MARCH1: AN EXPLORATION OF ITS DOMAINS AND UNDERSTANDING ITS ROLE IN THE MECHANISMS OF ANTIGEN-PRESENTATION AND THE POTENTIAL SEQUELA OF DYSREGULATION

By

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A Thesis Submitted to The Honors College
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Abstract

Current research of membrane-associated ring finger -C3HC4-1 (MARCH1) suggests that its role in mammalian immunity is more complex than was previously thought. MARCH1 is an E3 ubiquitin ligase expressed by antigen-presenting cells of the immune system, where it inhibits antigen presentation by downregulation of its substrates, MHC class II and CD86. We have studied how MARCH1 expression is regulated and determined that the protein levels are regulated, in part, through its inherent instability. We have shown that MARCH1 has distinct regions responsible for both its stability and function. The amino terminus contains a region needed for destabilization of the protein, while when amino acid residues 229-257 are missing; it appears to lead to a loss of function. Preliminary studies into dysregulation of antigen processing, initiated by loss of MARCH1 expression in mice, have shown inclinations toward a metabolically diseased state. Knock-out (KO) MARCH1 mice have increased amounts of visceral adipose tissue relative to wild type. In addition, our preliminary results, examining transcript levels, suggest that a pro-inflammatory environment may result from the loss of MARCH1 in these fat depots, potentially resulting in metabolic disease. Our results indicate that changes in MARCH1 levels lead to a potentially dysregulated inflammatory environment.
Introduction

Regions of MARCH1 Function and Stability

An organism's immune system acts as a cellular line of defense against many harmful diseases. It is through a complex system of signaling and cellular interactions that the immune system is able to orchestrate a highly targeted attack on foreign agents without affecting the host's normal tissues. The overall benefits of having a well-functioning immune system are now appreciated to go far beyond immunity's conventional role. There is a growing body of literature in support of the immune system having non-traditional roles in processes such as neurodegeneration, cardiovascular function and, particularly, metabolism1. This paper focuses on one immune molecule, MARCH1, and the domains responsible for its properties and function as well as its potential involvement in metabolic pathways.

The immune response is subject to stringent control mechanisms. One component of this regulation is the membrane-associated RING-CH-1 (MARCH1) protein. MARCH1 is expressed in antigen presenting cells (APCs) such as dendritic cells (DC), macrophages and B cells 2,3. DCs are “professional” APCs that are major players in the initiation of T cell responses. To achieve effective antigen-presentation, DCs use a number of cell surface and co-stimulatory molecules to enhance the interaction with T cells and increase priming potency 4. Upon activation by some foreign antigen, DCs become potent immune stimulators. These DCs undergo a process of maturation marked by an increase in specific cell surface markers, such as Major Histocompatibility Complex II (MHC II, found on APCs and
lymphocytes and is involved in mediating specific immune responses by presenting pathogen-derived-peptides), Cluster of Differentiation 86 (CD86, found on APCs and is involved in co-stimulatory activation of T-cells) and Cluster of Differentiation 80 (CD80, found on B cells and monocytes and is involved in co-stimulatory activation of T-cells). MARCH1 has been shown to be involved in this maturation event, regulating the surface expression of both MHC-II and CD86 in resting APCs. Induction of APC maturation results in a loss of MARCH1 expression and function, and subsequently leads to the development of an effective immune response. Better understanding of this maturation event can be realized by determining the domains of MARCH1 that are responsible for its function, stability and regulation.

MARCH1 is an E3 ubiquitin ligase, responsible for tagging (ubiquitinating) its specific target molecules for destruction. It belongs to a family of proteins which share homology with the K3 family of viral immune evasion proteins that inhibit the surface expression of proteins such as MHC-I and CD86. The K3 proteins contain two transmembrane domains following the amino terminal RING-CH domain (figure 1). The RING-CH domain of MARCH proteins is responsible for the interaction with their specific E2 ubiquitin conjugating enzyme. It has been experimentally determined that MHC class II and CD86 are targets of MARCH1 ubiquitination. The downregulation of MARCH1 is one of the major events needed for DCs to undergo maturation. In order for DCs to become mature they need to express high levels of MHC-II and CD86. MARCH1 needs to be downregulated, or inhibited, to allow for accumulation of these molecules at the surface of cells. Because cells are in need of a way to quickly mount an immune response, MARCH1
is tightly regulated at multiple levels to ensure rapid maturation and immune induction. One unique aspect of MARCH1 regulation is the inherent instability of the protein.

