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Increasing muscle mass to improve metabolism

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Skeletal muscle insulin resistance is a predictor of the development of type 2 diabetes and maintenance of adequate muscle glucose disposal in muscle may help to prevent diabetes. Lipodystrophy is a type of diabetes caused by a reduction of white adipose tissue and the adipokine leptin. Lipidemia, insulin resistance and hyperphagia develop as a consequence. In a recent study, we showed that increasing skeletal muscle mass by inhibiting signaling of myostatin, a transforming growth factor β (TGFβ) family member that negatively regulates muscle growth, prevents the development of diabetes in a mouse model of lipodystrophy. Muscle-specific myostatin inhibition also prevented hyperphagia suggesting muscle may regulate food intake. Here we discuss these results in the context of strategies to increase muscle insulin sensitivity as well as new findings about the effects of myostatin and other TGFβ family members on similar metabolic processes.

Keywords: myostatin, lipodystrophy, muscle hypertrophy, hyperphagia, diabetes, insulin resistance, leptin, adipose tissue, skeletal muscle, food intake

Abbreviations: ACVR2B, activin receptor type IIB; AgRP, agouti-related protein; BAT, brown adipose tissue; BMP, bone morphogenetic protein; DIO, diet-induced obesity; GLP-1, glucagon-like peptide 1; IGF, insulin-like growth factor; MSTN, myostatin; NPY, neuropeptide Y; POMC, pro-opiomelanocortin precursor; PYY, peptide YY; T2DM, type 2 diabetes mellitus; TGFβ, transforming growth factor β; WAT, white adipose tissue

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Introduction

Skeletal muscle is a crucial tissue for maintaining blood glucose control and energy balance. Muscle uses both glucose and fatty acids as fuel and serves as a source of amino acids for fuel utilization by other tissues during starvation. Glucose uptake into muscle is stimulated by contraction or by postprandial insulin secretion. Euglycemic-hyperinsulinemic clamp studies in humans demonstrate that the majority of whole body insulin-stimulated glucose uptake occurs in skeletal muscle where most of it is stored as glycogen or oxidized.[1,2] Intramyocellular lipid accumulation is correlated with insulin resistance and is detectable much earlier than the onset of type 2 diabetes mellitus (T2DM).[2] The precise role of mitochondrial lipid oxidation in the pathogenesis of muscle insulin resistance is still debated. Different and potentially overlapping mechanisms for the cause of lipid accumulation have been proposed including mitochondrial dysfunction leading to lipotoxicity and insulin resistance, incomplete lipid oxidation leading to harmful intermediate metabolites, or changes in lipid signaling, redox balance or ER stress.[2,3]

We are interested in the metabolic effects of a member of the transforming growth factor β (TGFβ) family member that negatively regulates skeletal muscle growth, myostatin (MSTN), particularly in regard to the progression, prevention, or treatment of common metabolic conditions. Recently, we published a study describing the prevention of diabetes and hyperphagia (excess food intake) in diabetic mice with lipodystrophy, a disease caused by a lack of white adipose tissue (WAT), by blocking signaling of MSTN in muscle.[4] Our results align with the growing realization that muscle hypertrophy has beneficial effects on glucose metabolism and may also implicate skeletal muscle in the regulation of energy intake by some as yet unknown mechanism. Here we will put our results in context with some current active areas of research into metabolic homeostasis.

Exercise and Exercise Mimetics for Fighting Insulin Resistance

We all know that we should exercise regularly even if we would rather not. It has long been appreciated that different types of exercise promote changes in skeletal muscle size and energy utilization. Skeletal muscle fibers are multinucleated cells that are classified by two properties, contractile and metabolic. Slow contracting oxidative fibers contain a high density of mitochondria and preferentially utilize fatty acids for fuel ("red" muscle) while fast contracting glycolytic fibers have fewer mitochondria and use relatively more glucose ("white" muscle). Endurance exercise training promotes a transition toward a slow oxidative phenotype in muscle and improved cardiovascular fitness and whole body insulin sensitivity. Molecular pathways promoting mitochondrial biogenesis and oxidative capacity in muscle have been proposed to be targets for endurance exercise mimetics to improve metabolism in patients incapable of exercise. However, this approach has been questioned, particularly by Muoio and Neufer in a recent and provocative review article.[3] As they describe, increasing fat oxidation or mitochondrial biogenesis in muscle can sometimes worsen insulin resistance. They review evidence suggesting that harmful intermediary metabolites are produced when increasing energy demand does not offset the increased energy production that occurs with higher levels of fat oxidation.

