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(54) Title: METALLOPROTEASE ACTIVATION OF MYOSTATIN, AND METHODS OF MODULATING MYOSTATIN
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It has been determined that metalloprotease cleavage of a myostatin pro peptide results in activation of a latent inactive myostatin to an active form. Accordingly, methods of identifying agents that modulate metalloprotease mediated activation of myostatin are provided, as are agents identified using such methods. Also provided are methods of modulating muscle growth in an organism by increasing or decreasing metalloprotease mediated cleavage of a myostatin pro peptide.



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(54) Title: METALLOPROTEASE ACTIVATION OF MYOSTATIN, AND METHODS OF MODULATING MYOSTATIN ACTIVITY

(57) Abstract: It has been determined that metalloprotease cleavage of a myostatin pro peptide results in activation of a latent inactive myostatin to an active form. Accordingly, methods of identifying agents that modulate metalloprotease mediated activation of myostatin are provided, as are agents identified using such methods. Also provided are methods of modulating muscle growth in an organism by increasing or decreasing metalloprotease mediated cleavage of a myostatin pro peptide.



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**METALLOPROTEASE ACTIVATION OF MYOSTATIN,
AND METHODS OF MODULATING MYOSTATIN ACTIVITY**

[0001] This invention was made in part with government support under Grant Nos. HD35887, AR47746, and GM63471 awarded by the National Institutes of Health. The United States government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The invention relates generally to metalloprotease regulation of myostatin activity, and more specifically to methods of using agonists or antagonists of the BMP-1/TLD family of metalloproteases to modulate myostatin activity including, for example, to regulate muscle development in an organism, to methods of identifying agonists and antagonists of such metalloproteases, and to agonists and antagonists so identified.

Background Information

[0003] Myostatin is a transforming growth factor- β (TGF- β) family member that is essential for proper regulation of skeletal muscle growth. Myostatin is a secreted protein that is expressed specifically by cells of the skeletal muscle lineage during embryonic development and in adult animals; low levels of myostatin mRNA also are present in fat cells in adults animals. During early embryogenesis, myostatin mRNA is detectable in the myotome compartment of developing somites. At later embryonic stages and in postnatal life, myostatin is expressed widely in all skeletal muscles that have been examined.

[0004] The function of myostatin was elucidated by gene targeting studies in mice. Mice lacking myostatin demonstrated a dramatic and widespread increase in skeletal muscle mass due to muscle fiber hyperplasia and hypertrophy, indicating that myostatin is a negative regulator of muscle growth. The myostatin gene is highly conserved across evolution, with the predicted mature myostatin protein sequence being identical among mice, rats, humans, chickens, turkeys, and pigs, and highly homologous even with respect to aquatic organisms. The function of myostatin also is conserved, with mutations in the myostatin gene correlating to the double muscling phenotype in cattle.

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[0005] The role of myostatin in regulating muscle growth and development indicates that methods and compositions that regulate myostatin activity can have a broad variety of applications, including, for example, for treating human diseases and for improving livestock production. With respect to human therapeutic applications, inhibitors of myostatin expression or function can provide a clinical benefit in the treatment of muscle wasting disorders such as muscular dystrophy, cachexia, and sarcopenia. In addition, myostatin deficient animals have a significant reduction in fat accumulation, and the loss of myostatin is protective against the development of obesity and type II diabetes in genetic models in mice. As such, modulation of myostatin activity also can be useful in the treatment of metabolic disorders such as obesity and type II diabetes. Further in this respect, inhibitors of myostatin expression or function not only can be useful for increasing the efficiency of livestock production, but also can result in the production of meat with a lower fat content.

[0006] Various strategies for manipulating the biological activities of myostatin have been described. Myostatin is synthesized as a precursor protein that undergoes proteolytic processing to generate an N-terminal fragment termed the "pro peptide" and a C-terminal fragment, a disulfide-linked dimer of which is the biologically active species. Currently described strategies for inhibiting myostatin activity have utilized molecules that can bind the myostatin C-terminal dimer and inhibit its activity. For example, myostatin binds two activin type II receptors, Act RIIA and Act RIIB, *in vitro*, and expression of a truncated dominant negative form of Act RIIB in transgenic mice resulted in the mice having increases in muscle mass comparable to that of transgenic myostatin knock out mice.

[0007] The myostatin pro peptide also has been used to inhibit myostatin activity. Following proteolytic processing, the myostatin pro peptide remains non-covalently associated with the C-terminal dimer and maintains the dimer in a latent, inactive state. The pro peptide has been shown to block the activity of the purified myostatin C-terminal dimer in various *in vitro* assays, and overexpression of the pro peptide in transgenic mice resulted in a phenotype characteristic of the myostatin null mutation. Follistatin is another protein that acts as a myostatin inhibitor. Follistatin can bind and inhibit the activity of a variety of TGF- β family members, including myostatin, and transgenic mice

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overexpressing follistatin in muscle have dramatic increases in muscle growth, consistent with inhibition of myostatin activity.

[0008] The above described inhibitors of myostatin each specifically interact with mature myostatin to inhibit its activity. While inhibiting the activity of a protein such as myostatin using an agent that directly interacts with the protein provides great specificity, such a method can require that all or most of the proteins be bound by the agent for the inhibitory effect to be manifest. An alternative way to inhibit the activity of a protein, particularly a protein that, itself must be activated by a second protein such as an enzyme in order for the first protein to be functional, is to target the second protein. Such a method can be advantageous because activating proteins such as enzymes generally are present at much lower levels than their substrates. As such, there is a greater likelihood that all or most of an activating protein such as an enzyme can be inhibited.

[0009] With respect to myostatin, at least two proteases are known to be involved in processing promyostatin, the primary gene product, into a signal peptide, a pro peptide and a C-terminal fragment, the latter of which forms homodimers that have biological myostatin activity. Unfortunately, these proteases also can act on a variety of other proteins and, therefore, agents that target and inhibit these proteases, for example, signal peptidase, likely would have diverse and deleterious effects if administered to a living organism. Thus, a need exists to identify biological molecules that are more specifically involved in regulating myostatin activation and activity. The present invention satisfies this need and provides additional advantages.

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SUMMARY OF THE INVENTION

[0010] The present invention is based on the identification of proteases that cleave myostatin pro peptide, including when the myostatin pro peptide is present in a complex with a myostatin C-terminal dimer. As such, the proteases can convert a latent inactive myostatin complex, which comprises a myostatin pro peptide associated with a C-terminal myostatin polypeptide, to active myostatin, which is a negative regulator of muscle growth and development. Such proteases, which are exemplified by the metalloprotease bone morphogenic protein-1/tolloid (BMP-1/TLD) family of proteins, provide targets for drugs that can increase or decrease the protease activity and, therefore, increase or decrease myostatin activity. Accordingly, the present invention provides agents that modulate metalloprotease mediated myostatin pro peptide cleavage and activation of myostatin, as well as methods of using such agents, for example, to modulate myostatin activity in an organism. Methods of identifying such agents also are provided.

[0011] The present invention relates to a method of modulating myostatin activation. Such a method can be performed, for example, by contacting a latent myostatin complex, which includes a myostatin pro peptide and a myostatin C-terminal fragment, particularly a C-terminal fragment dimer, with a metalloprotease that can cleave the myostatin pro peptide, and with an agent that can increase or decrease proteolytic cleavage of the pro peptide by the metalloprotease, thereby modulating myostatin activation. The metalloprotease can be any metalloprotease that can cleave the myostatin pro peptide, particularly when the pro peptide comprises a latent myostatin complex, including, for example, a BMP-1/TLD family member such as BMP-1, TLD, tolloid-like protein-1 (TLL-1), or tolloid-like protein-2 (TLL-2), particularly mammalian BMP-1/TLD family members such as mammalian (m) TLD (mTLD), mTLL-1, and mTLL-2.

[0012] A method of the invention can be used to increase the level of myostatin activation (i.e., above a baseline level of myostatin activation in the absence of an agent), for example, by contacting a latent myostatin complex and metalloprotease with an agent that increases proteolytic cleavage of the pro peptide by the metalloprotease; or can be used to decrease the level of myostatin activation (below a baseline level), for example, by contacting a latent myostatin complex and metalloprotease with an agent that decreases

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proteolytic cleavage of the pro peptide by the metalloprotease. The method can be performed *in vitro*, using, for example, cells or a tissue in culture, a cell extract, or substantially purified reagents, including substantially purified metalloprotease and/or latent myostatin complex; or can be performed *in vivo*, for example, in a cell or tissue, either of which can be *in situ* in an organism or isolated from an organism (e.g., a cell *ex vivo*, which can be in culture). Thus, the method can be performed by contacting a sample comprising a latent myostatin complex and metalloprotease (e.g., a tissue sample and/or a biological fluid) with an agent *in vitro*, or the contacting can be performed *in vivo*, for example, by administering the agent to a subject.

