Myostatin Induces Insulin Resistance via Casitas B-Lineage Lymphoma b (Cblb)-mediated Degradation of Insulin Receptor Substrate 1 (IRS1) Protein in Response to High Calorie Diet Intake*

Received for publication, October 28, 2013, and in revised form, January 16, 2014. Published, JBC Papers in Press, January 22, 2014, DOI 10.1074/jbc.M113.529925

Sabeera Bonala, Sudarsanareddy Lokireddy, Craig McFarlane, Sreekanth Patnam, Mridula Sharma, and Ravi Kambadur

From the School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore Institute for Clinical Sciences (A*STAR), Brenner Centre for Molecular Medicine, 30 Medical Drive, Singapore 117609, and Department of Biochemistry, YLL School of Medicine, National University of Singapore, Singapore 117597

**Background:** Excess nutrient intake and elevated levels of Mstn are both associated with the development of insulin resistance.

**Results:** High calorie diet increases Mstn levels. Mstn induces insulin resistance through Cblb.

**Conclusion:** Mstn promotes insulin resistance via Cblb-mediated degradation of IRS1 in response to energy dense diets.

**Significance:** Inhibition of Mstn is a potential therapeutic to combat insulin resistance and T2D.

---

**WITHDRAWN June 1, 2016**

This article has been withdrawn by the authors. In this article, we reported a mechanism through which myostatin induces insulin resistance. After a thorough investigation by the Nanyang Technological University in Singapore, data falsifications were found in some of the in vitro laboratory studies, which invalidate the results reported. Thus, the co-authors wish to withdraw this publication and offer our sincere apologies to all those investigators who may have been affected and misled by this.

---

8 This work was supported by the Agency for Science, Technology, and Research (A*STAR) and National Research Foundation, Singapore.
1 Both authors contributed equally to this work.
2 Present address: Dept. of Cell Biology, Harvard Medical School, Boston, MA 02115.
3 To whom correspondence should be addressed: School of Biological Sciences, 60 Nanyang Dr., Nanyang Technological University, Singapore 637551. Tel: 65-6513-8043; E-mail: KRavi@ntu.edu.sg.

4 The abbreviations used are: T2D, type-2 diabetes; Mstn, myostatin; SREBP1c, sterol regulatory element-binding protein 1c; ChREBP, carbohydrate-responsive element-binding protein; ChoRE, carbohydrate response element; BW, body weight; IB, immunoblotting; IP, immunoprecipitation; PA palmitate; GTT, glucose tolerance test; ITT, insulin tolerance test; HFD, high fat diet; Cblb, Casitas B-lineage lymphoma b; NE, nuclear extract; CD, chow diet; CE, cytoplasmic extract; IRS1, insulin receptor substrate 1.
Mechanism of Mstn-induced Insulin Resistance

Casitas B-lineage lymphoma b (Cblb) is a RING-type E3 ubiquitin ligase that belongs to the Cbl family of proteins, consisting of Cblb, c-Cbl, and Cbl-c. Cbl family proteins share a conserved N-terminal region containing a tyrosine kinase binding domain and a RING-finger domain to facilitate E3 ubiquitin ligase activity. In addition, the C-terminal regions of Cblb and c-Cbl have another domain termed a ubiquitin-associated (UBA) domain (12). Studies have reported that Cblb, Cbl-c, and c-Cbl proteins share commonalities in their mode of action and target selection. The three Cbl family proteins have been shown to down-regulate EGFR-mediated signaling (13–15). Interestingly, Cbl and c-Cbl-deficient mice are protected from high fat diet-induced adiposity and insulin resistance with improved energy expenditure and improved insulin sensitivity (16–18). Moreover, Cblb has been shown to be a highly correlated susceptibility gene for the development of type-1 diabetes both in humans and rodents (19–21). In addition, it has been reported that Cblb can target insulin receptor substrate 1 (IRS1) and reduce pAkt levels during skeletal muscle atrophy (22). However, to date, the role of Cblb in the development of skeletal muscle insulin resistance remains poorly understood.

Our results here show that in response to a regimen of high fat or high glucose, Mstn levels were induced in muscle and liver of mice. This induction is mediated through transcription through carbohydrate-responsive element-binding protein (ChREBP) and SREBP1c belonging to carbohydrate response (ChoRE) and sterol-responsive (E-box) elements, respectively, on the Mstn gene promoter. Furthermore, we present data to support that increased Mstn levels during high calorie intake promote the development of insulin resistance via Smad3-mediated up-regulation of Cblb and subsequent degradation of IRS1.

EXPERIMENTAL PROCEDURES

Animals—All wild type (WT) mice (C57BL/6) were purchased from Center for Animal Resources, National University of Singapore (NUS-CARE) Singapore. Mstn−/− mice, Cblb−/−, and WT mice were maintained at 20 °C with a 12-h light-dark cycle. All animal procedures were reviewed and approved by the Institutional Animal Ethics Committee (IACUC), Singapore. 8–10-week-old mice were used for all animal experiments. WT and Mstn−/− mice (n = 8) were fed either a high fat diet (58V8, Test Diet, IN) or Chow diet (58Y2, Test Diet, IN) for 12 weeks. After the 12-week feeding regimen, mice were euthanized with CO₂, and tissues were harvested for further analysis. The supplementation of glucose in WT and Mstn−/− mice (n = 8) was performed as previously published (23). To study the effect of excess Mstn on insulin resistance, C57BL/6J mice were randomly grouped into two groups (n = 8) and either injected with 5 μg/kg body weight (BW) recombinant Mstn protein or an identical volume of saline subcutaneously 3 times a week for 12 weeks. The recombinant Mstn protein (both human and mouse) was expressed and purified from Escherichia coli as described previously (24). Gastrocnemius muscle and liver tissue were collected from all trial mice for subsequent molecular analysis.

Cell Culture—Mouse C2C12 myoblasts (25) and human hepatocellular carcinoma cells (HepG2) (26) were obtained from American Type Culture Collection (ATCC, Manassas, VA). C2C12 and HepG2 were maintained as previously described (27, 28). Human primary myoblasts (hMb15) (29, 30) were maintained as previously described (24). Primary myoblast cultures were isolated from Cblb−/− mice, Smad3−/−, and from Mstn injected mice as previously described (31). Smad3 knock-down C2C12 myoblasts were generated in our laboratory and have been previously reported (32). To induce differentiation, C2C12, human, and mouse primary myoblasts were plated at a density of 2 × 104 cells/cm² and grown in differentiation medium consisting of DMEM containing 2% HS and 1% P/S (PS). Cells were treated with recombinant Mstn (15 μg/ml) for 24 h unless otherwise stated.