In order to determine the domains important to MARCH1 stability and function, N and C terminal truncations mutants were designed. We demonstrated that N-terminus truncations caused an increase in MARCH1 stability over wildtype, while C terminus truncations saw a loss of stability/expression. The function of these truncated proteins was also investigated. Our results showed that N-terminus truncations were functional, as long as the RING domain was left intact, and that a specific leucine residue (L215) is needed for the function of MARCH.

MARCH expression is accomplished via a very tightly regulated mechanism at both the transcriptional and post-translational levels. It has been shown that LPS-induced maturation causes transcriptional downregulation of MARCH1 mRNA expression. This downregulation was visualized via qPCR showing a 20-fold decrease in mRNA transcript levels 24 hours following LPS-induced maturation. Due to its instability, MARCH1 protein levels decrease over time after maturation due to the decrease in transcription. Another key regulator of MARCH1 transcription is the anti-inflammatory cytokine IL-10. Cells treated with IL-10 have shown an increase in MARCH1 mRNA and protein levels.

Dysregulation of MARCH1 and Metabolic Disease

While examining the structure, function, and regulation of MARCH1, we observed that our MARCH1 knockout (KO) mice were often larger (fatter) than their wildtype (WT) counterparts. This observation led us to investigate a potential link
between the immune system and metabolism. The immune system is suggested to play an integral role in metabolic processes. Immune cells have been shown to utilize unique metabolic pathways under certain conditions to control their fate. For example, it has been shown that T cells use serine/threonine kinases (AKT1-3, AMPK, mTOR, and LKB1), usually thought to be used as cellular nutrient sensors of levels of intracellular amino acid pools and carbohydrate, to control the fate switch from cytotoxic to memory CD8+ T cell. In addition, it has been shown that obesity is linked to a chronic state of low-grade inflammation. Adipose tissue is not only involved in energy storage but can act as an endocrine organ that secretes numerous factors. The dysregulated production of such factors due to obesity and excess adiposity can lead to pathology. Adipokines are the substances secreted from the numerous cells found in adipose tissue. The cells that contribute to the secretion of adipokines are: adipocytes, cells in the stromal-vascular fraction of adipose (including leukocytes), liver, and muscle cells. Leptin is a key adipokine which is secreted from adipocytes and functions to control one's appetite. Tumor Necrosis Factor (TNF) and Adiponectin are two other key adipokines, which have been shown to have antagonistic functions. TNF is secreted from the stromal vascular fraction of cells as well as adipocytes and functions to increase inflammation and antagonizes insulin signaling. Adiponectin on the other hand sensitizes targets to insulin and is anti-inflammatory. This interference with the signaling of insulin leads to the conclusion that adiposity and inflammation are responsible for at least some of the effects on the development of Type 2 diabetes (T2D).
Within the past 10-15 years there have been an increasing number of studies published linking type 2 diabetes and inflammation. T2D is the consequence of insulin-resistance and/or islet pancreatic Beta-cell (the cells responsible for insulin production) dysfunction 17. As a result, the body is unable to absorb glucose which then builds up in the blood 18, and cells are starved of energy, despite high blood glucose levels (hyperglycemia). Persistent hyperglycemia causes damage to nerves and blood vessels, which can result in complications such as heart disease, stroke, kidney disease, blindness, dental disease, and amputations 18. It has been noted, however, that many over-weight insulin-resistant individuals do not develop diabetes 18. In fact, only about one-third of those (over-weight) individuals develop diabetes. The patients that do develop T2D present some underlying cellular stressor which is either caused by or induces inflammation 18. This underlying inflammation causes an increase of specific circulating inflammatory factors, which have been shown to be elevated in patients with T2D 18. The key inflammatory factors have been defined include C-reactive protein and IL-6, which are secreted from adipose tissue and liver 18,19, and promote insulin desensitization leading to T2D 20,21. With this understanding of the relationship between the immune system and metabolic disease, our research aims to determine whether MARCH1-dependent APC maturation has some role in the inflammatory state and adiposity of the visceral adipose tissues (VAT), ultimately predisposing individuals to metabolic disease.