[0013] Free myostatin pro peptide, latent myostatin complex, and a metalloprotease that can cleave a myostatin pro peptide can be present intracellularly or extracellularly. However, the pro peptide or latent myostatin complex generally is not present in the same cells or cell type as the metalloprotease and, therefore, cleavage of myostatin pro peptide by the metalloprotease generally occurs extracellularly upon contact of the metalloprotease with the pro peptide. As such, contacting of an agent with the pro peptide, complex, and/or metalloprotease will depend in part on how the agent acts to modulate the cleavage. For example, where the agent can bind to and alter the conformation of the metalloprotease so as to inhibit its cleavage activity with respect to a myostatin pro peptide, cells that produce the metalloprotease can be contacted with the agent such that the secreted metalloprotease lacks such activity, or the agent can be administered to a medium into which the metalloprotease is secreted (e.g., into the bloodstream of a living organism) such that, upon contact with the agent in the medium, the cleavage of the pro peptide by the metalloprotease is reduced or inhibited. In comparison, where the agent acts, for example, to destabilize an interaction of the metalloprotease and the pro peptide, or where the agent acts as a competitive or non-competitive inhibitor of the metalloprotease with respect to the pro peptide, the agent generally is contacted with the medium in which the metalloprotease and pro peptide are likely to interact (e.g., the blood).

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[0014] In one embodiment, the agent decreases proteolytic activity of a metalloprotease that cleaves myostatin pro peptide from a latent myostatin complex, thereby reducing or inhibiting myostatin activation below a level of myostatin activation that occurs or would occur in the absence of the agent. Where such an agent is administered to a subject, the agent can result in increased muscle mass or decreased fat content or both in the subject. The subject can be any subject in which myostatin is expressed, particularly a vertebrate organism, for example, animals that are raised as a food source, such as a mammalian species (e.g., an ovine, porcine species, or bovine species), avian species (e.g., chickens or turkeys), or a piscine species (e.g., salmon, trout, or cod). The subject also can be a human subject, for example, a subject suffering from a muscular disorder (e.g., a dystonia or dystrophy), a subject suffering from wasting disorder (e.g., cachexia), or a subject suffering from clinical obesity or other metabolic disorder such as type II diabetes. In another embodiment, the agent increases proteolytic activity of a metalloprotease that cleaves myostatin pro peptide from a latent myostatin complex, thereby increasing myostatin activation above a level, if any, of myostatin activation that occurs or would occur in the absence of the agent. Where such an agent is administered to a subject, the agent can result in decreased muscle mass or increased fat content or both in the subject.

[0015] The present invention also relates to a method of increasing muscle mass in a subject. Such a method can be performed, for example, by administering to the subject an agent that reduces or inhibits proteolytic cleavage of a myostatin pro peptide by a protease that cleaves myostatin pro peptide, thereby preventing activation of latent myostatin in the cell and increasing muscle mass in the subject. The metalloprotease can be any metalloprotease, particularly a BMP-1/TLD family member such as BMP-1, TLD, TLL-1, or TLL-2, including mTLD, mTLL-1 and mTLL-2. The subject in which muscle mass is to be increased generally is vertebrate, for example, a domesticated or farm animal, including a mammal such as an ovine species, a porcine species, or a bovine species; an avian species such as a chicken or a turkey; or a piscine species; or can be a human subject.

[0016] The present invention further relates to a method for ameliorating a metabolic disorder in a subject. Such a method can be performed, for example, by administering to

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the subject an agent that reduces or inhibits the proteolytic cleavage of a myostatin pro peptide by a protease that cleaves myostatin pro peptide, thereby preventing activation of latent myostatin in the cell and ameliorating the metabolic disorder. The metabolic disorder can be any such disorder associated with increased or undesirable myostatin activation or activity, including, for example, a muscle wasting disorder such as is associated with muscular dystrophy, cachexia (e.g., associated with a cancer or acquired immunodeficiency disease), or sarcopenia; or a metabolic disorder such as clinical obesity or type 2 diabetes. The subject in which the metabolic disorder is ameliorated can be any subject, and generally is a vertebrate subject, for example, a domesticated animal such as a cat or dog, or an animal raised as a source of food (e.g., cattle, sheep, pigs, or fish); or can be a human subject. Amelioration of the disorder can be identified using any assay generally used to monitor the particular metabolic disorder, for example, a glucose tolerance test for diabetes, or a serum leptin assay for body fat analysis.

[0017] The present invention also relates to a method of identifying an agent that modulates metalloprotease mediated myostatin pro peptide cleavage and activation of latent myostatin. Such a screening method can be performed, for example, by contacting a myostatin pro peptide, a metalloprotease that can cleave the myostatin pro peptide, and a test agent, under conditions sufficient for cleavage of the pro peptide by the metalloprotease; and detecting a change in the amount of cleavage of the pro peptide in the absence of the test agent as compared to the presence of the test agent, thereby identifying the test agent as an agent that modulates metalloprotease mediated activation of the latent myostatin. The myostatin pro peptide can be in an isolated form, or can be a component of a latent myostatin complex that further contains a myostatin C-terminal fragment or a myostatin C-terminal dimer.

[0018] Where a test agent is identified as having metalloprotease mediated myostatin modulating activity, a screening assay of the invention can further include a step of determining an amount by which the agent increases or decreases myostatin pro peptide cleavage or myostatin activation. For example, where an agent is identified that increases the proteolytic activity of the metalloprotease above a basal level in a cell, a method of the invention can further include determining an amount by which the agent increases

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myostatin activation above the basal level. As such, a method of the invention provides a means to obtain agents or panels of agents that variously modulate myostatin activation by a metalloprotease. Such a method further provides a means to determine amounts of a particular agent useful for providing a desired level of myostatin activity.

[0019] A difference in the amount of cleavage of the pro peptide due to contact with a test agent can be detected, for example, by detecting the pro peptide or a cleavage product of the pro peptide using a method such as electrophoresis, chromatography, or mass spectrometry, which can detect a myostatin pro peptide or cleavage product thereof based on its size, charge, or both; an immunological based assay such as an immunoblot analysis, an enzyme-linked immunosorption assay (ELISA), or the like, which utilizes an antibody specific for the intact pro peptide or the cleaved pro peptide, but not an antibody that binds both the intact and the cleaved pro peptide; or a fluorescence based assay, including, for example, a fluorescence resonance energy transfer (FRET) assay, wherein fluorescence of the intact pro peptide is quenched, and the quenching is relieved upon cleavage of the pro peptide. Depending on the relative amount of intact myostatin pro peptide, pro peptide cleavage product, or a combination thereof that is detected, a test agent can be identified as an agent that increases or decreases metalloprotease mediated myostatin pro peptide cleavage and activation of the latent myostatin.

[0020] A difference in the amount of cleavage of the pro peptide also can be detected by detecting a change in binding of myostatin to a myostatin receptor *in vitro* or expressed on a cell surface, or by detecting a change in a myostatin mediated signal transduction in a cell expressing a myostatin receptor. Where the assay is a cell based assay, the cell can be one that expresses an endogenous myostatin receptor, for example, L6 myocytes, or can be a cell expressing a transgene encoding the myostatin receptor, for example, a cell transfected with a polynucleotide encoding an activin receptor such as an activin type II receptor. Myostatin mediated signal transduction can be detected at any level in the signal transduction pathway, including from binding of myostatin to a cell surface receptor to expression of a gene that is regulated due to myostatin binding to a myostatin receptor, wherein, in a screening assay of the invention, the signal transduction is dependent on metalloprotease mediated cleavage of a myostatin pro peptide and activation of a latent

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myostatin complex. As such, myostatin mediated signal transduction can be detected by detecting myostatin binding to a myostatin receptor using a receptor binding assay, or by detecting expression of a myostatin regulated gene, including, for example, a reporter gene, which can comprise, for example, a TGF- β regulatory element operatively linked to a polynucleotide encoding a detectable polypeptide. Accordingly, the present invention provides agents that modulate metalloprotease mediated myostatin pro peptide cleavage and myostatin activation, wherein the agents are identified using a screening assay of the invention. The present methods also are useful for confirming that an agent modulates metalloprotease mediated myostatin pro peptide cleavage and myostatin activation, including, if desired, the specific activity of the agent.