Muscle hypertrophy and increased strength, on the other hand, can be achieved by resistance exercises. It is now becoming appreciated that promoting hypertrophy of fast glycolytic muscle fibers also has metabolic benefits. In humans, lean mass is positively associated with reduced incidence of insulin resistance[5] or the metabolic syndrome,[6] a group of risk factors for cardiovascular disease and T2DM that includes elevated fasting glucose and triglyceride levels, hypertension, obesity and reduced HDL. Like endurance exercise, resistance exercise is also associated with improvements in glucose metabolism and both are now recommended for T2DM patients.[7] Genetically modified mice with increased muscle mass have reduced adipose mass and resistance to diet-induced obesity (DIO) and insulin resistance even when the manipulation is muscle-specific.[8,9] One explanation for this may be that insulin and insulin-like growth factor 1 (IGF1) signaling converges on the phosphatidyl inositol (PI) 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway to promote insulin-stimulated glucose disposal and anabolic growth in muscle.[10] Thus, signaling pathways that promote muscle hypertrophy are potential targets for resistance exercise mimetics that may have the added benefit of preventing or treating muscle insulin resistance.

Analogous with adipokines secreted from fat cells, muscle is increasingly recognized as a secretory tissue that produces potentially hundreds of its own "myokines" that can act locally or systemically.[11] Some of these are increased with exercise and may mediate the effects of exercise on other tissues. For example, as recently described by Spiegelman and colleagues, irisin from muscle promotes brown adipose tissue (BAT) differentiation and thermogenesis in WAT.[12] In contrast, *MSTN* expression in muscle is often reduced with exercise but increased with insulin resistance or morbid obesity.[13] Furthermore, repeated injection of MSTN can cause insulin intolerance in mice.[14]

Loss of function mutations in the *MSTN* gene or treatment with soluble MSTN antagonists greatly increases skeletal muscle size.[15] *Mstn* gene deletion or transgenic overexpression of a dominant negative activin receptor type IIB (ACVR2B, also known as ActRIIB) during development causes an increase in muscle fiber number (hyperplasia) and diameter (hypertrophy) in mice.[8,16] *Mstn* mutant muscle from mice or the Belgian Blue cattle breed also contains more fast glycolytic fiber types than normal muscle.[17-19] In contrast, MSTN inhibition in adults causes hypertrophy without hyperplasia.[8] Although fiber types do not seem to be changed, gene expression analysis shows a reduction in expression of slow isoforms of structural genes and oxidative genes suggesting that fast glycolytic fibers are hypertrophied more than the slow oxidative fibers.[20] MSTN antagonists administered postnatally can therefore be considered resistance exercise mimetics for therapeutic intervention for a variety of muscle wasting diseases.

An additional effect of exercise training is increased vascularity which may promote increased oxygen, nutrient and insulin delivery to muscle fibers. Capillary proliferation, a well-established effect of endurance exercise training,[21] has also been demonstrated in rodent muscles that are hypertrophied by functional overload due to ablation of synergistic muscles.[22-24] In contrast, there does not seem to be an increase in capillary density in muscle with *MSTN* gene deletion. *Mstn* null mice bred to a high growth genetic background (DUHi) or Belgian Blue cattle have reduced capillary density.[19,25] Another detailed study using *Mstn*$^{-/-}$ mice, however, found normal capillary density taking fiber type into consideration and normal perfusion of muscle during electro-stimulated exercise in vivo.[17] One interesting observation from this study is that post-exercise hyperfusion is prolonged compared with WT mice, similar to what is seen in short distance trained runners compared with long distance trained runners.[26] Thus, this effect may be a characteristic of fast glycolytic muscle. Vascularization in mice treated with postnatal MSTN inhibitors has not yet been determined.