Model
It is believed that the MARCH1 KO mice have Macrophages and other APCs that are able to mature faster due to their loss of MARCH1 (they are “pre-activated”). When APCs are mature they secrete pro-inflammatory factors and have high antigen-presentation, leading to activation of CD4 T helper cells. In response, activated T cells secrete pro-inflammatory cytokines, which along with those secreted from the APC can target adipocytes, causing them to increase production of their own inflammatory molecules. These combined responses can trigger low-grade systemic inflammation, which may influence the development of insulin resistance and T2D. This paper explores the different aspects of MARCH1’s structure and function, as well as investigates MARCH1’s effects on Metabolism.

**Materials/Methods:**

*Mice.* C57BL/6 mice were bred in-house and were housed in the animal facilities at the University of Arizona. All experiments involving animals were performed according to guidelines set forth by the Institutional Animal Care and Use Committee. Mice were maintained on Global Teklad 2019 chow (Harlan) containing 22% calories from fat. For high-fat diet experiments, mice were fed Teklad TD.06415 containing 45% calories from fat.
**Antibodies.** Mouse anti-HA (influenza hemagglutinin epitope) antibody (Ab) (clone 6E2) was acquired from Cell Signaling Technology. Rat anti-CD86 (clone GL1) was purchased from R&D Systems. Rat anti-mouse MHC class II (clone M5/114.15.2), rat anti-mouse invariant chain (In-1), hamster anti-CD80 (16-10A1), and hamster anti-CD11c (clone N418) were obtained from BD Biosciences. Mouse anti-CD86 (B7.2) Ab (clone PO3.1) was obtained from eBioscience.

**Cell Lines and Cell Culture.** WT3 is a C57BL/6-derived mouse embryo fibroblast cell line which was previously obtained from Dr. T. Hansen (Washington University School of Medicine)\(^{22}\). DC2.4 is a DC-like cell line derived from C57BL/6 mice and was supplied by Dr. K. Rock (University of Massachusetts Medical School)\(^{23}\). Bone marrow-derived DC (BMDCs) were generated from a culture of C57BL/6 bone marrow cells incubated 6 days in the presence of 10 ng/ml IL-4 and 10 ng/ml GM-CSF (PeproTech) as described (Jabbour 2009 ref). All cell lines were cultured in complete RPMI 1640 (Mediatech) containing 10% FCS (HyClone), 1 mM HEPES (Invitrogen), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin, all of which were purchased from Mediatech.

**DNA Constructs.** Mouse MARCH1 cDNA was cloned from C57BL/6 spleen cDNA and an amino terminal HA epitope tag was added (Jabbour 2009 ref). Various MARCH1 point mutants used in the experiments were generated using Site-directed mutagenesis using QuickChange XL mutagenesis kit from Stratagene. MARCH1 C-
and N-terminal double truncation mutants were created using PCR and primers used in previous experiments and are depicted in related figures. All sequences of constructs were confirmed by DNA sequence analysis.

*Transfections and Transductions.* Transient transfection was performed using FuGene 6 reagent (Roche Diagnostics) according to the supplier's instructions. For retroviral vector production, 293T cells were transfected with viral components and mutant-protein expression constructs. Virus was concentrated by ultracentrifugation as in 24. Ectropic retroviral vector infection of WT3’s was used to generate stable cell lines, which were drug selected for over one week.