[0021] The present invention also relates to an agent that modulates metalloprotease mediated activation of latent myostatin. The agent can be an agonist or an antagonist of metalloprotease mediated activation of latent myostatin, and can reduce or inhibit metalloprotease mediated activation of latent myostatin, or can increase metalloprotease mediated activation of latent myostatin. An agent that modulates metalloprotease mediated activation of latent myostatin can be any type of molecule, including, for example, a peptide agent, a polynucleotide agent, an antibody agent, or a small organic molecule agent.

[0022] An agent that modulates metalloprotease mediated activation of latent myostatin is exemplified herein by a peptide agent. A peptide agent can include, for example, a peptide portion of a myostatin polypeptide, or a derivative of such a peptide portion of myostatin. In one embodiment, a derivative of a peptide portion of myostatin is a peptide that corresponds to a myostatin pro peptide. In one aspect of this embodiment, the derivative is a pro peptide having a mutation of the metalloprotease cleavage site, for example, a substitution, deletion, or insertion of an amino acid at or in sufficient proximity to the cleavage site such that the metalloprotease has increased or decreased cleavage activity with respect to the peptide agent. In another aspect of this embodiment, the derivative of a peptide portion of myostatin is a peptide agent that reduces or inhibits metalloprotease mediated activation of latent myostatin. The agent that modulates metalloprotease mediated activation of latent myostatin can be operatively linked to a

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second molecule, which facilitates the action or activity of the agent, or increases or decreases the stability of the agent in a particular environment. For example, a peptide agent can be stabilized by operatively linking the peptide agent to a polypeptide such as an Fc domain of an antibody molecule, thereby increasing the half-life of the peptide agent *in vivo*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Figure 1 demonstrates that incubation of the myostatin complex (MSTN; C-terminal myostatin dimer and pro peptide) with mTLL-1 resulted in a dramatic increase in expression of a luciferase reporter gene (stippled bar; see Example 2), the expression of which is regulated in transfected rhabdomyosarcoma cells upon contact of the cells with active myostatin. Only background expression was observed in cells contacted with myostatin complex, alone (solid bar), or with mTLL-1, alone (hatched bar).

[0024] Figure 2 shows a standard curve generated using the luciferase reporter assay, wherein the transfected cells (see Figure 1, above) were contacted with the specified amounts of active purified C-terminal myostatin dimer (diamonds). Control luciferase activity (no myostatin) is shown by the circles.

[0025] Figures 3A to 3E show determination of cleavage of the myostatin pro peptide by BMP-1/TLD family of proteinases.

[0026] Figures 3A and 3B show detection of a pro peptide degradation product in CHO cell conditioned media. Conditioned media prepared from CHO cells expressing the pro peptide (Figure 3A) or wild type and mutant forms of pro peptide/Fc fusion proteins (Figure 3B) were analyzed by SDS-PAGE followed by western blot analysis using antibodies directed against either the myostatin pro peptide (Figure 3A) or IgG (Figure 3B). Note that mutation of D76 to A resulted in loss of the degradation product.

[0027] Figure 3C shows purification of wild type and mutant pro peptide/C-terminal dimer complexes. Protein complexes were analyzed by SDS-PAGE in the presence or absence of β -mercaptoethanol followed by western blot analysis, as indicated. Note that like the wild type pro peptide, the D76A mutant pro peptide purified in a complex with the

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C-terminal dimer. The pro peptide degradation product did not co-purify with and was thus not part of the complex. Bands denoted by the asterisk indicate misfolded myostatin species, which were evident under non-reducing conditions.

[0028] Figures 3D and 3E show cleavage of the pro peptide by BMP-1/TLD proteinases. Wild type and mutant complexes were incubated with purified proteinases and analyzed by SDS-PAGE followed by western blotting using antibodies directed against the pro peptide. Incubations were carried out with 1 µg latent complex and 250 ng proteinase for 16 hours at 37°C, except that in Figure 3D, the samples were incubated with an additional 250 ng BMP-1 for 4 more hours. In Figure 3E, lanes labeled “no enzyme” indicate samples incubated for 16 hours at 37°C in the absence of enzyme. Note that all enzymes were capable of generating the cleavage product and that the D76A mutant protein was completely resistant to cleavage.

[0029] Figures 4A to 4D show activation of latent myostatin activity by BMP-1/TLD proteinases. In Figures 4B to 4D, black bars represent wild type, and gray bars represent D76A mutant complexes. Note that although heat treatment activated both the wild type and mutant complexes (Figure 4B), each proteinase was capable of activating only the wild type complex (Figures 4C and 4D). * $p < 0.05$, ** $p < 0.01$.

[0030] Figure 4A shows activation of pGL3-(CAGA)₁₂-luciferase reporter gene activity by purified myostatin C-terminal dimer.

[0031] Figure 4B shows activation of the myostatin pro peptide/C-terminal dimer latent complex by heat treatment. Control (no myostatin (MSTN)) is indicated.

[0032] Figure 4C and 4D show activation of the myostatin pro peptide/C-terminal dimer latent complex by BMP-1/TLD proteinases. The samples used for the reporter assays in Figures 4C and 4D are the same samples shown in Figures 3D and 3E, respectively.

[0033] Figure 5 shows inhibition of reporter gene activity by wild type and mutant pro peptide/Fc fusion proteins *in vitro*. A204 cells transfected with the reporter construct

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were incubated with 10 ng/ml of purified myostatin C-terminal dimer and various concentrations of wild type (dark) or D76A mutant (light) pro peptide/Fc fusion protein. Note that the wild type and mutant proteins were equally effective in blocking myostatin activity.

DETAILED DESCRIPTION OF THE INVENTION

[0034] The present invention is based on the identification of proteases that cleave myostatin pro peptide, including when the pro peptide is present in a complex with a myostatin C-terminal dimer, thereby converting latent inactive myostatin complex to active myostatin. Proteases having such myostatin pro peptide cleaving activity are exemplified by the metalloprotease bone morphogenic protein-1/tolloid (BMP-1/TLD) family of proteins. As such, the proteases provide targets and reagents for identifying drugs that can increase or decrease the protease activity, or can increase or decrease myostatin pro peptide cleavage mediated by the proteases, and, therefore, increase or decrease myostatin activity.

[0035] Myostatin (growth differentiation factor-8; GDF-8) is expressed as a pre-proprotein, promyostatin, which includes a signal peptide (amino acid residues about 1 to 20), the myostatin pro peptide domain (amino acid residues about 20 to 262 or 263) and the myostatin C-terminal domain (amino acid residues about 267 or 268 to 375). Promyostatin polypeptides and encoding polynucleotides are highly conserved evolutionarily (see McPherron and Lee, *Proc. Natl. Acad. Sci., USA* 94:12457, 1997; GenBank Acc. Nos. AF019619, AF019620, AF019621, AF019622, AF019623, AF019624, AF019625, AF019626, and AF019627; U.S. Pat. No. 5,994,618, each of which is incorporated herein by reference). Promyostatin polynucleotides and encoded polypeptides are exemplified herein by human promyostatin (SEQ ID NOS:1 and 2; pro peptide is amino acid residues about 20 to 263), bovine promyostatin (SEQ ID NOS:3 and 4; pro peptide is amino acid residues about 20 to 262), chicken promyostatin (SEQ ID NOS:5 and 6; pro peptide is amino acid residues about 20 to 262), and zebrafish promyostatin (SEQ ID NOS:7 and 8; pro peptide is amino acid residues about 20 to 262).

[0036] Myostatin is activated by two proteolytic cleavage events - a first removing the signal sequence (approximately the first 20 N-terminal amino acid residues of promyostatin; see, for example, SEQ ID NO:2), and a second at a tetrabasic processing site (at about amino acid residues 263 to 266 of promyostatin) - resulting in the generation of a 26 kDa N-terminal pro peptide (approximately amino acid residues 20 to 262 or 263) and a 12.5 kDa C-terminal peptide (approximately amino acid residue 266 or 267 to the C-terminus); a dimer of the C-terminal peptide is biologically active. Upon secretion from cells, the myostatin C-terminal dimer is maintained in a latent, inactive state due to its remaining bound to the myostatin pro peptide (Lee and McPherron, *Proc. Natl. Acad. Sci., USA* 98:9306-9311, 2001). The latent myostatin complex that circulates in the blood of adult mice can be activated *in vitro* by treatment with acid (Zimmers et al., *Science* 296:1486-1488, 2002).