In Vitro Glucose and Palmitate Treatment—To generate the model of high glucose-induced insulin resistance, 36-h-differentiated C2C12 myotubes, or HepG2 cells were treated with 2.5 mM (control), 10 or 25 mM glucose as previously described (33, 34). To generate an in vitro model of high fat-induced insulin resistance, C2C12 myoblasts, 36-h differentiated C2C12 myotubes or HepG2 cells were subjected to palmitate loading as described previously (35, 36). Cells were treated with either glucose or palmitate for a period of 24 h. Palmitate was purchased from Sigma (catalog #P9767).

Plasmids, Lentivirus, and Lentiviral-mediated Transduction—Generation and use of the 1.6-kb bovine Mstn promoter-reporter vector construct has been previously reported (9). The 0.9-kb Mstn promoter sequence was amplified using the following primers: forward (5′-GCT AGC ATG AGA AAC TGG CAA AGG AAG-3′) and reverse (5′-AAG CTA AGG CTG GCC ACA CCA G-3′). The amplified product was subcloned into the pGL3-basic (pGL3b) luciferase reporter vector and verified by sequencing. FLAG tagged-ChREBP and HA tagged-Mlxγ overexpression vectors were gifts of Prof. Howard C. Towle (Center for Diabetes research, University of Minnesota, Minneapolis/St. Paul, MN) (37). The lentiviral packaging plasmid pCMV-dR8.2 dvpr (8455), envelope plasmid pCMV-VSVG (8454), and the FLAG-tagged SREBP1c overexpression vector (ID 32017) were purchased from Addgene. The ChREBP-shRNA (catalog #RHS4533) vectors were purchased from Open Biosystems, Huntsville, AL 35806. SREBP1c-shRNA vectors were purchased from Origene (catalog #TG514167). The Cblb (hCblb)-specific overexpressing lentiviral particles (catalog #OHS5899-202619951) were purchased from Open Biosystems. Generation of ChREBP and SREBP1c shRNA lentiviral particles and lentiviral-mediated

MARCH 14, 2014 • VOLUME 289 • NUMBER 11 • JOURNAL OF BIOLOGICAL CHEMISTRY 7655

WITHDRAWN
transduction of myoblasts/myotubes was performed as previously described (38).

**Transfection and Luciferase Reporter Assays**—Co-transfection of plasmids into myoblasts using LF2000 has been previously described (38). After transfection, myoblasts were subsequently differentiated into myotubes for further analysis. Assessment of *Mstn* promoter-reporter (1.6 and 0.9 kb) activity was performed as previously described (38).

**Quantitative Real Time PCR and Primer Sequences**—Tissue and whole cell RNA was extracted using TRIzol reagent as per the manufacturer’s protocol (Invitrogen). Synthesis of cDNA, quantitative real time PCR, and subsequent data analysis was performed as previously described (31). The gene-specific primers used in this manuscript are available upon request.

**Protein Isolation, Immunoblotting (IB), and Immunoprecipitation (IP)**—Protein isolation, quantification, gel electrophoresis, and target protein detection were performed as previously published (31). Co-immunoprecipitation analysis of Cblb and IRS1 interaction was performed as previously described (40). For the detection of ChREBP and SREBP-1c, nuclear and cytoplasmic extracts were prepared using the NE-PER® Nuclear and Cytoplasmic Extraction kit from Thermo Fisher Scientific, Rockford, IL (catalog # 78833). The nuclear (50 µg) and cytoplasmic (50 µg) proteins prepared from cells and tissues were subjected to 10% SDS-PAGE. The details of the antibodies used in this manuscript are available upon request.

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts were prepared as described above. The 32P double-stranded oligonucleotides, which contain E-box sequences specific for the Mstn promoter, were commercially synthesized by Sigma. The sequences of the Mstn promoter-specific ChoRE element are forward (5’-ATA CTG CTT GGT GAC TTG TGA-3’) and reverse (5’-CAG GGA GTC CTG TAT ACT G-3’). The E-box probes used are as follows: E-box #1 forward (5’-ATA CTG CTT GGT GAC TTG TGA-3’); E-box #2 forward (5’-AGA TCC GCA CTC CAA GTC TTA AAG GA-3’) and reverse (5’-GTT AAA ACC CTG TCT GTC ACA AG-3’); E-box #3 forward (5’-ATA CTG TCT GTT GAC TTG TGA-3’); and E-box #3 forward (5’-CAA GGA GTC CTG TCT ATAG A-3’). The mutant E-box (mut-ChoRE) probe lacks the 5’ most E-box sequence (TGTAA) and its sequence is 5’-AGA TCT GCA CTC CAA GTC TTA AAG GA-3’.

**Chromatin Immunoprecipitation (ChIP)**—ChIP was performed using myoblasts transfected with the 1.6-kb bovine *Mstn* promoter alone or together with FLAG-ChREBP and HA-MIxy in the absence (2.5 mm) or presence of glucose (25 mm). ChIP was also performed on C2C12 myoblasts transfected with the FLAG-SREBP1c construct and treated with or without 0.25 mm palmitate (PA). ChIP was performed as described previously (38). The sequences of the primers used to detect the *Mstn* promoter-specific ChoRE element are forward (5’-AAA AAG CCC CAT TCT CTG CT-3’) and reverse (5’-TGC CCA TTT TTC TGC TTC TC-3’). The sequences of the primers used to detect the *Mstn* promoter-specific E-box sequences are: E-box #1 forward (5’-ATA CTG CTT GGT GAC TTG TGA-3’) and reverse (5’-CAG GGA GTC CTG TAT ACT G-3’); E-box #2 forward (5’-AGA TCT GCA CTC CAA GTC TTA AAG GA-3’) and reverse (5’-GTT AAA ACC CTG TCT GTC ACA AG-3’); E-box #3 forward (5’-ATA CTG TCT GTT GAC TTG TGA-3’); and E-box #3 forward (5’-CAA GGA GTC CTG TCT ATAG A-3’).