*SDS-PAGE and Immunoblotting.* Cell lysates were generated by lysis in 1% IGEPAL CA-630 (NP-40) (from Sigma-Aldrich) dissolved in 50 mM Tris, 150 mM NaCl (pH 7.4) buffer (TBS), supplemented with 0.3 mM PMSF, 20 mM iodoacetamide, 10 M MG132 (all from Sigma-Aldrich), and protease inhibitor cocktail III (Calbiochem). Protein content was determined using the BCA protein assay from Thermo Scientific, following centrifugation to remove nuclei. Diluted samples were separated by electrophoresis on either 7% or 3-8% NuPAGE Tris-Acetate or 10% or 4-12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 1 h with 5% dried milk, 0.1% Tween 20 (Sigma- Aldrich), and 0.01% SDS in D-PBS. Membranes were then washed three times with 0.1% Tween 20, 0.01% SDS in D-PBS. Immunoblotting of membrane was performed using the appropriate dilution
for primary Ab overnight on rocker at 4°C. Membranes were then washed three times, and incubated with appropriate biotin-conjugated secondary Abs for 1 h, followed by incubation with streptavidin-conjugated HRP (Zymed Laboratories) for 1 h. Membranes were then incubated in SuperSignal West Femto ECL (Thermo Scientific) and visualized using ChemiDoc XRS (Bio-Rad) digital imaging system.

**Flow Cytometry.** Cells were harvested and prepared for staining. Ab staining was performed in staining buffer (1% BSA, 0.1% sodium azide in D-PBS). Fc-receptor blocking was performed using anti-CD16/32 antibody (clone 2.4G2; BD Biosciences or as hybridoma supernatants). Cells were incubated on ice for 30 min. in primary antibody diluted in staining buffer. Samples were washed three times and incubated with appropriate fluorochrome-conjugated secondary antibody covered on ice for at least 30 min. Cells were again washed three times and resuspended in 1X D-PBS and fixed with an equal volume of 1% paraformaldehyde (in D-PBS). Cells were then analyzed using a FACSCalibur cytometer (BD Biosciences) and data was analyzed using FlowJo (Treestar) software.

*Reverse transcription (RT) PCR.* Conditions were as follows: 100°C for denaturation, followed by 10 minutes of 25°C for annealing, 55°C for 30 minutes for extension, ending with 85°C for reverse transcriptase enzyme inactivation. Reagents used were from Roche Transcriptor First Strand cDNA Synthesis Kit. Roche Random Hexamer primers were used according to the supplier’s instructions.
Real-Time quantitative RT-PCR (qPCR). Real-time quantitative PCR was performed on a Roche LightCycler® 480 Real-Time PCR System using LightCycler® 480 SYBR Green I Master Mix (Roche, Indianapolis, USA) at 45 cycles with cDNA from 200 ng of starting RNA per reaction. mRNA for each target was quantified with the ΔΔCt method as described²⁵ using LightCycler 480 software version 1.5.0.39 (Roche USA). Transcript levels were normalized to HPRT as an endogenous control. Each reaction was performed in triplicate. Primers used were obtained from Fischer Scientific.

Primers used for qPCR:

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Results

Mapping regions of MARCH1 function and stability

To extend our previous findings, we generated a number of additional mutant MARCH1 mutant proteins. It had previously been shown that when the 21’-WKKKSK-25’ block was mutated to alanine residues; a distinct banding pattern (different from wild type) was noted by immunoblot. It was also shown that this block mutation caused a decrease in function of the resulting proteins (results not shown). Therefore, individual point mutations within this block were generated, converting the specific each amino acid to an alanine while all other residues remained unchanged. The generated mutant constructs were transfected into 293T cells, and mutant proteins were analyzed by immunoblot (results not shown). None of the individual point mutations alone resulted in the abnormal banding pattern previously seen in the block mutant. To assess the function of the mutants, we stained transfected cells for levels of the MARCH1 substrate, CD86; there was not a marked increase in the levels of CD86 for the individual point mutants, in contrast to the poor function (increased CD86) noted for the block WKKKSK mutation (results not shown). These results indicate that something is uniquely changed when the entire block is mutated which disrupts function (and possibly the folding) of the protein that cannot be attributed to a single amino acid residue.