[0037] Mice in which the myostatin gene has been knocked out show increased muscle mass, and further exhibit a significant reduction in fat accumulation with increasing age as compared to wild type littermates (McPherron and Lee, *J. Clin. Invest.* 109:595-601, 2002). Conversely, over-expression of myostatin *in vivo* produces the signs and symptoms characteristic of the muscle wasting syndrome, cachexia (Zimmers et al., *supra*, 2002). The muscle wasting observed in mice having increased levels of circulating myostatin can be partially reversed by introducing myostatin binding agents such as the myostatin pro peptide and follistatin to the mice (Zimmers et al., *supra*, 2002). These results confirmed that the observed muscle wasting was due to increased myostatin, and indicate that methods for decreasing the level of active myostatin or otherwise reducing or inhibiting myostatin activity can be useful for ameliorating muscle wasting. In view of the highly conserved nature of myostatin among species as diverse as fish and humans, these results indicate that myostatin also can be involved in the cachexia associated with various disorders in humans, including, for example, cancer, acquired immunodeficiency syndrome (AIDS), and sepsis, as well as in neuromuscular disorders such as muscular dystrophy (see Gonzalez-Kadavid et al., *Proc. Natl. Acad. Med., USA* 95:14938-14943, 1998).

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[0038] Proper skeletal muscle function also is involved in maintaining normal glucose metabolism, and skeletal muscle resistance to insulin stimulated glucose uptake is the earliest manifestation of non-insulin dependent (type 2) diabetes (see McPherron and Lee, *supra*, 2002). In two mouse models of obesity and diabetes, loss of myostatin prevented an increase in adipose tissue mass with age and attenuated the obese and diabetic phenotype in the mouse models (McPherron and Lee, *supra*, 2002). As such, methods that modulate myostatin activity also can be useful for reducing body fat in an individual, and for treating disorders associated with abnormal muscle function or obesity, for example, type 2 diabetes.

[0039] As disclosed herein, the myostatin pro peptide, either in a free form or when part of a complex with the myostatin C-terminal dimer, can be cleaved by members of the BMP-1/TLD family of metalloproteases, and such cleavage releases the myostatin C-terminal dimer from the inhibitory effects of the pro peptide, thus generating active myostatin. As such, the BMP-1/TLD proteases provide a target for drugs that can modulate myostatin activity and, therefore, increase or decrease muscle mass or reduce or prevent obesity in an organism. Accordingly, the invention provides methods of identifying agents that modulate metalloprotease mediated myostatin pro peptide cleavage, and that modulate metalloprotease mediated activation of latent myostatin.

[0040] A screening method of the invention can be performed, for example, by contacting a myostatin pro peptide, a metalloprotease that can cleave the myostatin pro peptide, and a test agent, under conditions sufficient for cleavage of the pro peptide by the metalloprotease; and detecting a change in the amount of cleavage of the pro peptide in the absence of the test agent as compared to the presence of the test agent, thereby identifying the test agent as an agent that modulates metalloprotease mediated myostatin pro peptide cleavage. The myostatin pro peptide can be in an isolated form, or can be a component of a latent myostatin complex that further contains a myostatin C-terminal fragment or a myostatin C-terminal dimer.

[0041] A metalloprotease examined according to a screening assay of the invention can be any protease that cleaves a myostatin pro peptide, particularly a metalloprotease that

cleaves the pro peptide when it is in a latent myostatin complex with a C-terminal myostatin fragment or dimer thereof, such that active myostatin is generated from the latent myostatin complex. Such metalloproteases are exemplified by the BMP-1/TLD family of metalloproteases, which includes four mammalian proteins, BMP-1 (Wozney et al., *Science* 242:1528-1534, 1988), mammalian Tolloid (mTLD; Takahara et al., *J. Biol. Chem.* 269:32572-32578, 1994), mammalian Tolloid-like-1 (mTLL-1; Takahara et al., *Genomics* 34:157-165, 1996), and mammalian Tolloid-like-2 (mTLL-2; Scott et al., *Devel. Biol.* 213:283-300, 1999). The BMP-1/TLD family of metalloproteases, in turn, are members of a larger family of proteins, the astacin family, which includes proteases that are expressed in various vertebrate and invertebrate organisms, including, for example, *Xenopus* (Xolloid; UVS.2), fish (choriolysin H and L; zebrafish Tolloid), sea urchin (BP-10 and SpAN), and hydra (HMP-1; see, for example, Li et al., *Proc. Natl. Acad. Sci., USA* 93:5127-5130, 1996). As such, the screening assays of the invention can be practiced using any of various metalloproteases and, therefore, allow an identification of agents that can be useful, for example, for modulating myostatin activation in a variety of different organisms.

[0042] BMP-1 and mTLD are encoded by alternatively spliced mRNAs from a single gene (Takahara et al., *supra*, 1994), whereas mTLL-1 and mTLL-2 are encoded by distinct genes. The BMP-1/TLD family of proteases is known to have a role in regulating the activity of at least three classes of substrates. First, BMP-1, mTLD, and mTLL-1 are capable of processing procollagen precursors into the mature monomers required for assembly into the multimeric fibers that are normally present in the extracellular matrix (Kessler et al., *Science* 271:360-362, 1996; Li et al., *supra*, 1996). Second, BMP-1, mTLD, mTLL-1 and mTLL-2 each can process pro-lysyl oxidase into the mature, biologically active enzyme (Uzel et al., *J. Biol. Chem.* 276:22537-22543, 2001). Third, BMP-1 and mTLL-1 can cleave chordin (Scott et al., *supra*, 1999), which normally binds various members of the BMP subgroup of the TGF- β superfamily and maintains them in a latent state (Blader et al., *Science* 278:1937-1940, 1997; Marques et al., *Cell* 91:417-26, 1997; Piccolo et al., *Cell* 91:407-416, 1997). Cleavage of chordin by these metalloproteases releases the BMP from the inhibitory effect of chordin. As such, BMP-1

and TLL-1 are believed have a role in modulating the effects of the BMPs during a variety of morphogenic processes. As disclosed herein, BMP-1/TLD family members, including BMP-1, mTLD, mTLL-1 and mTLL-2 also can cleave the myostatin pro peptide, either in its free form or when bound to the myostatin C-terminal dimer (latent myostatin complex), wherein cleavage of the pro peptide results in activation of the myostatin C-terminal dimer (see Examples 1 and 2).

[0043] A test agent that can be examined according to a method of the invention can be any type of molecule, including, for example, a peptide, peptide derivative such as a peptide hydroxamate or a phosphinic peptide, peptoid, polynucleotide, or small organic molecule (see Example 3). Thus, the term "test agent" is used broadly herein to mean any compound that is being examined for agonist or antagonist activity with respect to metalloprotease mediated myostatin pro peptide cleavage or myostatin activation. Although the method generally is used as a screening assay to identify previously unknown molecules (test agents) that can act as agonist or antagonist agents, the method also can be used to confirm that an agent known to have a particular activity in fact has the activity, for example, in standardizing the activity of the agent; and can be used to screen derivatives or other modified forms or mimics of such known agents.

[0044] A screening method of the invention conveniently can be adapted to high throughput analysis and, therefore, can be used to screen combinatorial libraries of test agents, which can be a library of random test agents, biased test agents, or variegated test agents (see, for example, U.S. Pat. No. 5,571,698), in order to identify those agents that can modulate metalloprotease mediated cleavage of a myostatin pro peptide and, therefore, myostatin activity. Methods for preparing a combinatorial library of molecules that can be tested for a desired activity are well known in the art and include, for example, methods of making a phage display library of peptides, which can be constrained peptides (see, for example, U.S. Pat. No. 5,622,699; U.S. Pat. No. 5,206,347; Scott and Smith, *Science* 249:386-390, 1992; Markland et al., *Gene* 109:13-19, 1991); a peptide library (U.S. Pat. No. 5,264,563); a library of peptide derivative compounds such as a hydroxamate compound library, reverse hydroxamate

compound library, a carboxylate compound library, thiol compound library, a phosphinic peptide library, or phosphonate compound library (see, for example, Dive et al., *Biochem. Soc. Trans.* 28:455-460, 2000; Ye and Marshall, *Peptides: The Wave of the Future* (Lebl and Houghten, ed.; American Peptide Society, 2001)); a peptidomimetic library (Blondelle et al., *Trends Anal. Chem.* 14:83-92, 1995); a nucleic acid library (O'Connell et al., *Proc. Natl. Acad. Sci., USA* 93:5883-5887, 1996; Tuerk and Gold, *Science* 249:505-510, 1990; Gold et al., *Ann. Rev. Biochem.* 64:763-797, 1995); an oligosaccharide library (York et al., *Carb. Res.* 285:99-128, 1996; Liang et al., *Science* 274:1520-1522, 1996; Ding et al., *Adv. Expt. Med. Biol.* 376:261-269, 1995); a lipoprotein library (de Kruif et al., *FEBS Lett.* 399:232-236, 1996); a glycoprotein or glycolipid library (Karaoglu et al., *J. Cell Biol.* 130:567-577, 1995); or a chemical library containing, for example, drugs or other pharmaceutical agents (Gordon et al., *J. Med. Chem.* 37:1385-1401, 1994; Ecker and Crooke, *BioTechnology* 13:351-360, 1995).