**RESULTS**

**Mstn Is Up-regulated upon High Glucose Treatment and High Fat Diet Feeding**—Quantitative real time PCR and IB analysis revealed that *Mstn* expression was significantly up-regulated in response to high glucose and palmitate treatment in C2C12 myotubes and HepG2 human hepatocytes (Fig. 1, A–D) when compared with control-treated cells. The increase in *Mstn* due to high glucose was both time- and dose-dependent (Fig. 1A). In addition to elevated *Mstn*, high glucose and palmitate stimulation resulted in increased expression of mtP38 protein, a marker of oxidative stress (Fig. 1B). Additionally, the increase in *Mstn* expression was accompanied by a decrease in ChREBP activity, as measured by luciferase reporter assays (Fig. 1C). Moreover, the increase in *Mstn* expression was correlated with a decrease in the expression of the glucose transporters (GLUT4 and GLUT1) and an increase in the expression of the insulin receptor (IRS1) (Fig. 1D). These results indicate that high glucose and palmitate treatment induces a metabolic switch from glucose uptake to glycolysis, resulting in increased *Mstn* expression and oxidative stress. This switch is mediated by the downregulation of ChREBP activity, which ultimately leads to reduced glucose uptake and increased insulin resistance. The data suggest that *Mstn* expression is a key determinant of insulin resistance and that interventions targeting *Mstn* expression may be effective in addressing insulin resistance. Further studies are needed to elucidate the molecular mechanisms underlying the regulation of *Mstn* expression and its role in metabolic diseases.
tate treatment led to reduced levels of pAkt, which is consistent with impaired insulin signaling (Fig. 1, C and D). We next validated these results in vivo. Upon 12 weeks of injections with high glucose, WT mice gained BW (Fig. 2 A), exhibited increased fat pad weights (Fig. 2 B) and increased liver weights (Fig. 2 C), and developed insulin resistance, as measured by glucose tolerance testing (GTT) (Fig. 2 G) and insulin tolerance testing (ITT) (Fig. 2 H). Similarly, WT mice gained significant BW, fat mass, and liver weight upon high fat diet (HFD) feeding (Fig. 2, D–F) and developed insulin resistance, as measured by GTT (Fig. 2 J) and ITT (Fig. 2 F). A list of the full biochemical data relating to high glucose and HFD trials is provided in Table 1. Consistent with the in vitro experiments above (Fig. 1, A–D), molecular and biochemical analysis confirmed that treating mice with either high glucose or HFD induces the expression of Mstn (Fig. 1, E–H) concomitant with reduced pAkt levels (Fig. 1, E and F). Although IB analysis confirmed increased Mstn levels in skeletal muscle and liver in response to high glucose (Fig. 1 E) or HFD (Fig. 1 F), ELISA also revealed significantly increased Mstn levels in serum collected from high glucose (Fig. 1 G) and HFD (Fig. 1 H)-treated WT mice. These data demonstrate that nutrient-rich diets induce Mstn both in vitro and in vivo.

**Mechanism of Mstn-induced Insulin Resistance**

**FIGURE 1.** **High glucose and palmitate loading increases Mstn expression in vitro and in vivo.** Mstn mRNA expression in glucose (Glu)-treated (A) or in PA-treated (B) cells, normalized to GAPDH. Shown is IB analysis of Mstn, pAkt, and total Akt in glucose-treated (C) or in PA-treated (D) cells. Tubulin levels were assessed to ensure equal loading. Shown is IB analysis of Mstn, pAkt, and total Akt protein levels in muscle (M) and liver (L) tissue from WT mice injected with either saline or glucose (E) or in mouse (M) and liver (L) tissue from WT mice fed on CD or HFD (F). Tubulin levels were assessed to ensure equal loading (n = 4, for each group). All graphs display the mean ± S.E. *, p < 0.05; **, p < 0.01, and ***, p < 0.001. G, ELISA of Mstn levels in serum from WT mice injected with either saline or glucose (n = 8, for each group). H, ELISA of Mstn levels in serum from WT mice fed either CD or HFD (n = 8 for each group).

**ChREBP and SREBP1c Induce Mstn Expression in Response to High Glucose and Fatty Acid Loading, Respectively**—Analysis of the Mstn gene promoter revealed the presence of a putative ChoRE (Fig. 3 A, upper panel). Subsequent promoter-reporter analysis revealed that the 1.6-kb Mstn upstream element was sufficient for robust activation by high glucose (Fig. 3A, lower panel). Partial deletion of the ChoRE element, however,
Mechanism of Mstn-induced Insulin Resistance

resulted in a complete loss of Mstn promoter-reporter activity in response to high glucose (Fig. 3A, lower panel). ChREBP is a well-characterized transcription factor that in response to glucose binds to ChoREs in target genes to regulate expression (41–44). Consistent with this, we found increased ChREBP translocation into the nucleus upon high glucose treatment in vitro (Fig. 3B, upper panel) and in muscle and liver tissues of WT mice injected with high glucose (Fig. 3B, lower panel). Furthermore, EMSAs revealed enhanced dose-dependent interaction between nuclear extracts (NE) from high glucose treated myoblasts and the wild type ChoRE (wt-ChoRE) sequence found in the Mstn promoter region (Fig. 3C, upper panel), which was ablated by incubation with a mutated ChoRE (mut-ChoRE) (Fig. 3C, lower panel). This showed specificity of the protein-DNA interaction, overexpression of either Mlxγ increased Mstn promoter-reporter activity (Fig. 4A), and enhanced upon treatment with high glucose (Fig. 4B). In addition, overexpression of SREBP1c dramatically enhanced Mstn promoter-reporter activity (Fig. 4D). Similar to high glucose, treatment with palmitate also increased Mstn promoter-reporter activity (Fig. 4A). SREBP1c has been previously shown to regulate Mstn promoter activity in 3T3L1 cells (4). In agreement with this, we observed strong nuclear translocation of SREBP1c in cells treated with palmitate (Fig. 4B) and in muscle and liver tissues of WT mice fed with HFD (Fig. 4C). Furthermore, overexpression of SREBP1c blocked palmitate-mediated induction of Mstn promoter-reporter activity (Fig. 4E), which was further enhanced upon treatment with palmitate. Consistent with this, knock down of SREBP1c expression (Fig. 4, F and G) blocked palmitate-mediated induction of Mstn promoter-reporter activity in muscle cells (Fig. 4H). It is well documented that SREBP1c can bind to E-box motifs to regulate target gene activation (45, 46). Analysis of the Mstn gene promoter revealed the presence of five E-box motifs consisting of three different consensus E-box sequences (Fig. 4I). With this in mind we performed EMSAs using the 3 different E-box sequences contained within the Mstn 1.6-kb promoter region on nuclear extracts from palmitate-treated hMb15 myoblasts. Results revealed that a specific band shift was seen upon incubation with labeled oligo containing Mstn E-box #1 sequence (5′-CACCAGG-3′) (Fig. 4J), whereas no band shift was observed upon incubation.