In addition, we extend prior work mapping key domains within MARCH1 that are responsible for its stability and function. Figure 1 diagrammatically shows all of the previously existing and newly created truncation mutants of MARCH1. The stability of the proteins was initially evaluated via immunoblot. Figure 2 shows the expression of the proteins in transfected 293T cells. Consistent with what was previously seen⁹, the
expression level for the Δ 1-66 was greatly increased relative to WT, as was Δ1-40, though to a lesser extent. A decrease in expression was still noted amongst the ΔC terminus truncations, with both Δ229-279 and Δ257-279 barely visible via immunoblot. When the stabilizing N terminus truncations were added to each of the ΔC mutants, the effect was an increase in protein levels (reflecting enhanced stability). As expected the Δ1-66 caused the greatest increase in protein levels, and Δ1-40 showed a marked increase as well. This suggests that the N terminus is responsible for stability independent from the C terminus. The various truncation mutants were expressed in DC2.4 cells, a DC cell line, to evaluate the functionality of each protein. Figure 3 shows the flow cytometric analysis of a few of the stabilized double truncation mutants. Results were consistent with what was expected, that the C terminus is responsible for function of the protein. When the larger block of the C terminus was removed (229-279) the ability of MARCH1 to down-regulate CD86 was lost, even when the protein was expressed at relatively high levels due to the stabilizing effect of the N terminal deletion. When a stabilizing mutation was combined with a smaller C terminal truncation (which retains some function) the activity of the protein was restored back to wild type levels.

*Dysregulation of MARCH1 (KO mice) and Metabolic Disease*

Our investigations on MARCH1 regulation indicate that this protein is subjected to multiple levels of stringent regulation. Therefore, we have begun to study the consequences of altered MARCH1 expression, using knock-out mice (KO). In the course of our structure-function work on MARCH1, we noticed that or MARCH1 KO mice sometimes exhibited an “overweight” phenotype. To determine whether the obese
phenotype observed in the MARCH1 KOs was consistent, we followed age- and gender-matched littermate mice over time on normal and high-fat chow(s). Figure 4 shows that KOs showed a steeper the change in body weight of the percent starting weight relative to WT when on this high-fat diet, in one of two experiments. However, weight gain is a relatively crude indicator of disease progression, so we began examining additional parameters. We looked at the type of adipose tissue that the mice were storing, visceral adipose (VAT) or subcutaneous adipose tissue (SAT), and found that KO mice had a significant increase in VAT-to-body mass ratio relative to WT (Figure 5).

In an effort to gauge the inflammatory state of the VAT, we examined cellularity using flow cytometry on the stromal vascular fraction of VAT in WT and KO mice, focusing on the percentage of macrophage infiltration (Figure 6). The KO mice showed signs of increased macrophage infiltration into the VAT over WT. After observing that there were more immune cells in the tissue in this experiment, we then looked at what cytokines and other factors were being produced that could be contributing to this inflamed tissue state. Quantitative reverse transcriptase PCR (qRT-PCR) was used to analyze gene expression in WT vs. KO VAT. Figure 7 shows various inflammatory markers from whole VAT and their relative expression levels in a preliminary experiment. An increase of expression of MCP-1 (monocyte chemoattractant protein 1-produced mainly by macrophages and is responsible for recruiting monocytes, memory T cells, and dendritic cells to sites of tissue injury and infection), IL-6 (produced by macrophages, T-cells and adipocytes and leads to an increased immune response) and IL-10 (produced mainly by monocytes and a lesser extent by lymphocytes and is responsible for suppressing ongoing immune responses) in KO over WT was observed. In a second
experiment with age- and sex-matched KO and WT mice, we looked specifically at the stromal vascular fraction of VAT (figure 8). Here, only leptin mRNA levels were shown to be higher in KO mice, though some of the other markers trended higher but did not reach statistical significance.

Adipose tissue inflammation in the KO mice supports a role for MARCH1 in the homeostasis of VAT and a potential role in a metabolic disease related to overweight individuals with chronic inflammation, such as type 2 diabetes (T2D). T2D was investigated as a possible outcome of MARCH1 dysregulation. Figures 9 and 10 present models of the progression of insulin resistance in individuals with increased adipose mass and how the immune system is thought to be involved. Obesity and chronic inflammation lead to self-perpetuating macrophage infiltration into the tissue, which can then lead to insulin resistance by ill-defined mechanisms. As a preliminary test of this model, we measured blood glucose levels in KO and WT mice. Figure 11 shows that KO and heterozygous mice showed elevated blood glucose levels (mild hyperglycemia) over WT. This led us to place MARCH1 within our inflammatory model of T2D, seen in figure 12.