Blocking MSTN signaling in muscle also affects whole body metabolism. Mice with *Mstn* gene deletion, transgenic mice with overexpression of a dominant negative ACVR2B, or transgenic mice expressing any of several naturally occurring MSTN binding proteins are muscular and resistant to the metabolic consequences of high-fat feeding including obesity, insulin resistance and atherosclerosis.[8,9] These mice also have increased insulin-stimulated activation of Akt and increased whole body and muscle insulin-stimulated glucose uptake even on standard chow.[8] Soluble MSTN inhibitors prevent diet-induced obesity (DIO) and insulin resistance when given at the onset of high-fat diet feeding but have been ineffective at inducing weight loss in mice made obese prior to MSTN inhibition.[27-31] The better outcomes obtained when anti-MSTN treatment is given at the same time as a high-fat diet is initiated is possibly because maximal muscle hypertrophy is achieved

within a few weeks, a time frame preceding much of the diet-induced weight gain. Thus MSTN inhibition may be more useful for preventing rather than treating obesity.

In contrast to inhibiting MSTN in obese mice, inducing constitutive activation of Akt1 in skeletal muscle from obese mice results in weight loss and improved glucose tolerance.[32] The magnitude of the muscle hypertrophy alone cannot account for the difference of weight loss in these two models suggesting that signaling downstream of the Akt pathway is responsible for the weight loss in the constitutively active Akt transgenic mice. These authors later identified one potential candidate, the insulin sensitizer fibroblast growth factor 21, which is increased in muscle and in the bloodstream in constitutively active Akt mice.[33] Many myokines have yet to be defined functionally, and this is likely to be an area of intense activity in the near future particularly in regard to crosstalk with other tissues.

Muscle Hypertrophy in Lipodystrophic Mice

MSTN inhibitors are currently in development for treating muscle wasting conditions. They may also be beneficial for improving some aspects of metabolic dysfunction even though they have been unsuccessful at inducing weight loss in obese rodents. Notably, glucose tolerance and fasting glucose were improved in *ob/ob* mice treated with anti-MSTN antibodies.[28] We are interested in testing whether inhibiting this pathway and increasing muscle mass could prevent or treat diabetes. Because adiposity is lower in genetically modified mice made muscular early in development even on a standard diet, we cannot distinguish whether insulin sensitivity is improved because of changes in metabolism in muscle itself or from a secondary effect of preventing the development of obesity. In addition, mice with DIO generally do not become very diabetic. As an alternative, we turned to a mouse model of lipodystrophic diabetes.

Lipodystrophy is caused by rare genetic mutations or by highly active anti-retroviral therapy for HIV infection.[34,35] The absence of adequate adipose storage sites causes overflow of lipid into circulation and ectopic deposition in tissues which is thought to lead to insulin resistance, and, in some cases, diabetes.[2] As Shulman and colleagues have pointed out, despite the absence of obesity, the metabolic problems in lipodystrophy are similar to obesity demonstrating the importance of lipid dysregulation in the development of insulin resistance.[2] The lack of WAT also reduces production of adipokines such as leptin which normally induces peripheral fat oxidation and inhibits food intake.

Our study describes the inhibition of MSTN signaling in A-ZIP/F-1 mice (hereafter called A-ZIP), a mouse model of generalized lipodystrophy.[4] Made over 10 years ago by Charles Vinson's laboratory, A-ZIP transgenic mice overexpress a dominant negative leucine zipper construct that blocks the DNA binding domain of the C/EBP family of B-ZIP domain-containing transcription factors that are required for adipogenesis.[36] A fat-specific aP2 (fatty acid binding protein 4) promoter was used to express this transgene which resulted in near complete inhibition of white adipocyte differentiation. We crossed A-ZIP mice to mice expressing a muscle-specific dominant negative ACVR2B which we call Muscle-DN mice. We found that the severe diabetes characteristic of A-ZIP mice was completely abolished, and insulin sensitivity was restored. In A-ZIP mice, muscle and hepatic triglyceride content is elevated prior to the onset of diabetes.[37] Normal lipid levels were found in our double transgenic mice, and in vivo assays demonstrated that the reduced lipidemia appeared to be due more to reduced lipogenesis rather than increased lipid oxidation. Consistent with our results, resistance exercise increased lean mass, reduced circulating triglyceride levels and improved insulin sensitivity in a small trial of HIV-associated lipodystrophy patients.[38]