TABLE 1

| Measurements                  | Saline | Glucose | Units |
|-------------------------------|--------|---------|-------|
| Plasma triglycerides          | 152 ± 4.1 | 284.3 ± 2.1* | mg/dl |
| Plasma adiponectin            | 10 ± 1.2  | 16 ± 2.20*   | ng/ml |
| Plasma leptin                 | 2.8 ± 0.5 | 14 ± 2.4*    | ng/ml |
| Blood glucose                 | 6 ± 1.3   | 9 ± 1.11*    | mmol/liter |
| Plasma insulin                | 137 ± 6.0 | 352 ± 4.0*   | mg/ml |
| Plasma cholesterol            | 100 ± 3.3 | 263 ± 4.9*   | mg/dl |

* p < 0.01.  ** p < 0.05.  *** p < 0.001.
with labeled oligos containing either Mstn E-box #2 (5'-CAAATG-3') or Mstn E-box #3 sequences (5'-CAGGTG-3') (Fig. 4F). Moreover, we observed a super shift upon the addition of an anti-SREBP1c-specific antibody, confirming the SREBP1c interaction with Mstn E-box #1 sequence (5'-CACTTG-3') in the Mstn promoter region (Fig. 4K). ChIP analysis further confirmed that SREBP1c does indeed bind to the Mstn E-box #1 sequence (5'-CACTTG-3') of the Mstn promoter and that this interaction was enhanced in response to palmitate treatment (Fig. 4L).

Mechanism of Mstn-induced Insulin Resistance

Mstn Induces Degradation of IRS1 Protein by Activating the E3 Ligase Cblb—To find out the possible mechanisms through which Mstn can induce insulin resistance, microarray was performed on Mstn-treated myotubes (Fig. 5A). The differential gene expression changes observed in response to Mstn treatment are listed in Table 2 (up-regulated) and Table 3 (down-regulated). We identified that Cblb, a ubiquitin E3 ligase, was up-regulated in response to Mstn treatment (Table 2). Interestingly, Cblb has been shown to degrade IRS1 protein during muscle atrophy and is linked with the development of type-1 diabetes.
FIGURE 4. The SREBP1c transcription factor is critical for fatty acid regulation of Mstn expression. A, Mstn promoter-reporter luciferase activity in PA-treated C2C12 myotubes. Shown is IB analysis of SREBP1c protein levels in CE and NE prepared from muscle (M) and liver (L) tissues of CD (−) or HFD (+) fed mice (C). D, IB analysis of SREBP1c and Mstn expression in control, empty vector, or FLAG-SREBP1c-transfected 96-h differentiated myotubes. E, Mstn promoter-reporter luciferase activity in C2C12 myotubes transfected with either FLAG-SREBP1c or pCMV4 constructs and treated with PA. F, SREBP1c mRNA expression in myotubes infected with shSREBP1c or shCon expressing lentivirus. G, IB analysis of SREBP1c protein levels in CE and NE from myoblasts infected with shSREBP1c or shCon expressing lentivirus. H, Mstn promoter-reporter luciferase activity in C2C12 myotubes infected with shSREBP1c or shCon expressing lentivirus and treated with PA. I, schematic showing the five different consensus E-box motifs identified in the Mstn promoter. Consensus E-box motifs are highlighted in red and underlined. J, EMSA performed in the absence (−) or presence (+) of NE from PA-treated C2C12 myoblasts. K, EMSA performed in the absence (−) or presence (+) of NE from PA-treated C2C12 myoblast with (+) or without (−) an anti-SREBP1c antibody. L, ChIP assay of SREBP1c interaction with Mstn promoter in C2C12 myoblasts treatment with (+) or without (−) PA. SREBP1c interaction with the β-actin gene promoter in the absence (−) or presence (+) of PA was also performed as a negative control (lower panel). Input DNA is shown for all ChIP assays. The ChIP data are representative of three independent experiments. Tubulin levels were assessed to ensure equal loading in all IBs. All IB images and graphs are representative of at least two independent experiments. All luciferase activities were normalized to Renilla and are expressed as fold change relative to respective controls (A, E, and H). All graphs display the mean ± S.E. of at least two independent experiments. *, p < 0.05; **, p < 0.01.
diabetes in rats and in humans (19–22, 47). However, to date the role of Cblb in the development of insulin resistance remains unknown. Hence we considered Cblb as a valid candidate through which Mstn may regulate insulin sensitivity. Cblb expression was validated through quantitative real time PCR and IB analysis, and our results revealed that Mstn treatment significantly increased Cblb mRNA levels in C2C12 myotubes and HepG2 cells (Fig. 5B, upper panel). The increased levels of Cblb were associated with reduced IRS1 and pAkt protein levels in the presence (+) or absence (−) of Mstn (Fig. 5B, lower panel). IB analysis of Cblb, IRS1, pAkt, and total Akt in muscle and liver tissues derived from WT and Mstn−/− mice (lower panel) is shown.