Discussion

Mapping regions of MARCH1 function and stability

Due to MARCH1’s emerging role in antigen presentation and metabolism, it is important to continue to expand our knowledge of this highly regulated protein. When looking at the domains important for MARCH1’s stability and function the 21-WKKS-25 block is still not fully understood. It was expected that the W21A point mutant would be the residue responsible for the novel banding pattern seen on immunoblot and the
noted decrease of function of the protein. That was not the case, however. Each individual point mutation had no effect on the protein like that of the block mutation. This could suggest that when the entire block is mutated to alanine it causes the protein to fold differently decreasing its ubiquitin ligase activity or its binding affinity to its substrates. As a next step, we would specifically test the block mutation’s binding affinity for substrates via protein complex immunoprecipitation. Given that the altered banding pattern of the block mutation correlates with decreased MARCH1 function, we believe it is important to determine how residues in this area alter the properties of MARCH1.

Looking at the N and C terminus regions for domains affecting stability, our results were consistent with previous experiments. The proteins lacking either Δ1-66 or Δ1-40 displayed increased steady-state levels (related to stability) over wild type. When these N-terminal truncations were added to the destabilized C-terminal Δ229-279 and Δ257-279 proteins, their stability was increased. This shows that there is something unique about the amino end of MARCH1, involving it in the stability/regulation of the protein, which acts independently of the destabilizing effects seen by the loss of the C terminus. Knowing that a portion of the N terminus is responsible for the stability of MARCH1 we then tested the function of the mutant proteins, focusing on the stabilized proteins lacking the Δ1-66 or Δ1-40. The fact that no matter if the Δ1-66 or Δ1-40 was removed along with the Δ229-279 the protein was still non-functional showed us that the decreased ligase activity previously seen was not due to the protein being unstable but that something essential to MARCH1’s activity is removed when this C-terminal region is missing. However when the Δ1-66 truncation is added to the Δ257-279 protein, the resulting protein was expressed stably and functioned similar to that of wild type. This
finding allowed for the inference that there is something within residues 229-257 of MARCH1 that is needed for its ubiquitin ligase action. This region may be required for MARCH1’s interaction with its substrates, as suggested previously 2.

Dysregulation of MARCH1 and Metabolic Disease

Beyond the cell biology of MARCH1, our findings suggested a relationship between MARCH1 function and metabolic disease. The discovery that KO mice increased in weight gain over WT and that the fat was being stored viscerally rather than subcutaneously was indicative of a diseased state. VAT is known to act like an endocrine organ, secrete higher amounts of adipokines than subcutaneous adipose tissue 27. This hyper-secretion of adipokines/cytokines may be the cause of the marked difference between KO and WT. Looking at the difference in adipokine levels between KO and WT, the only significant differences were seen in MCP-1, IL-6, IL-10 and leptin. Elevated MCP-1 will cause an increase in immune cell recruitment to the location, which can add to the relative inflammatory state of the tissue. IL-6 and IL-10 would be trying to counteract each other’s effects; one by increasing inflammation (IL-6) and one by decreasing inflammation (IL-10). It is noteworthy that IL-10 is known to induce MARCH1 expression 10. Thus, in our KO mice, some of the anti-inflammatory effects of IL-10 will be missing. The higher amounts of leptin in the tissue suggest a few possibilities. Leptin is normally present to decrease appetite and induce satiety. This increase in the KO is most likely due to the body having increased storage of adipose viscerally; the extra VAT is trying to communicate to the hypothalamus to decrease appetite. Leptin also plays a role in immune regulation having anti-apoptotic effects on many immune cells, as well as increasing cytokine production and macrophage activation
Therefore this increased leptin can also be contributing to the pro-inflammatory environment.

The general sum of all of these cytokines leads to the adipose tissue being in a state of pro-inflammation. This inflammatory state is somehow leading to insulin resistance. The elevated blood glucose levels in KO and heterozygous mice are suggestive of this insulin resistance but not of fully progressed T2D. Further experiments are ongoing in this area trying to piece together the relationship of pro-inflammatory environments leading to insulin resistance. Piecing together the complete pathway of how inflammation (IL-6 and increased adipose) induces insulin desensitization will allow us to better understand MARCH1’s potential role in induction of a metabolically diseased state.