[0045] Polynucleotides can be particularly useful as agents that can modulate metalloprotease mediated myostatin pro peptide cleavage or myostatin activation because nucleic acid molecules having binding specificity for cellular targets, including cellular polypeptides, exist naturally, and because synthetic molecules having such specificity can be readily prepared and identified (see, for example, U.S. Pat. No. 5,750,342).

The term "polynucleotide" is used broadly herein to mean a sequence of two or more deoxyribonucleotides or ribonucleotides that are linked together by a phosphodiester bond. As such, the term "polynucleotide" includes RNA and DNA, which can be a gene or a portion thereof, a cDNA, a synthetic polydeoxyribonucleic acid sequence, or the like, and can be single stranded or double stranded, as well as a DNA/RNA hybrid. A polynucleotide can be a naturally occurring nucleic acid molecule, which can be isolated from a cell, or a synthetic molecule, which can be prepared, for

example, by methods of chemical synthesis or by enzymatic methods such as by the polymerase chain reaction (PCR).

[0046] A polynucleotide agent (or test agent) can contain nucleoside or nucleotide analogs, or a backbone bond other than a phosphodiester bond. In general, the nucleotides comprising a polynucleotide are naturally occurring deoxyribonucleotides, such as adenine, cytosine, guanine or thymine linked to 2'-deoxyribose, or ribonucleotides such as adenine, cytosine, guanine or uracil linked to ribose. However, a polynucleotide also can contain nucleotide analogs, including non-naturally occurring synthetic nucleotides or modified naturally occurring nucleotides. Such nucleotide analogs are well known in the art and commercially available, as are polynucleotides containing such nucleotide analogs (Lin et al., *Nucl. Acids Res.* 22:5220-5234, 1994; Jellinek et al., *Biochemistry* 34:11363-11372, 1995; Pagratis et al., *Nature Biotechnol.* 15:68-73, 1997).

[0047] The covalent bond linking the nucleotides of a polynucleotide generally is a phosphodiester bond. However, the covalent bond also can be any of numerous other bonds, including a thiodiester bond, a phosphorothioate bond, a peptide-like bond or any other bond known to those in the art as useful for linking nucleotides to produce synthetic polynucleotides (see, for example, Tam et al., *Nucl. Acids Res.* 22:977-986, 1994; Ecker and Crooke, *BioTechnology* 13:351360, 1995).

The incorporation of non-naturally occurring nucleotide analogs or bonds linking the nucleotides or analogs can be particularly useful where the polynucleotide is to be exposed to an environment that can contain a nucleolytic activity, including, for example, a tissue culture medium or upon administration to a living subject, since the modified polynucleotides can be less susceptible to degradation.

[0048] A polynucleotide comprising naturally occurring nucleotides and phosphodiester bonds can be chemically synthesized or can be produced using recombinant DNA methods, using an appropriate polynucleotide as a template. In comparison, a polynucleotide comprising nucleotide analogs or covalent bonds other than phosphodiester bonds generally will be chemically synthesized, although an enzyme such

as T7 polymerase can incorporate certain types of nucleotide analogs into a polynucleotide and, therefore, can be used to produce such a polynucleotide recombinantly from an appropriate template (Jellinek et al., *supra*, 1995).

[0049] Similarly, peptides, as exemplified herein (see Examples 3 and 4) can be useful as agents for modulating metalloprotease mediated myostatin activation, or as test agents to screen for such activity. Peptide agents (or test peptides) can contain one or more D-amino acids and/or L-amino acids; and/or one or more amino acid analogs, for example, an amino acid that has been derivatized or otherwise modified at its reactive side chain. In addition, one or more peptide bonds in the peptide can be modified, and a reactive group at the amino terminus or the carboxy terminus or both can be modified. Peptides containing D-amino acids, or L-amino acid analogs, or the like, can have improved stability to a protease, an oxidizing agent or other reactive material the peptide may encounter in a biological environment, and, therefore, can be particularly useful in performing a method of modulating metalloprotease mediated myostatin activation as disclosed herein. As disclosed herein, the stability of a peptide agent (or test agent) also can be improved by generating (or linking) a fusion protein comprising the peptide and a second polypeptide (e.g., an Fc domain of an antibody) that increases the half-life of the peptide agent *in vivo* (see Example 4; see, also, U.S. Patent Application Publication No. US 2003/0104406 A1). Peptides also can be modified to have decreased stability in a biological environment, if desired, such that the period of time the peptide is active in the environment is reduced.

[0050] Test agents also can be antibodies that are raised against and specifically bind one or more epitopes of a metalloprotease that cleaves a myostatin pro peptide; or against an epitope of the pro peptide, which can be an isolated pro peptide or a pro peptide component of a latent myostatin complex; or a complex of the metalloprotease and pro peptide. As used herein, the term "antibody" is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies. The term "binds specifically" or "specific binding activity" or the like, when used in reference to an antibody, means that an interaction of the antibody and a particular epitope has a dissociation constant of at least about 1×10^{-6} M, generally at least about

1×10^{-7} M, usually at least about 1×10^{-8} M, and particularly at least about 1×10^{-9} M or 1×10^{-10} M or less. As such, Fab, F(ab')₂, Fd and Fv fragments of an antibody that retain specific binding activity are included within the definition of an antibody. In addition to specifically binding a particular epitope, an antibody agent modulates the protease cleavage activity of a metalloprotease for a myostatin pro peptide, including increasing or decreasing such activity.

[0051] The term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains (see Huse et al., *Science* 246:1275-1281, 1989).

These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known (Winter and Harris, *Immunol. Today* 14:243-246, 1993; Ward et al., *Nature* 341:544-546, 1989; Harlow and Lane, *Antibodies: A laboratory manual* (Cold Spring Harbor Laboratory Press, 1999); Hilyard et al., *Protein Engineering: A practical approach* (IRL Press 1992); Borrabeck, *Antibody Engineering*, 2d ed. (Oxford University Press 1995)).

[0052] A panel of test agent antibodies conveniently can be obtained by immunizing an animal using a peptide portion of a myostatin pro peptide or of a metalloprotease, particularly a BMP-1/TLD family member. Where such a peptide portion of the pro peptide or metalloprotease is non-immunogenic, it can be made immunogenic by coupling the hapten to a carrier molecule such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), or by expressing the peptide portion as a fusion protein. Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art (see, for example, by Harlow and Lane, *supra*, 1999). Methods for raising polyclonal antibodies, for example, in a rabbit, goat, mouse or other mammal, are well known in the art (see, for example, Green et al., "Production of Polyclonal

Antisera," in *Immunochemical Protocols* (Manson, ed., Humana Press 1992), pages 1-5; Coligan et al., "Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters," in *Curr. Protocols Immunol.* (1992), section 2.4.1).

In addition, monoclonal antibodies can be obtained using methods that are well known and routine in the art (see, for example, Kohler and Milstein, *Nature* 256:495, 1975; see, also, Harlow and Lane, *supra*, 1999).

For example, spleen cells from a mouse immunized with a myostatin receptor, or an epitopic fragment thereof, can be fused to an appropriate myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using labeled antigen to identify clones that secrete monoclonal antibodies having the appropriate specificity, and hybridomas expressing antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of the antibodies. A recombinant phage that expresses, for example, a single chain antibody that modulates metalloprotease mediated cleavage of myostatin pro peptide also provides an antibody that can be used for preparing standardized kits.

[0053] Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well established techniques, including, for example, affinity chromatography with Protein-A SEPHAROSETM gel, size exclusion chromatography, and ion exchange chromatography (Coligan et al., *supra*, 1992, see sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; see, also, Barnes et al., "Purification of Immunoglobulin G (IgG)," in *Meth. Molec. Biol.* 10:79-104 (Humana Press 1992)).

Methods of *in vitro* and *in vivo* multiplication of monoclonal antibodies are well known in the art. Multiplication *in vitro* can be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally replenished by a mammalian serum such as fetal calf serum or trace elements and growth sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication

in vivo can be carried out by injecting cell clones into mammals histocompatible with the parent cells, for example, syngeneic mice, to cause growth of antibody producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

[0054] Therapeutic applications for antibody agents identified according to a screening assay of the invention also are provided. Where the therapeutic procedure is for treating a human subject, the antibodies can be derived from a subhuman primate antibody (see, for example, Goldenberg et al., *Intl. Publ. WO 91/11465*, 1991; and Losman et al., *Intl. J. Cancer* 46:310, 1990). A

therapeutically useful antibody for human treatment also can be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are known (see, for example, Orlandi et al., *Proc. Natl. Acad. Sci., USA* 86:3833, 1989).