FIGURE 5. Mstn up-regulates Cblb, an ubiquitin E3 ligase in skeletal muscle. A, heat map representation of gene expression changes in 96-h differentiated myotubes treated with Mstn at different time points. B, Cblb mRNA expression in control and Mstn-treated cells normalized to GAPDH (upper panel). IB analysis of Cblb, IRS1, pAkt, and total Akt in cells transfected with (+) or without (−) Mstn all (upper panel). Bottom, IB analysis of Cblb, IRS1, pAkt, and total Akt in muscle and liver tissues derived from WT and Mstn−/− mice (lower panel) is shown. C, IB analysis of IRS1 protein immunoprecipitated with Cblb in C2C12 myotubes treated with (+) or without (−) Mstn. Bottom, IB analysis of ubiquitinated IRS1 protein levels in C2C12 myotubes treated with (+) or without (−) Mstn in the presence (+) or absence (−) of the proteasome inhibitor MG132. The graph shows densitometric analysis for IRS1 protein in arbitrary units (A.U). Tubulin levels were assessed in all IBs to ensure equal loading. All IB images and graphs are representative of at least two independent experiments. All graphs display the mean ± S.E. *, p < 0.05.

Mechanism of Mstn-induced Insulin Resistance

Cblb Expression Is Essential for Mstn-induced Insulin Resistance—We next treated mice with Mstn, and GTT and ITT confirmed that Mstn-treated WT mice developed insulin resistance (Fig. 6, A and B). Mstn treatment resulted in increased BW (Fig. 6C) with significantly increased epididymal fat mass (Fig. 6D) and reduced tibialis anterior and quadriceps muscle weights (Fig. 6E) despite normal comparable food intake between the groups (Fig. 6F).

A, heat map representation of gene expression changes in 96-h differentiated myotubes treated with Mstn at different time points. B, Cblb mRNA expression in control and Mstn-treated cells normalized to GAPDH (upper panel). IB analysis of Cblb, IRS1, pAkt, and total Akt in cells transfected with (+) or without (−) Mstn all (upper panel). Bottom, IB analysis of Cblb, IRS1, pAkt, and total Akt in muscle and liver tissues derived from WT and Mstn−/− mice (lower panel) is shown. C, IB analysis of IRS1 protein immunoprecipitated with Cblb in C2C12 myotubes treated with (+) or without (−) Mstn. Bottom, IB analysis of ubiquitinated IRS1 protein levels in C2C12 myotubes treated with (+) or without (−) Mstn in the presence (+) or absence (−) of the proteasome inhibitor MG132. The graph shows densitometric analysis for IRS1 protein in arbitrary units (A.U). Tubulin levels were assessed in all IBs to ensure equal loading. All IB images and graphs are representative of at least two independent experiments. All graphs display the mean ± S.E. *, p < 0.05.
Mechanism of Mstn-induced Insulin Resistance

Table 2

| GenBank accession | Gene symbol | Description | Fold-change of genes listed (p < 0.05) |
|-------------------|-------------|-------------|----------------------------------------|
| NM_178886         | Ldraf3      | Low density lipoprotein receptor class A domain containing 3 | 1.62 5.15 6.33 4.58 1.80 |
| NM_198612         | Glb8d4      | Glycosyltransferase 8 domain containing 4 | 1.12 3.24 3.75 4.19 4.05 |
| NM_199011         | Dgkq        | Diacylglycerol kinase, θ | 1.07 1.37 2.92 3.63 3.47 |
| NM_175475         | Cyp26b1     | Cytochrome P450, family 26, subfamily b, polypeptide 1 | 1.54 2.31 2.64 2.45 3.03 |
| NM_173782         | Ubtd2       | Ubiquitin domain containing 2 | 1.28 1.41 2.30 4.87 6.44 |
| NM_134072         | Akr1c14     | Aldo-keto reductase family 1, member C14 | 1.24 1.38 1.56 2.63 3.07 |
| NM_008555         | Ppap2b      | Phosphatidic acid phosphatase type 2B | 2.79 2.01 3.35 5.18 4.99 |
| NM_030612         | Nfkbia      | Nuclear factor of kappa T-cell activation | 1.08 2.00 3.70 2.84 2.68 |
| NM_029415         | Slc10a6     | Solute carrier family 10 (sodium/bile acid cotransporter family), member 6 | 1.13 3.03 4.56 3.42 2.76 |
| NM_025367         | Sphk1       | Sphingosine kinase 1 (Sphk1), transcript variant 2 | 1.29 2.36 1.87 1.80 2.15 |
| NM_024406         | Fabp4       | Fatty acid-binding protein 4, adipocyte | 1.10 1.10 3.70 2.06 2.97 |
| NM_024406         | Fabp4       | Fatty acid-binding protein 4, adipocyte | 1.27 1.52 2.07 2.25 2.30 |
| NM_021894         | Capn12      | Calpain 12 | 1.07 1.38 2.07 2.29 3.63 |
| NM_021398         | S10c3a      | Solute carrier family 43, member 3 | 1.45 2.42 1.42 1.56 4.69 |
| NM_001058         | Angpt4      | Angiopoietin like 4 | 1.52 2.41 1.77 2.48 2.08 |
| NM_019804         | B4galnt1    | UDP-Gal:αGalNAc β-1,4-galactosyltransferase, polypeptide 4 | 1.47 2.78 7.69 9.41 11.07 |
| NM_017373         | Nfkb1       | Nuclear factor of kappa T-cell activation, light polypeptide gene enhancer in B-cells inhibitor, α | 1.35 1.33 1.96 4.08 5.30 |
| NM_012833         | Pdk4        | Pyruvate dehydrogenase kinase, isozyme 4 | 5.82 140.13 193.28 176.22 163.74 |
| NM_013526         | Gf16        | Growth differentiation factor 6 | 1.15 1.21 1.55 2.20 2.60 |
| NM_013495         | Cnpl       | CARNITINE PALMITOLYLTRANSFERASE 1A, liver (CnplA), nuclear gene encoding mitochondrial protein | 1.15 1.49 1.74 2.03 2.01 |

Data showing that Cblb resulted in a rescue of Mstn-mediated degradation of IRS1 and loss of pAkt (Fig. 6). Moreover, the absence of Cblb prevented the enhanced ubiquitination of IRS1 observed after treatment with Mstn (Fig. 6). Collectively, these data reveal that increased Mstn induces insulin resistance through a mechanism involving Cblb-mediated loss of IRS1 protein.