Limitations of the Findings and Future Work

Many of the findings in this paper were preliminary in nature and are currently under further investigation. When looking at the weight gain experiments, we noted the increased KO over WT only in certain experiments. Comparing VAT-to-body weight ratios for male and female KO and WT, only female KOs showed in increase over WT. In the male experiment, WT actually had a higher VAT: body weight. Although this trend is promising these results indicate there is clearly more to the system than we currently understand. This goes for the mRNA levels as well. In one gene expression experiment with adipose tissue, many inflammatory modulators were found to be elevated in KO over WT. But when a similar experiment was repeated with more closely matched (age and gender) mice only one adipokine was significantly elevated. Thus, more experiments
are required with larger numbers of well-matched groups to determine the impact of MARCH1 on these parameters.

Continuing forward, better controlled experiments reexamining many of these finding are underway. We are controlling for diet and housing conditions. We have expanded our mRNA expression assay to screen for more inflammatory markers to try and better understand the cellular mechanisms at work. We have also begun examining liver sections histologically for signs of steatosis (abnormal fat accumulation).

Attributions and Acknowledgments

The author contributed to experimental results that went into the production of Figures 1, 2, 3, 6, 7, and 8. Figures 4-12 were created and data was compiled by Candida Bhagwandin and Lonnie Lybarger. A special thanks to Dr. Lonnie Lybarger, Kathleen Corcoran, Michael Whalen and Candida Bhagwandin for all of their help and support in the production of this paper.

Reference


22. Pretell, J., Greenfield, R. S. & Tevethia, S. S. Biology of simian virus 40 (SV40) transplantation antigen (TrAg). V In vitro demonstration of SV40 TrAg in SV40


**Figure Legends**

**Figure 1:** Depiction of mutant MARCH1 constructs used in domain analysis. This shows a depiction of various truncation mutants, all of which contain an amino terminal HA tag. Those in red were previously generated and sequenced in Jabbour, et al., 2009. The same primers and constructs were used to create the novel double truncation mutants, depicted in black.

**Figure 2:** MARCH1 domains that affect protein stability. Protein lysates from WT3 fibroblast stable cell lines carrying the mutant MARCH1 constructs were separated by electrophoresis and then transferred to PVDF membrane and blotted for MARCH1. Staining for the HA tag on MARCH1 shows the relative steady-state levels of each protein, which is known to correlate to their stability (Jabbour, et al., 2009).

**Figure 3:** MARCH1 domains that affect function. Flow cytometric analysis was performed on DC2.4 cells expressing MARCH1 truncation mutants. Cells were stained for cell surface CD86 and intra-cellular MARCH1 (HA). Each plot shows
whether the expressed MARCH1 mutant is functional in decreasing surface CD86 levels.

Figure 4: The effects of high fat diet on MARCH1 knock out and wild type mice. Graphs show change in body weight over time on high-fat diets. Red lines represent mean value for four male knock out mice, while the blue lines represent the mean value for four wild type male mice. (A) shows the percent change in body weight for 4 male KO/WT beginning at 13 weeks of age. (B) shows the change in body weight of the percent starting weight for 4 male KO/WT beginning at 28 weeks of age. (C) shows the percent coefficient of variation in the weight gain values from A (% CV of the means of each group at each week).

Figure 5: The amount visceral adipose tissue in knock out and wildtype mice as a function of body mass. Graphs show change over time of the ratio of visceral adipose tissue to body mass for (A) males and (B) females. Linear regression analysis was performed to determine the slopes and elevations of the lines.

Figure 6: Analysis of the stromal vascular fraction of adipose tissue by flow cytometry. Flow cytometric analysis of a female wild type mouse and MARCH1 knock out male mouse, both 8 months old. Stromal vascular fraction was collected after collagenase treatment and was stained with the indicated antibodies for flow cytometry. CD11b is an integrin component which is expressed on the surface of various leukocytes including macrophages. F4/80 is a member of the GPCR-family of proteins and is expressed selectively on the cell surface of macrophages. The gated area in upper right quadrant of each graph depicts a population that expresses high enough levels of each marker, which indicates it is a macrophage. Percentages are given of macrophages (CD11b+, F4/80+) within the leukocyte light-scatter gate.