Several groups have shown that *Mstn*$^{-/-}$ mice have higher insulin-stimulated glucose uptake in muscle along with the increased lean mass and glycolytic fiber types.[8] Taken together with the reduction in muscle triglyceride content in the double A-ZIP/Muscle-DN mutant mice, these results suggest that increased glucose utilization by muscle may prevent increases in ectopic lipid deposition. Along these lines, deletion of the nuclear receptor *Nur77*, a positive regulator of genes for glucose utilization particularly in muscle, reduces glucose disposal and increases muscle lipid accumulation and insulin resistance when fed a high-fat diet.[39]

BAT and Energy Expenditure in Lipodystrophic Mice

Another potential means of treating obesity or diabetes receiving a lot of attention recently is increasing energy expenditure by promoting thermogenesis in BAT. A-ZIP mice still have a small amount of BAT, and the Muscle-DN transgene is expressed at low levels in BAT.[40] Using another transgenic line that expressed the dominant negative ACVR2B in adipocytes under control of the aP2 promoter (Fat-DN mice)[40] to block MSTN signaling in BAT, however, failed to prevent diabetes or hyperphagia in A-ZIP mice.

While our manuscript was in press, Fournier et al. of Novartis reported evidence that MSTN directly inhibits brown adipogenesis in primary cultures of BAT preadipocytes.[41] Blocking the receptor in culture promoted adipogenesis, although not mitochondrial biogenesis, suggesting that at least one ACVR2B ligand is produced by BAT. They also treated mice with an anti-ACVR2B antibody with a dose that only increased muscle mass ~15% to minimize passive increases in energy expenditure from increased body weight. Anti-ACVR2B treatment increased energy expenditure, an effect that was completely blocked by analysis at thermoneutrality. This latter result strongly suggests that thermogenesis, rather than increased muscle mass and body weight, accounted for the higher energy expenditure at room temperature.

These results have prompted us to wonder whether our two DN receptor transgenic lines are blocking MSTN signaling cell-autonomously in different cell types within BAT. If so, the persistence of diabetes in Fat-DN, A-ZIP mice may not in fact rule out BAT-mediated improvement in metabolism in Muscle-DN, A-ZIP mice. In addition, the discovery of irisin demonstrates muscle-BAT crosstalk, so BAT function in A-ZIP, Muscle-DN mice,

as well as various models of anti-MSTN therapy, must be examined in more detail. However, we think that an increase in brown adipogenesis is unlikely to be the primary factor in the metabolic improvements in Muscle-DN, A-ZIP mice. BAT in double transgenic mice weighed 20% of WT BAT and was even smaller than A-ZIP BAT. Furthermore, energy expenditure was not increased in double mutant mice compared with WT, Muscle-DN or A-ZIP single mutants. Consistent with this, although mean body temperature was not different from A-ZIP mice, the standard deviation of this measurement was increased by 4-fold in double A-ZIP, Muscle-DN mutants suggesting a possible thermogenesis defect (unpublished data).

TGFβ Family Members and Food Intake

In addition to the complete prevention of diabetes in lipodystrophic mice, double mutant mice had another striking, but unexpected, phenotype—normal food intake. Like the *ob/ob* mouse, food intake in the A-ZIP mouse is increased ~2-fold due to the loss of the central effects of leptin. We did not predict an effect on food intake because the dominant negative ACVR2B receptor construct contains a transmembrane domain and is driven by a muscle-specific promoter. In addition, there have also been no descriptions of altered food intake in mice with mutations in the *MSTN* gene or with pharmacologic inhibition of MSTN to our knowledge. This decrease in hyperphagia might be a partial explanation for the improved lipidemia in A-ZIP, Muscle-DN mice. Pair feeding A-ZIP mice to WT levels of food intake, however, does not improve insulin resistance or reduce hyperglycemia.[42]

Energy intake is regulated by multiple sites in the central nervous system (CNS). So far, the hypothalamus has been the most thoroughly investigated. Within the hypothalamus, the orexigenic neurons expressing the peptides agouti-related protein (AgRP) and neuropeptide Y (NPY) and the anorexigenic neurons expressing pro-opiomelanocortin precursor (POMC) are clearly important for regulating energy intake.[43] Anorectic input to the CNS is received from metabolically important tissues such as WAT (leptin), the pancreas (insulin, glucagon), and the gut [glucagon-like peptide 1 (GLP1), peptide YY (PYY), etc.].[43] The gut also produces the orexigenic hormone ghrelin. Some of these signals may be mediated by afferent vagal or enteric neuronal signaling rather than acting directly on the CNS. Additionally, fatty acids and glucose are sensed by the brain and may regulate food intake.