Mstn Signal through Smad3 to Regulate Cblb Expression—Mstn has been previously shown to signal through Smad3 to elicit biological function (24). Consistent with this, in silico analysis revealed the presence of several putative Smad3 binding motifs in the Cblb promoter region (Fig. 7A). To test whether Smad3 is important in Mstn regulation of Cblb, we initially assessed the levels of active phosphorylated Smad3 in Mstn-treated myotubes compared to control treated myotubes at all time points in the microarray.
response to high glucose (Fig. 7B) and HFD regimen (Fig. 7C). Consistent with elevated Mstn levels (Fig. 1, A–H) the abundance of phosphorylated Smad3 was increased in response to both high glucose (Fig. 7B) and HFD regimen in vivo (Fig. 7C). Furthermore, treatment with Mstn failed to up-regulate Cblb mRNA expression (Fig. 7, D and F) or protein levels (Fig. 7, E and G) in both Smad3 knock down myotubes (Fig. 7, D and E) and in primary myoblasts isolated from Smad3−/− mice (Fig. 7, F and G) when compared with respective controls. In addition, Mstn-mediated loss of IRS1 and pAkt levels was also rescued in both Smad3 knock down myotubes and in primary myoblasts isolated from Smad3−/− mice when compared with respective controls (Fig. 7, E and G). Taken together these data suggest that Smad3 has an indispensable role in Mstn-mediated activation of Cblb at the transcript level.

**Glucose and Fatty Acids Require Mstn-Cblb Signaling to Promote Insulin Resistance**—To ascertain whether or not the Mstn-Cblb pathway plays a role in the development of insulin resistance in response to high glucose and HFD regimen, we next assessed the expression of Cblb, IRS1, and pAkt in high glucose- and palmitate-treated C2C12 myotubes and HepG2 cells in the presence or absence of Mstn. In agreement with the elevated Mstn levels detected in response to in vitro treatment with high glucose or palmitate (Fig. 1, A–D), we also noted increased levels of Cblb and reduced IRS1 and pAkt expression in C2C12 myotubes and HepG2 cells after treatment with high glucose (Fig. 8A) and palmitate (Fig. 8C). However, siRNA-mediated knock down of Mstn in C2C12 myotubes and HepG2 cells treated with high glucose (Fig. 8B) or palmitate (Fig. 8D) led to reduced Cblb levels, concomitant with a
rescue in the levels of IRS1 and pAkt, which is consistent with improved insulin sensitivity. In agreement with the in vitro analysis above, whereas high glucose and HFD treatment resulted in increased Cblb levels in both skeletal muscle and liver isolated from WT mice (Fig. 8, E and F), when compared with respective controls (saline or chow diet (CD)-fed), no increase in Cblb levels was noted in Mstn/H11002/H11002 mice fed either high glucose or HFD (Fig. 8, E and F). Moreover, the levels of IRS1 and pAkt in both skeletal muscle and liver were comparable between CD-fed controls and high glucose or HFD-treated Mstn/H11002/H11002 mice (Fig. 8, E and F). However, in contrast, a dramatic reduction in the levels of both IRS1 and pAkt was observed in WT mice during high glucose and HFD treatment (Fig. 8, E and F), which was consistent with both the high levels of Cblb protein (Fig. 8, E and F) and the development of insulin resistance observed in WT mice (Fig. 2, G–J). Furthermore, overexpression of Cblb through lentiviral-mediated transduction in hMb15 myoblasts reduced both basal and insulin-stimulated pAkt levels together with IRS1 protein (Fig. 8G). On the other
hand, enhanced basal and insulin stimulated pAkt levels, and IRS1 abundance was observed in myoblasts derived from Cblb−/− mice when compared with myoblasts isolated from control Cblb+/* mice (Fig. 8H). Taken together these data confirm that the Mstn-Cblb pathway appears to play a critical role during the induction of insulin resistance in response to both high glucose and HFD regimen and that Cblb plays a major role in inhibiting insulin signaling.
Mechanism of Mstn-induced Insulin Resistance

**DISCUSSION**

Energy-dense diets that are high in fat, protein, and sugar are associated with a risk of obesity and T2D (48). Insulin resistance is key predictor of T2D and is associated with both non-obese and obese pathological conditions (49). However, the underlying molecular mechanism(s) that initiates the development of insulin resistance during continued high calorie intake is poorly characterized. Previous work by Hittel et al. (50) has revealed that injection of exogenous Mstn protein into mice leads to the development of insulin resistance. However, the role of Mstn in initiating insulin resistance in response to high calorie intake has not been studied. Here we show a conclusive mechanism through which energy-rich diets in mice induce high levels of Mstn, which subsequently results in the targeted degradation of the critical insulin-signaling molecule IRS1 by up-regulation of the ubiquitin E3 ligase Cblb.