Figure 7: Gene expression analysis of wild type and MARCH1 knock out visceral adipose tissue by qPCR. Reverse transcriptase quantitative PCR used to look at mRNA transcript levels in each tissue sample. (A) shows the specific gene/transcript in question and what cells it is found in: A-adipocytes, T_{reg}-regulatory T cell, Mφ-macrophage, M1-M1 activated macrophage, M2-M2 activated macrophage, Tc-cytotoxic T cell, T-T cell. (B) shows each mRNA expression level normalized to a housekeeping gene (HPRT). RNA was prepared from visceral adipose tissue of 25 week-old female mice (body weight: WT= 36 g; KO = 32.1 g). Numbers above the bars represent fold-change for each primer set in the knock out vs wild type sample. Arrows indicate notable changes. N.D. = no signal detected.

Figure 8: Gene-expression analysis of wild type and MARCH1 knock out stromal vascular fraction of adipose tissue by qPCR. Reverse transcriptase quantitative PCR used to look at mRNA transcript levels in each tissue sample. The relative mRNA expression levels of each gene normalized to a housekeeping gene (HPRT) is shown. RNA was prepared from 14-week-old female mice fed a high-fat diet for one week. Weights were not significantly different but knock out showed an
increase in VAT: body mass over wild type. Significant differences in expression demarcated with an asterisk (*=p<0.05).

Figure 9: **Model for obesity induced insulin resistance.** Simplified depiction of systemic events leading to the development of Type 2 diabetes (T2D).

Figure 10: **Cellular events in the control and progression of Type 2 Diabetes.** Simplified depiction of cellular events leading to the development of Type 2 diabetes (T2D).

Figure 11: **MARCH1 effects on blood glucose levels.** Shows blood glucose readings of wild type (blue), heterozygous (black), and knock out (red) mice taken monthly starting at 8 weeks of age. Male and female littermates were used. Mice were fasted for four hours before readings via standard glucose meter. Significant differences, determined by pair-wise t-tests, are demarcated by asterisk (*=p<0.05, ****=p<0.0001).

Figure 12: **Model for MARCH1-dependent induction of insulin resistance.** Diagrammatic depiction of the cells and factors involved in the healthy and diseased state within adipose tissue, and how MARCH1 may be involved in this regulatory circuit.
Figure 1

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<th>TM2</th>
<th>Wildtype</th>
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<td>TM2</td>
<td>ΔN 1-40  AC 229-279</td>
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</table>

Figure 2

Legend:
- ΔN 1-40
- ΔN 1-66
- AC 229-279
- AC 257-279
- W104A RING Mutant
Figure 3

Empty Vector Control

Wildtype

Δ1-66

Δ1-66 Δ229-279

Δ1-40 Δ229-279

Δ1-40 Δ257-279

Figure 4

A

B

C

% change in body weight

Weeks of high-fat diet

Δ body weight (% of starting)

Slopes: p = 0.672

Slopes: p = 0.003

Slopes: p < 0.0001
Figure 7

A

Adiponectin
FoxP3
MHCII
F4/80
CD206
MCP-1
IL-6
IL-10
MARCH1

B

Normalized mRNA expression

Adiponectin FoxP3 MHCII F4/80 CD206 MCP1 IL-6 IL-10 MARCH1

Figure 8

WT/KO (F), 14wks
Figure 9

Normal

Nutrients
Glucose
Insulin
Stored (Energy)
Insulin Sensitive

Disease

Nutrients
Glucose
Insulin
Storaid (Energy)
Insulin Resistance
Chronic hyperglycemia
T2D
Kidney damage
Heart disease
Retina damage
Neuropathy (amputations)

Figure 10

Normal Adipose tissue

Resident Mφ (anti-inflammatory)
Infiltration Regulated

• CONTROLS:
  • IL-10 → MARCH1
  • Maintain insulin sensitivity

Obesity/Genetic factors

Chronic Inflammation
(Mφ infiltration)

M2 → M1

Insulin resistance and Type II Diabetes
Figure 11

Figure 12