To try to determine the mechanism of this reduced food intake in double mutant mice, we proceeded to round up many of the usual suspects. The expression levels of *AgRP*, *Npy* and *Pomc* were not significantly different between genotypes so we are unsure what part of the CNS might be affected in double mutant mice. In double mutants, levels of the hormones ghrelin, PYY, GLP-1 and glucagon were similar to WT mice. One means for improving diabetes and hyperphagia in lipodystrophic mice is leptin replacement,[42] and it has been suggested that *Mstn*$^{-/-}$ mice are more sensitive to the effects of injected leptin on food intake suppression.[44] We found, however, that diabetes and hyperphagia were rescued in triple mutant A-ZIP, Muscle-DN, *ob/ob* mice demonstrating that the metabolic improvements caused by inhibiting MSTN signaling in muscle of A-ZIP mice are leptin-independent.

Could an ActRIIB ligand(s) regulate food intake by acting directly on the CNS? Many TGFβ family members are expressed in the brain including MSTN,[45] many bone morphogenetic proteins (BMPs)[46,47] and the activins.[48] Furthermore, there are a few descriptions in the literature of effects of TGFβ family members on food or water intake. In *C. elegans*, TGFβ signaling in neurons may negatively regulate food intake.[49] Another family member, macrophage inhibitory cytokine-1, is expressed during inflammation and inhibits food intake in mice by targeting POMC and NPY neurons possibly through binding to TGFBR2.[50]

Researchers from Genentech, Inc. reported that activin, a ligand for ACVR2B with a similar downstream signaling pathway and redundant functions to MSTN in muscle, increased water consumption with no effect on food intake when infused into the hypothalamus of rats.[51] This effect was not seen with inhibin, another family member that antagonizes activin by competing for receptor binding. These authors also mention unpublished data they gathered showing a reduction in food intake and an increase in water intake by continuous subcutaneous infusion in rats given much higher doses of activin than used for hypothalamus infusion (~10-fold). Mice null for the *inhibin* α gene develop gonadal tumors and cachexia, which is thought to be due to elevated activin levels.[52] Food intake is reduced in these mice, and both food intake and muscle mass are normalized by treatment with a soluble ACVR2B.[53] *Inhibin* α$^{-/-}$ mice have hepatocellular necrosis and pathological changes in the glandular stomach that are mediated by a different receptor, ACVR2.[52] Therefore, these experiments do not distinguish a toxic effect of elevated activin levels vs. a regulatory gut-brain network modulated by activin to explain the reduced food intake in *inhibin* α$^{-/-}$ mice. In contrast, systemically overexpressing MSTN causes cachexia without a decrease in food intake or similar effects on liver and gut.[54] Inhibiting ACVR2B signaling in muscle may induce feedback on the levels of one or more of the shared secreted antagonists so activin signaling in Muscle-DN, A-ZIP mice will need to be examined.

We were especially excited to see a report from Tseng and colleagues describing the effects of BMP7 on food intake.[47] Their interest in the pro-adipogenic effects of BMP7 on BAT led them to analyze energy intake and expenditure in BMP7-treated mice. They found that DIO mice treated with adenovirus to systemically express BMP7 lost fat mass within a week mainly due to decreased appetite. By a series of intracerebroventricular administration experiments they show that BMP7 caused an acute decrease in food intake in WT with an increase in *Pomc* expression and cFOS immunoreactivity in the hypothalamus, an indication of neuronal activation. They further showed that the BMP7-induced satiation required intact mTOR signaling in the hypothalamus and is leptin-independent.