The myokine, Mstn, belongs to the TGF-β super family and primarily functions to control muscle growth and development (51). However, recent reports have shown that Mstn also plays a role in regulating muscle metabolism; in fact, either inhibition of Mstn or lack of Mstn reduces fat accumulation and enhances insulin sensitivity (24, 52). Our laboratory characterized increased AMP-activated protein kinase and peroxisome proliferator-activated receptor signaling in Mstn−/− muscle as mechanisms behind the increased fat oxidation observed in Mstn−/− mice (24). Moreover, recent evidence also reveals that elevated levels of Mstn in muscle in both human and mouse models are associated in obesity, type-1 and type-2 diabetes (5, 7, 53). Therefore, we considered Mstn to be an excellent candidate that could potentially respond to nutrient signals to further regulate muscle metabolism. Indeed we observed that in vitro treatment of C2C12 and HepG2 cells with high glucose or fatty acid (54–56). In addition, fatty acid regulation of gene transcription through ChREBP is not a new concept; in fact studies have reported that in response to high glucose treatment, ChREBP binds and activates genes involved in lipogenesis (54–56). Therefore, we considered Mstn to be an excellent candidate that could potentially respond to nutrient signals to further regulate muscle metabolism. Indeed we observed that in vitro treatment of C2C12 and HepG2 cells with high glucose or fatty acid (54–56). In addition, fatty acid regulation of gene transcription through ChREBP is not a new concept; in fact studies have reported that in response to high glucose treatment, ChREBP binds and activates genes involved in lipogenesis (54–56). Therefore, we considered Mstn to be an excellent candidate that could potentially respond to nutrient signals to further regulate muscle metabolism. Indeed we observed that in vitro treatment of C2C12 and HepG2 cells with high glucose or fatty acid (54–56). In addition, fatty acid regulation of gene transcription through ChREBP is not a new concept; in fact studies have reported that in response to high glucose treatment, ChREBP binds and activates genes involved in lipogenesis (54–56). Therefore, we considered Mstn to be an excellent candidate that could potentially respond to nutrient signals to further regulate muscle metabolism. Indeed we observed that in vitro treatment of C2C12 and HepG2 cells with high glucose or fatty acid (54–56). In addition, fatty acid regulation of gene transcription through ChREBP is not a new concept; in fact studies have reported that in response to high glucose treatment, ChREBP binds and activates genes involved in lipogenesis (54–56). Therefore, we considered Mstn to be an excellent candidate that could potentially respond to nutrient signals to further regulate muscle metabolism. Indeed we observed that in vitro treatment of C2C12 and HepG2 cells with high glucose or fatty acid (54–56). In addition, fatty acid regulation of gene transcription through ChREBP is not a new concept; in fact studies have reported that in response to high glucose treatment, ChREBP binds and activates genes involved in lipogenesis (54–56). Therefore, we considered Mstn to be an excellent candidate that could potentially respond to nutrient signals to further regulate muscle metabolism. Indeed we observed that in vitro treatment of C2C12 and HepG2 cells with high glucose or fatty acid (54–56). In addition, fatty acid regulation of gene transcription through ChREBP is not a new concept; in fact studies have reported that in response to high glucose treatment, ChREBP binds and activates genes involved in lipogenesis (54–56). Therefore, we considered Mstn to be an excellent candidate that could potentially respond to nutrient signals to further regulate muscle metabolism. Indeed we observed that in vitro treatment of C2C12 and HepG2 cells with high glucose or fatty acid (54–56). In addition, fatty acid regulation of gene transcription through ChREBP is not a new concept; in fact studies have reported that in response to high glucose treatment, ChREBP binds and activates genes involved in lipogenesis (54–56). Therefore, we considered Mstn to be an excellent candidate that could potentially respond to nutrient signals to further regulate muscle metabolism. Indeed we observed that in vitro treatment of C2C12 and HepG2 cells with high glucose or fatty acid (54–56). In addition, fatty acid regulation of gene transcription through ChREBP is not a new concept; in fact studies have reported that in response to high glucose treatment, ChREBP binds and activates genes involved in lipogenesis (54–56). Therefore, we considered Mstn to be an excellent candidate that could potentially respond to nutrient signals to further regulate muscle metabolism. Indeed we observed that in vitro treatment of C2C12 and HepG2 cells with high glucose or fatty acid (54–56). In addition, fatty acid regulation of gene transcription through ChREBP is not a new concept; in fact studies have reported that in response to high glucose treatment, ChREBP binds and activates genes involved in lipogenesis (54–56).
been shown that during skeletal muscle atrophy Cblb targets IRS1 protein for degradation via the ubiquitin proteasome pathway (22). However, to date, the importance of Cblb during the development of insulin resistance and for factors that regulate Cblb expression during obesity are poorly understood. Subsequent quantitative PCR and IB analysis independently confirmed that Mstn indeed induces Cblb expression not only in vitro but also in mice in vivo. Consistent with the E3 ligase function of Cblb, induction of Cblb by Mstn led to increased association of Cblb with IRS1 and subsequent ubiquitination of IRS1 protein. The Mstn-Cblb-IRS1 pathway was further confirmed when Mstn failed to reduce IRS1 protein levels either in the absence of Cblb or in the presence of the proteasome inhibitor MG132. IRS1 is a key molecule in the insulin-signaling pathway (58–60) and is highly expressed in skeletal muscle and white adipose tissue (61). Consistent with this, deletion of IRS1 in mice induces severe insulin resistance (62–64). Moreover, reduced IRS1 mRNA and/or protein levels are detected in subjects with T2D (65, 66). Therefore, Mstn/Cblb-mediated loss of IRS1 would most certainly contribute to the insulin resistance phenotype observed in response to increased Mstn levels.

Importantly, studies have also reported that serine phosphorylation of IRS1, in contrast to the tyrosine phosphorylation of IRS1 normally observed after insulin treatment, is associated with T2D (67, 68). In addition, serine phosphorylation of IRS1 has been shown to promote both enhanced degradation of IRS1 through the ubiquitin proteasome pathway (69) and development of insulin resistance. However, it is noteworthy to mention that in the current study we did not assess the serine phosphorylation status of IRS1; as such, development of insulin resistance, due to increased serine phosphorylation of IRS1 in response to high calorie diet and Mstn treatment cannot be ruled out. Nevertheless, through several independent investigations we have clearly shown that initiation of the Mstn-Cblb pathway leads to degradation of IRS1.

We hypothesized that Mstn-Cblb degradation of IRS1 would further result in hypophosphorylation of Akt and development of insulin resistance. In agreement with this, overexpression of Cblb resulted in loss of IRS1 and reduced pAkt levels, whereas the absence of Cblb blocked IRS1 degradation and increased the levels of pAkt under basal conditions and in response to insulin. These data strongly suggest increased insulin sensitivity in the absence of Cblb. In addition, we observed a greater increase in Mstn and Cblb protein levels in both skeletal muscle and liver tissues of mice treated with either high glucose or HFD, with an associated decrease in IRS1 protein, reduced phosphorylation of Akt, and impaired insulin sensitivity in WT mice.
mice but not in Mstn−/− mice. These data demonstrate that in the absence of Mstn, high glucose and HFD feeding failed to activate Cblb in both liver and muscle tissues. These observations also support a role for Mstn in promoting insulin resistance in liver in response to high glucose and HFD feeding, which is in fact quite consistent with a previously published report demonstrating that lack of Mstn protects the liver from diet-induced insulin resistance (39). Although Wilkes et al. (39) speculated that the improved insulin sensitivity observed in the liver may be due to reduced TNF-α levels, our data presented here suggests that loss of Mstn may also lead to reduced activation of Cblb in liver and improved insulin sensitivity.

In summary, we for the first time report that energy-rich diets, specifically high glucose and high fat, signal through ChREBP in liver and improved insulin sensitivity.

