, Y., Ueno, T., Umemura, M., Umemiya-Shirafuji, R., Unni, V. K., Vaccaro,
Vanhorebeek, I., Vaquero, E. C., Velasco, G., Velill, T., Vicencio, J. M., Vierstra, R. D.,
Vila, M., Vindou, C., Viola, G., Viscomi, M. T., Voitekovich, E. O., von Haefen, C.,

~ 109 ~


~ 110 ~
Curriculum Vitae

Marcus Seldin

Education:

- **2014-Present**: University of California, Los Angeles, Postdoctoral fellow in the Department of Cardiology under the supervision of Dr. Jake Lusis.
- **2009-2014**: Johns Hopkins University School of Medicine, PhD Candidate in the Department of Cellular and Molecular Physiology
- **2004–2008**: University of California, Irvine, B.S. in Biological Sciences, minor in History with emphasis on Middle Eastern studies

Employment and other positions:

- **2011-Present**: Co-Chair, Board of Directors to Hand in Hand, Baltimore: Hand in Hand Baltimore is a non-profit organization dedicated to reducing juvenile recidivism in the Baltimore area. Hand in Hand provides those under 18 who are charged as adults with mental health services, academic counseling, case management and mentoring services during their residence at the detention center and upon their release.
- **2009-2014**: JHGSA Physiology Program Representative: Serves as the Cellular and Molecular Physiology graduate program representative for the Johns Hopkins Graduate Student Association (GSA). The GSA is the school-wide student body which represents all graduate students in terms of policies and events
- **2008-2009**: Laboratory Technician, Department of Neurological Surgery, University of California, Irvine: Conducting research on the role of Aquaporin-4 in the onset of several CNS disorders.

Awards

- **2014**: Johns Hopkins Young Investigators Paul Erlich Award Recipient: Myonectin, A novel skeletal muscle nutrient sensor which regulates postprandial metabolism
- **2011**: Johns Hopkins Urban Health Institute Graduate-Community Grant Recipient: Discovering Me: Partnering with young men charged as adults to create selfworth and positive self-image!
- **2008**: Excellence in Research Award Recipient, Department of Biological Sciences, University of California, Irvine: Expression of Aquaporin-4 in the adult mouse brain
- **2007-2008**: Undergraduate Research Opportunities Fellow, University of California, Irvine Grant Recipient: Expression of Aquaporin-4 in the Epileptic Mouse Brain
- **2007**: University of California, Irvine Grant Recipient, Summer 2007 SURP Fellow: Expression of mAQP4 in the Epileptic Brain

~ 111 ~
Posters and presentations:

- **2009**: Society for Neuroscience Annual Convention: Seldin, MM; Rajneesh KF; Hsu, MS; Binder, DK. Regulation of angiogenic factors in the mouse intrahippocampal kainic acid model of epileptogenesis
- **2008**: American Epilepsy Society Annual Meeting: Lee, D; Hsu, M; Seldin, M; Nanduri, A; Binder, D. The functional role of Aquaporin-4 in Epileptogenesis
- **2008**: Excellence in Research Symposium, UCI: Seldin, MM; Binder DK. Expression of Aquaporin-4 in the adult mouse brain
- **2007**: Society for Neuroscience Annual Convention: Hsu, MS; Alfonso, VH; Seldin, MM, Binder, DK. Expression of the Glial water channel, aquaporin-4 (AQP4) in the mouse brain

Peer-reviewed Publications:

- Peterson JM, Wei Z, Seldin MM, Byerly MS, Aja S, Wong GW. CTRP9 transgenic mice are protected from diet-induced obesity and metabolic dysfunction. Am J Physiol Regul Integr Comp Physiol. 2013 305(5):R522-33

~ 112 ~


Research Mentoring

• Medije Mashkulli – Medical Student Summer Intern, New York College of Osteopathic Medicine; 2009

• Jung Woo Wren Kim – Department of Physiology PhD candidate, Johns Hopkins University; 2010

• Hoa Jia - Department of Physiology PhD candidate, Johns Hopkins University; 2011

• Kevin Stanson – Honors Undergraduate Summer Fellow, *University of Pittsburgh; 2012*

• Ying-Lin Stefanie Tan – Department of Biochemistry, Cellular and Molecular Biology PhD candidate, Johns Hopkins University; 2011

• Victoria Kuhns- Department of Physiology PhD student, Johns Hopkins University; 2012

• Oluwaseun Ogunbona – Department of Physiology PhD student, Johns Hopkins University; 2013

• Risa Wolf – Clinical Fellow, Department of Pediatric Endocrinology, Johns Hopkins University; 2014
Hannah Little - *Department of* Biochemistry, Cellular and Molecular Biology PhD candidate, Johns Hopkins University; 2014