Techniques for producing humanized monoclonal antibodies also are known (see, for example, Jones et al., *Nature* 321:522, 1986; Riechmann et al., *Nature* 332:323, 1988; Verhoeven et al., *Science* 239:1534, 1988; Carter et al., *Proc. Natl. Acad. Sci., USA* 89:4285, 1992; Sandhu, *Crit. Rev. Biotechnol.* 12:437, 1992; and Singer et al., *J. Immunol.* 150:2844, 1993).

Alternatively, the antibodies can be derived from human antibody fragments isolated from a combinatorial immunoglobulin library (see, for example, Barbas et al., *METHODS: A Companion to Methods in Immunology* 2:119, 1991; Winter et al., *Ann. Rev. Immunol.* 12:433, 1994).

[0055] The antibodies also can be derived from human monoclonal antibodies, which, for example, can be obtained from transgenic mice that have been genetically modified to

produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are well known (see, for example, by Green et al., *Nature Genet.* 7:13, 1994; Lonberg et al., *Nature* 368:856, 1994; and Taylor et al., *Intl. Immunol.* 6:579, 1994), and commercial sources of human antibodies are available (Abgenix, Inc.; Fremont CA).

[0056] Antigen binding fragments of an antibody can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment, F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly (see, for example, Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647; Nisonhoff et al., *Arch. Biochem. Biophys.* 89:230, 1960; Porter, *Biochem. J.* 73:119, 1959; Edelman et al., *Meth. Enzymol.* 1:422 (Academic Press 1967) ; see, also, Coligan et al., *supra*, 1992, see sections 2.8.1-2.8.10 and 2.10.1-2.10.4).

[0057] Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light/heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques can also be used, provided the fragments specifically bind to the antigen that is recognized by the intact antibody. For example, Fv fragments comprise an association of V_H and V_L chains; this association can be

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noncovalent (Inbar et al., *Proc. Natl. Acad. Sci., USA* 69:2659, 1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (Sandhu, *supra*, 1992). Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow et al., *METHODS: A Companion to Methods in Enzymology* 2:97, 1991; Bird et al., *Science* 242:423-426, 1988; Ladner et al., U.S. Pat. No. 4,946,778; Pack et al., *BioTechnology* 11:1271-1277, 1993; each of which is incorporated herein by reference; see, also Sandhu, *supra*, 1992. Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (see, for example, Larrick et al., *METHODS: A Companion to Methods in Enzymology* 2:106, 1991, which is incorporated herein by reference).

[0058] A difference in the amount of cleavage of the pro peptide due to contact with a test agent can be detected, for example, by detecting the pro peptide and/or a cleavage product of the pro peptide using a method such as electrophoresis, chromatography, or mass spectrometry (see, for example, Thies et al., *Growth Factors* 18:251-259, 2001, which is incorporated herein by reference), which can detect a myostatin pro peptide or cleavage product thereof based on its size, charge, or both; an immunological based assay such as an immunoblot analysis, an enzyme-linked immunosorption assay (ELISA), or the like, which utilizes an antibody specific for the intact pro peptide or the cleaved pro peptide, but not both; or a fluorescence based assay, including, for example, a fluorescence resonance energy transfer (FRET) assay, wherein fluorescence of the intact pro peptide is quenched, and the quenching is relieved upon cleavage of the pro peptide.

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Where an increased amount of a cleavage product of the pro peptide is detected in the presence of (or following contact with) the test agent as compared to an amount of cleavage product in the absence of the test agent, the test agent is identified as an agent that can increase metalloprotease mediated activation of the latent myostatin. Similarly, where a decreased amount of the pro peptide is detected in the presence of (or following contact with) the test agent as compared to an amount of pro peptide in the absence of the test agent, the test agent is identified as an agent that can increase metalloprotease mediated activation of the latent myostatin. Conversely, where a decreased amount of a cleavage product of the pro peptide is detected in the presence of (or following contact with) the test agent as compared to an amount of cleavage product in the absence of the test agent, the test agent is identified as an agent that can decrease metalloprotease mediated activation of the latent myostatin. Where a greater amount of the pro peptide is detected in the presence of (or following contact with) the test agent as compared to an amount of pro peptide in the absence of the test agent, the test agent is identified as an agent that can decrease metalloprotease mediated activation of the latent myostatin. Such activity can be confirmed using a cell based or animal assay by detecting, for example, a change in myostatin mediated signal transduction activity due to the agent.

[0059] A difference in the amount of cleavage of the pro peptide also can be detected by detecting a change in binding of myostatin to a myostatin receptor, or by detecting a change in a myostatin mediated signal transduction in a cell expressing a myostatin receptor. Cells useful for performing a screening assay of the invention include, for example, cells from mammals, birds, fish, yeast, or *Drosophila*. Such functional assays can directly indicate that a test agent modulates metalloprotease mediated myostatin activation. A cell useful for such a method can be one that expresses an endogenous myostatin receptor, for example, L6 myocytes, or can be a cell genetically modified, transiently or stably, to express a transgene encoding the myostatin receptor, for example, an activin receptor such as an activin type II receptor (Thies et al., *supra*, 2001). Myostatin mediated signal transduction can be detected at any level in the signal transduction pathway, including from binding of myostatin to a cell surface receptor to expression of a gene that is regulated by myostatin, which, in a screening assay of the

invention, is dependent on metalloprotease mediated cleavage of a myostatin pro peptide and myostatin activation.

[0060] Metalloprotease mediated myostatin activation and consequent myostatin mediated signal transduction can be detected by measuring myostatin binding to a myostatin receptor using a receptor binding assay, which can be an *in vitro* assay or cell based assay. Metalloprotease mediated myostatin activation and consequent myostatin mediated signal transduction also can be detected by measuring expression of a myostatin regulated gene, which can be a reporter gene comprising, for example, a TGF- β regulatory element operatively linked to a polynucleotide encoding a detectable label. Expression of the reporter gene can be detected, for example, by detecting an RNA transcript of the reporter gene sequence, or by detecting a polypeptide encoded by the reporter gene or an activity of the encoded polypeptide. A polypeptide reporter can be, for example, β -lactamase, chloramphenicol acetyltransferase, adenosine deaminase, aminoglycoside phosphotransferase, dihydrofolate reductase, hygromycin-B phosphotransferase, thymidine kinase, β -galactosidase, luciferase, or xanthine guanine phosphoribosyltransferase, and can be detected, for example, by detecting radioactivity, luminescence, chemiluminescence, fluorescence, enzymatic activity, or specific binding due to the reporter polypeptide, or survival in a selective medium of cells expressing the reporter polypeptide. Methods for introducing a transgene such as a polynucleotide encoding a myostatin receptor or a reporter gene under conditions such that a polypeptide encoded by the transgene can be expressed are disclosed herein or otherwise known in the art.

[0061] Generally, a reporter gene includes a coding sequence, which encodes the reporter polynucleotide or polypeptide, operatively linked to one or more transcription and, as appropriate, translation regulatory elements, and can be contained in a vector, particularly an expression vector. If desired, the coding sequence can further encode an operatively linked peptide tag such as a His-6 tag, which can be detected using a divalent cation such as nickel ion, cobalt ion, or the like; a FLAG epitope, which can be detected using an anti-FLAG antibody (see, for example, Hopp et al., *BioTechnology* 6:1204, 1988,; U.S. Pat. No. 5,011,912); a c-myc

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epitope, which can be detected using an antibody specific for the epitope; biotin, which can be detected using streptavidin or avidin; glutathione S-transferase, which can be detected using glutathione; or an Fc domain of an antibody, which can be detected using Protein A or an anti-Fc antibody, either of which, can, but need not, be detectably labeled or attached to a solid support or, in turn, detected using a second antibody. As such, it will be recognized that various means for detecting a particular tagged molecule also can be used to isolate the tagged molecule.

[0062] As used herein, the term "operatively linked" means that two or more molecules are positioned with respect to each other such that they act as a single unit and effect a function attributable to one or both molecules or a combination thereof. For example, a polynucleotide sequence encoding a reporter polypeptide can be operatively linked to a regulatory element, in which case the regulatory element confers its regulatory effect on the polynucleotide similarly to the way in which the regulatory element would effect a polynucleotide sequence with which it normally is associated with in a cell. A first polynucleotide coding sequence also can be operatively linked to a second (or more) coding sequence such that a chimeric polypeptide can be expressed from the operatively linked coding sequences. The chimeric polypeptide can be a fusion polypeptide, in which the two (or more) encoded peptides are translated into a single polypeptide (see, e.g., Example 4), i.e., are covalently bound through a peptide bond; or can be translated as two discrete peptides that, upon translation, can associate with each other to form a stable complex.