Overlap in the signaling pathways between MSTN and BMP7 is intriguing in light of these results. In addition to

the activins and MSTN, BMP7 can signal through ACVR2B although different co-receptors (type I receptors) and downstream mediators are activated, and MSTN and BMP7 can antagonize each other by competing for ACVR2B binding.[41,55] IGF1 activates mTOR/Akt signaling to cause hypertrophy in myotubes which is inhibited by MSTN signaling.[10,56] Could BMP7 and MSTN antagonize each other in the hypothalamus, and could increased BMP7 signaling be causing reduced food intake in A-ZIP, Muscle-DN mice? Four lines of evidence suggest that this is not likely to be the explanation for reduced hyperphagia in A-ZIP, Muscle-DN mice. First, the inhibitory effect of BMP7 on appetite is abolished when a lentiviral vector expressing shRNA to *BMP receptor 2* is administered to the hypothalamus,[47] but MSTN is not known to signal through BMPR2. Furthermore, food intake is not affected by ACVR2B antibody treatment in mice on standard chow or by MSTN antibody treatment in *ob/ob* mice.[28,41] Second, *Pomc* expression is not increased in A-ZIP, Muscle-DN mice.[47] Third, the nature of the MSTN mutants used in our study do not support a direct role for MSTN signaling in regulating food intake: The dominant negative ACVR2B was expressed from a muscle-specific promoter, and we could not detect transgene expression in the hypothalamus or whole brain. These mice still produce MSTN. Fourth and most important, A-ZIP mice in a *Mstn*$^{-/-}$ background phenocopy the reduced blood glucose and food intake of the A-ZIP, Muscle-DN mice. Thus, the inhibition of MSTN signaling in muscle or, more generally, increased muscle mass, seems to be the key factor in promoting the improved metabolic phenotypes including normalizing food intake rather than any hypothetical direct effects of MSTN itself on the CNS.

Conclusions and Future Directions

We have shown that MSTN inhibition in muscle can prevent both diabetes and hyperphagia in mice with no WAT and that this effect is independent of leptin. The positive effects on insulin sensitivity found in our model described here and in other mice with muscle hypertrophy, particularly transgenic mice expressing constitutively active Akt,[32] highlight the importance of glycolytic metabolism in preventing lipid accumulation and insulin resistance in muscle.

These results also suggest that muscle may produce a factor that regulates food intake. This feedback to the CNS could be via a direct muscle-brain axis through an unknown myokine, although not necessarily a peptide, or an indirect mechanism by muscle signals acting on another tissue that relays satiety signals to the brain. Although it seems plausible that muscle would send a food intake signal to the CNS due to its proportion of body weight, energy needs, and importance to whole body metabolic homeostasis, it is surprising that increased muscle mass would reduce, rather than increase, food intake. Further studies are needed to determine whether this effect is specific to certain types of metabolic dysfunction such as in hyperphagia, diabetes, or in the absence of adipose tissue or adipokines. Treating lipodystrophic mice with pharmacologic MSTN inhibitors will be necessary to determine whether this approach can treat existing diabetes and hyperphagia. If so, acute effects should be more amenable to analysis to elucidate the potential mechanisms involved.

What are other possible mechanisms for the improved metabolic phenotype in A-ZIP, Muscle-DN mice? If muscle hypertrophy prevents the development of insulin resistance in the brain secondary to preventing elevated circulating lipid and glucose, food intake could be inhibited by restoring insulin action in the CNS. However, this would not explain why leptin is no longer required. Other contenders are glucocorticoids which are well-known to affect insulin resistance, muscle wasting, and food intake, especially considering that *Mstn*$^{-/-}$ mice are resistant to glucocorticoid-induced muscle wasting.[57]

So far, unlike models with increased lipid oxidation in muscle, hypertrophic models have consistently demonstrated improved insulin sensitivity or glucose metabolism particularly when muscling precedes metabolic challenge. Although MSTN inhibitors are a potential resistance exercise mimetic, it is doubtful that the FDA will approve them for use by otherwise healthy people for prevention of diabetes and obesity. The accumulating evidence suggests, however, that they may bring additional metabolic benefits for patients taking them to treat muscle wasting. For the rest of us, it might be a good idea to hit the weight room as well as the treadmill.

Disclosure of Potential Conflicts of Interest

Under a licensing agreement between Pfizer and the Johns Hopkins University, A.C.M. is entitled to a share of royalty received by the University on sales of the factor described in this paper. The terms of these arrangements are being managed by the University in accordance with its conflict of interest policies.

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