REFERENCES

1. Brand-Miller, J. C., Holt, S. H., Pawlak, B. E., and McMillan, J. (2002) Glycemic index and obesity. Am. J. Clin. Nutr. 76, 2815–2855
2. Bray, G. A., Smith, S. R., de Jonge, L., Xie, H., Rood, J., Martin, C. K., Most, M., Brock, C., Mancuso, S., and Redman, L. M. (2012) Effect of dietary protein content on weight gain, energy expenditure, and body composition during overeating. A randomized controlled trial. JAMA 307, 47–55
3. Winzil, M. S., and Ahren, B. (2004) The high-fat diet-fed mouse. A model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. Diabetes 53, S215–S219
4. Allen, D. L., Cleary, A. S., Speaker, K. J., Lindsay, S. F., Uyenishi, J., Reed, J. M., Madden, M. C., and Mehan, R. S. (2008) Myostatin, activin receptor-like IIb, and follistatin-like–3 gene expression are altered in adipose tissue and skeletal muscle of obese mice. Am. J. Physiol. Endocrinol. Metab. 294, E918–E927
5. Hettel, D. S., Berggren, J. R., Shearer, J., Boyle, K., and Houmard, J. A. (2009) Increased secretion and expression of myostatin in skeletal muscle from extremely obese women. Diabetes 58, 30–38
6. Brandt, C., Nielsen, A. R., Fischer, C. P., Hansen, J., Pedersen, B. K., and Plomgaard, P. (2012) Plasma and muscle myostatin in relation to type 2 diabetes. PLoS ONE 7, e37236
7. Chen, Y., Cao, L., Ye, J., and Zhu, D. (2009) Upregulation of myostatin gene expression in streptozotocin-induced type 1 diabetes mice is attenuated by insulin. Biochem. Biophys. Res. Commun. 388, 112–116
8. Ma, K., Mallidis, C., Bhasin, S., Mahabadi, V., Arata, J., Gonzalez-Cadavid, N., Arias, J., and Salehian, B. (2003) Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expres-

sion. Am. J. Physiol. Endocrinol. Metab. 285, E363–E371
9. Spiller, M. P., Kambadur, R., Jeangplong, F., Thomas, M., Martyn, J. K., Bass, J. J., and Sharma, M. (2002) The myostatin gene is a downstream target gene of basic helix-loop-helix transcription factor MyoD. Mol. Cell. Biol. 22, 7066–7082
10. Grzelkowska-Kowalczyk, K., Wieteska-Skrzeczyńska, W., Grabiec, K., and Tokarska, J. (2013) High glucose-mediated alterations of mechanisms important in myogenesis of mouse C2C12 myoblasts. Cell Biol. Int. 37, 29–35
11. Nakazato, K., Hirose, T., and Song, H. (2006) Increased myostatin synthesis in rat gastrocnemius muscles under high-fat diet. Int. J. Sport. Nutr. Exerc. Metab. 16, 153–165
12. Swaminathan, G., and Tsygankov, A. Y. (2006) The Cbl family proteins. Ring leaders in regulation of cell signaling. J. Cell Physiol. 209, 21–43
13. Ettenberg, S. A., Keane, M. M., Nau, M. M., Frankel, W., Lang, M. L., Pierce, J. H., and Lipkowitz, S. (1999) cbl-b inhibits epidermal growth factor receptor signaling. Oncogene 18, 1855–1866
14. Levkowitz, G., Waterman, H., Ettenberg, S. A., Kat, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yard, Y. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol. Cell 4, 1029–1040
15. Kim, M., Tsuch, S. Y., Sugano, S., Hiroi, M., and Yamamoto, T. (1999) Molecular characterization of a novel cbl-family gene, cbl-c. Gene 223, 82–91
16. Moler-Gonzalez, M. C., Land, W. Y., James, D. E., and Cooney, G. J. (2004) c-Cbl-deficient mice have reduced adiposity and higher energy expenditure, and improved peripheral insulin action. J. Clin. Invest. 114, 1326–1333
17. Yaffe, D., and Saxel, O. (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. J. Clin. Invest. 56, 229–235
18. Spiller, M. P., Couzens, M., Herzog, H., Thien, L., Waller, K., Murphy, M. A., Bowtell, D. D., James, D. E., and Cooney, G. J. (2006) Genetic ablation of the c-Cbl ubiquitin ligase domain gene of basic helix-loop-helix transcription factor receptor signaling. J. Clin. Invest. 114, 229–235
Mechanism of Mstn-induced Insulin Resistance

66. Kovacs, P., Hanson, R. L., Lee, Y. H., Yang, X., Kobes, S., Permana, P. A., Bogardus, C., and Baier, L. J. (2003) The role of insulin receptor substrate-1 gene (IRS1) in type 2 diabetes in Pima Indians. *Diabetes* **52**, 3005–3009

67. Bouzakri, K., Roques, M., Gual, P., Espinosa, S., Guebre-Egziabher, F., Riou, J. P., Laville, M., Le Marchand-Brustel, Y., Tanti, J. F., and Vidal, H. (2003) Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. *Diabetes* **52**, 1319–1325

68. Morino, K., Petersen, K. F., Dufour, S., Befroy, D., Frattini, J., Shatzkes, N., Neschen, S., White, M. F., Bilz, S., Sono, S., Pypaert, M., and Shulman, G. I. (2005) Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J. Clin. Invest.* **115**, 3587–3593

69. Pederson, T. M., Kramer, D. L., and Rondinone, C. M. (2001) Serine/threonine phosphorylation of IRS-1 triggers its degradation. Possible regulation by tyrosine phosphorylation. *Diabetes* **50**, 24–31

WITHDRAWN
June 1, 2016
Myostatin Induces Insulin Resistance via Casitas B-Lineage Lymphoma b (Cblb)-mediated Degradation of Insulin Receptor Substrate 1 (IRS1) Protein in Response to High Calorie Diet Intake
Sabeera Bonala, Sudarsanareddy Lokireddy, Craig McFarlane, Sreekanth Patnam, Mridula Sharma and Ravi Kambadur

J. Biol. Chem. 2014, 289:7654-7670.
doi: 10.1074/jbc.M113.529925 originally published online January 22, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M113.529925

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 69 references, 30 of which can be accessed free at http://www.jbc.org/content/289/11/7654.full.html#ref-list-1

WITHDRAWN June 1, 2016