[0063] A polynucleotide such as a reporter gene can be contained in a vector, which can facilitate manipulation of the polynucleotide, including introduction of the polynucleotide into a target cell. The vector can be a cloning vector, which is useful for maintaining the polynucleotide, or can be an expression vector, which contains, in addition to the polynucleotide, regulatory elements useful for expressing the polynucleotide and, where the polynucleotide encodes a polypeptide, for expressing the encoded peptide in a particular cell. An expression vector can contain the expression elements necessary to achieve, for example, sustained transcription of the encoding polynucleotide, or the

regulatory elements can be operatively linked to the polynucleotide prior to its being cloned into the vector.

[0064] An expression vector (or the polynucleotide) generally contains or encodes a promoter sequence, which can provide constitutive or, if desired, inducible, tissue specific, or developmental stage specific expression of the encoding polynucleotide, a poly-A recognition sequence, and a ribosome recognition site or internal ribosome entry site, or other regulatory elements such as an enhancer, which can be tissue specific. The vector also can contain elements required for replication in a prokaryotic or eukaryotic host system or both, as desired. Such vectors, which include plasmid vectors and viral vectors such as bacteriophage, baculovirus, retrovirus, lentivirus, adenovirus, vaccinia virus, semliki forest virus and adeno-associated virus vectors, are well known and can be purchased from a commercial source (Promega, Madison WI; Stratagene, La Jolla CA; GIBCO/BRL, Gaithersburg MD) or can be constructed by one skilled in the art (see, for example, *Meth. Enzymol.* Vol. 185, Goeddel, ed. (Academic Press, Inc., 1990); Jolly, *Canc. Gene Ther.* 1:51-64, 1994; Flotte, *J. Bioenerg. Biomemb.* 25:37-42, 1993; Kirshenbaum et al., *J. Clin. Invest.* 92:381-387, 1993).

[0065] A polynucleotide encoding a reporter polypeptide can be operatively linked, for example, to a tissue specific regulatory element, for example, a muscle cell specific regulatory element, wherein expression of the reporter polypeptide is restricted to the muscle cells in an individual, or to muscle cells in a mixed population of cells in culture, for example, an organ culture. Muscle cell specific regulatory elements include, for example, the muscle creatine kinase promoter (Sternberg et al., *Mol. Cell. Biol.* 8:2896-2909, 1988) and the myosin light chain enhancer/promoter (Donoghue et al., *Proc. Natl. Acad. Sci., USA* 88:5847-5851, 1991).

[0066] Viral expression vectors can be particularly useful for introducing a polynucleotide into a cell, including, if desired, into a cell in a subject. Viral vectors provide the advantage that they can infect host cells with relatively high efficiency and can

infect specific cell types. Viral vectors have been developed for use in particular host systems, particularly mammalian systems and include, for example, retroviral vectors, other lentivirus vectors such as those based on the human immunodeficiency virus (HIV), adenovirus vectors, adeno-associated virus vectors, herpesvirus vectors, vaccinia virus vectors, and the like (see Miller and Rosman, *BioTechniques* 7:980-990, 1992; Anderson et al., *Nature* 392:25-30 Suppl., 1998; Verma and Somia, *Nature* 389:239-242, 1997; Wilson, *New Engl. J. Med.* 334:1185-1187, 1996).

[0067] A polynucleotide such as a reporter gene or a polynucleotide agent, which can be contained in a vector, can be introduced into a cell by any of a variety of methods (Sambrook et al., *Molecular Cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press 1989); Ausubel et al., *Current Protocols in Molecular Biology* (John Wiley and Sons, Baltimore, MD 1987, and supplements through 1995)).

Such methods include, for example, transfection, lipofection, microinjection, biolistic methods, electroporation and, with viral vectors, infection; and can include the use of liposomes, microemulsions or the like, which can facilitate introduction of the polynucleotide into the cell and can protect the polynucleotide from degradation prior to its introduction into the cell. The selection of a particular method will depend, for example, on the cell into which the polynucleotide is to be introduced, as well as whether the cell is isolated in culture, or is in a tissue or organ in culture or *in situ*.

[0068] Where a test agent is identified as having myostatin modulating activity, the screening assay can further include a step of determining an amount by which the agent increases or decreases myostatin activation. For example, where an agent is identified that increases the proteolytic activity of the metalloprotease for the myostatin pro peptide above a baseline level of activity in a particular system, for example, in an *in vitro* assay using purified reagents or *in vivo* in a subject, the method can further include determining an amount by which the agent increases myostatin activation above the basal level. As such, different agents or panels of agents can be obtained that increase or decrease myostatin activation by a metalloprotease in a relatively defined amount. Such a method

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further provides a means to determine amounts of a particular agent useful for providing a desired level of myostatin activity. As such, the present invention provides agents and panels of agents that modulate metalloprotease mediated myostatin activation, such agents being useful as medicaments to modulate myostatin activation in a subject, for example, in a subject having a metabolic disorder such as muscular dystrophy, muscle wasting, obesity, or type 2 diabetes.

[0069] Accordingly, the invention provides methods of modulating metalloprotease mediated myostatin activation. As used herein, the term "modulate," when used in reference to an effect on metalloprotease mediated cleavage of myostatin pro peptide or metalloprotease mediated myostatin activation, means that the amount of pro peptide cleavage or myostatin activation either is increased or is reduced or inhibited. The terms "increase" and "reduce or inhibit" are used in reference to the effect of an agent on a baseline level of metalloprotease mediated myostatin pro peptide cleavage or myostatin activation. The baseline level of activity can be a level of cleavage or activation that is identified as occurring in an *in vitro* assay using purified pro peptide and metalloprotease under defined conditions, or using a biological sample such as a cell or tissue extract obtained from a subject, which can, but need not, be a normal healthy individual; or a level of cleavage or activation that occurs *in vivo* in a subject. The terms "reduce or inhibit" are used together herein because it is recognized that, in some cases, the level of metalloprotease mediated myostatin pro peptide cleavage or myostatin activation can be reduced below a level that can be detected by a particular assay. As such, it may not be determinable using such an assay as to whether, for example, a low level of myostatin pro peptide cleavage remains, or whether such cleavage is completely inhibited.

[0070] A method of modulating metalloprotease mediated myostatin pro peptide cleavage or myostatin activation can be performed, for example, by contacting a latent myostatin complex, which includes a myostatin pro peptide and a myostatin C-terminal fragment, particularly a C-terminal fragment dimer, with a metalloprotease that can cleave the myostatin pro peptide, and with an agent that can increase or decrease proteolytic cleavage of the pro peptide mediated by the metalloprotease. The metalloprotease can be any metalloprotease that can cleave the myostatin pro peptide, particularly when the

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pro peptide comprises a latent myostatin complex, including, for example, a BMP-1/TLD family member such as BMP-1, mTLD, mTLL-1, or mTLL-2. The agent can act in any way to modulate metalloprotease mediated cleavage of the myostatin pro peptide, including, for example, by increasing or decreasing the proteolytic activity of the metalloprotease, by competing with the pro peptide for the metalloprotease, by facilitating contact of the metalloprotease and a latent myostatin complex comprising the pro peptide, or by inducing a conformational change in the latent myostatin complex such that it is a less fit (or more fit) substrate for the metalloprotease.

[0071] A method of modulating metalloprotease mediated myostatin activation can be practiced with respect to any subject that expresses myostatin, including vertebrates and invertebrates. For example, the subject can be a human, mouse, cow, pig, sheep, goat, dog, cat, chicken, turkey, zebrafish, salmon, finfish, other aquatic organisms and other species. Examples of aquatic organisms include those belonging to the class *Piscina*, such as trout, char, ayu, carp, crucian carp, goldfish, roach, whitebait, eel, conger eel, sardine, flying fish, sea bass, sea bream, parrot bass, snapper, mackerel, horse mackerel, tuna, bonito, yellowtail, rockfish, fluke, sole, flounder, blowfish, filefish; those belonging to the class *Cephalopoda*, such as squid, cuttlefish, octopus; those belonging to the class *Pelecypoda*, such as clams (e.g., hardshell, Manila, Quahog, Surf, Soft-shell); cockles, mussels, periwinkles; scallops (e.g., sea, bay, calloo); conch, snails, sea cucumbers; ark shell; oysters (e.g., *C. virginica*, Gulf, New Zealand, Pacific); those belonging to the class *Gastropoda* such as turban shell, abalone (e.g. green, pink, red); and those belonging to the class *Crustacea* such as lobster, including but not limited to Spiny, Rock, and American; prawn; shrimp, including but not limited to *M. rosenbergii*, *P. styllrolls*, *P. indicus*, *P. jeponious*, *P. monodon*, *P. vannemel*, *M. ensis*, *S. melantho*, *N. norvegious*, cold water shrimp; crab, including, but not limited to, Blue, rook, stone, king, queen, snow, brown, dungeness, Jonah, Mangrove, soft-shelled; squilla, krill, langostinos; crayfish/crawfish, including, but not limited, to Blue, Marron, Red Claw, Red Swamp, Soft-shelled, white; *Annelida*; *Chordata*, including, but not limited to, reptiles such as alligators and turtles; *Amphibia*, including frogs; and *Echinodermata*, including, but not limited to, sea urchins.

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[0072] A method of modulating metalloprotease mediated myostatin activity can be performed *in vitro* or *ex vivo* using cells or a tissue in culture, a cell or tissue extract, a biological fluid such as a serum or plasma sample, or substantially purified reagents, including substantially purified metalloprotease and/or latent myostatin complex (see, for example, Thies et al., *supra*, 2001). Where the method is performed *in vitro*, the agent can be contacted with sample comprising the metalloprotease and latent myostatin complex by adding the agent to the sample, which generally is in a culture medium or other buffered solution. For example, where the method is performed using cells in culture, the agent can be added to the culture medium such that it contacts the metalloprotease and/or pro peptide, one or both of which can be present in cells in the culture or secreted into the medium. The agent can be selected such that it is soluble in the sample medium, or can be formulated to increase solubility, if desired.

[0073] A method of modulating myostatin activation also can be performed *in vivo*, including in a living subject, including with respect to cells or a tissue *in situ* in a subject. In general, such a method is performed by administering the agent to the subject and, therefore, the agent generally is formulated in a composition suitable for administration to the subject. As such, compositions containing an agent that can modulate metalloprotease mediated myostatin activation are provided, such compositions including the agent in a pharmaceutically acceptable carrier. Such compositions are useful as medicaments for treating a subject suffering from a muscular and/or metabolic disorder as disclosed herein, and are useful for administration to animals such as farm animals used for labor or as food products.

[0074] A composition for administration to a living subject generally includes the agent in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters. A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the agent. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as

ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the physico-chemical characteristics of the agent to be administered, and on the route of administration of the composition, which can be, for example, orally or parenterally such as intravenously, and by injection, intubation, or other such method known in the art. The composition also can contain one or more additional reagent, including, for example, nutrients or vitamins or, where the composition is administered for a therapeutic purpose, a diagnostic reagent or therapeutic agent relevant to the disorder being treated.

[0075] The agent can be incorporated within an encapsulating material such as into an oil-in-water emulsion, a microemulsion, micelle, mixed micelle, liposome, microsphere or other polymer matrix (see, for example, Gregoriadis, *Liposome Technology* Vol. 1 (CRC Press, Boca Raton, FL 1984); Fraley, et al., *Trends Biochem. Sci.* 6:77 (1981)).

Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer. "Stealth" liposomes (see, for example, U.S. Pat. Nos. 5,882,679; 5,395,619; and 5,225,212)

are an example of such encapsulating materials particularly useful for preparing a composition useful for practicing a method of the invention, and other "masked" liposomes similarly can be used, such liposomes extending the time that the therapeutic agent remains in the circulation. Cationic liposomes, for example, also can be modified with specific receptors or ligands (Morishita et al., *J. Clin. Invest.* 91:2580-2585 (1993)).

[0076] The route of administration of a pharmaceutical composition containing an agent that modulates metalloprotease mediated myostatin activation will depend, in part, on the chemical structure of the molecule. Polypeptides and polynucleotides, for example, are not particularly useful when administered orally because they can be degraded in the digestive tract. However, methods for chemically modifying polypeptides, for example, to render them less susceptible to degradation by endogenous proteases or more absorbable

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through the alimentary tract are well known (see, for example, Blondelle et al., *supra*, 1995; Ecker and Crook, *supra*, 1995). In addition, a peptide agent can be prepared using D-amino acids, or can contain one or more domains based on peptidomimetics, which are organic molecules that mimic the structures of peptide domains; or based on a peptoid such as a vinylogous peptoid.

[0077] A composition as disclosed herein can be administered to a subject by various routes including, for example, orally or parenterally, such as intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly, intraperitoneally, intrarectally, intracisternally or by passive or facilitated absorption through the skin using, for example, a skin patch or transdermal iontophoresis, respectively. Furthermore, the composition can be administered by injection, intubation, orally or topically, the latter of which can be passive, for example, by direct application of an ointment, or active, for example, using a nasal spray or inhalant, in which case one component of the composition is an appropriate propellant.

[0078] The pharmaceutical composition can be formulated as an oral formulation, such as a tablet, or a solution or suspension form; or can comprise an admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications, and can be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, or other form suitable for use. The carriers, in addition to those disclosed above, can include glucose, lactose, mannose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening or coloring agents and perfumes can be used, for example a stabilizing dry agent such as triulose (see, for example, U.S. Pat. No. 5,314,695).

[0079] The total amount of an agent to be administered in practicing a method of the invention can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment

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protocol, in which multiple doses are administered over a prolonged period of time. It will be recognized that the amount of the pharmaceutical composition, for example, to treat obesity in a subject depends on many factors including the age and general health of the subject as well as the route of administration and the number of treatments to be administered. In view of these factors, the skilled artisan would adjust the particular dose as necessary. In general, the formulation of the composition and the routes and frequency of administration are determined, initially, using Phase I and Phase II clinical trials.

[0080] A method of the invention can be used to increase the level of myostatin activation (i.e., above a baseline level of myostatin activation in the absence of an agent), for example, by contacting a latent myostatin complex and/or metalloprotease with an agent that increases proteolytic activity of the metalloprotease; or can be used to decrease the level of myostatin activation (below a baseline level), for example, by contacting a latent myostatin complex and/or metalloprotease with an agent that decreases metalloprotease mediated proteolytic activity of myostatin pro peptide. The agent can be one that decreases proteolytic activity of a metalloprotease that cleaves myostatin pro peptide of a latent myostatin complex, thereby reducing or inhibiting myostatin activation below a level of myostatin activation that occurs or would occur in the absence of the agent. Where such an agent is administered to a subject, the agent can result in increased muscle mass or decreased fat content or both in the subject. For example, the subject can be a human subject suffering from a muscle wasting disorder, wherein increased muscle mass can ameliorate the signs and symptoms of the disorder.

Alternatively, the agent can be one that increases metalloprotease mediated proteolytic cleavage of myostatin pro peptide from a latent myostatin complex, thereby increasing myostatin activation above a level, if any, of myostatin activation that occurs or would occur in the absence of the agent. Where such an agent is administered to a subject, the agent can result in decreased muscle mass or increased fat content or both in the subject. Such a subject can be, for example, an undesirable organism such as an invasive fish species or rodents, wherein decreased muscle mass and/or increased fat content places the invasive species at a competitive disadvantage in the environment.

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[0081] Accordingly, in one embodiment, the invention provides a method of increasing muscle mass or reducing the fat content or both of a subject by modulating proteolytic cleavage of a myostatin pro peptide by a metalloprotease such as a BMP-1/TLD family metalloprotease. Such a method can be performed, for example, by administering to the subject an agent that reduces or inhibits the proteolytic activity of a protease that cleaves myostatin pro peptide, thereby preventing activation of latent myostatin and increasing muscle mass in the subject. The subject in which muscle mass is to be increased can be any subject in which myostatin is expressed, particularly a vertebrate organism, including domesticated animals (e.g., a feline or canine species), farm animals or animals that are raised as a food source, including mammalian species (e.g., an ovine, porcine, or bovine species), avian species (e.g., chickens or turkeys), and piscine species (e.g., salmon, trout, or cod). For example, where such a method is performed on an organism that is useful as a food source, the protein content of the food can be increased, the cholesterol level can be decreased, and the quality of the foodstuff can be improved. Thus, a method of the invention can be performed on any eukaryotic organism that expresses myostatin and relies on metalloprotease mediated cleavage of myostatin pro peptide to activate myostatin, including a vertebrate organism, for example, mammalian, avian or piscine organism, or an invertebrate organism, for example, a mollusk, echinoderm, gastropod or cephalopod. In one embodiment, the subject is a human subject, for example, a subject suffering from a metabolic disorder such as a muscular disorder (e.g., a dystonia or dystrophy), a wasting disorder (e.g., cachexia), clinical obesity, or type 2 diabetes.

[0082] As such, the invention also provides a method for ameliorating a metabolic disorder in a subject by administering an agent that modulates metalloprotease mediated myostatin activation in the subject. As used herein, the term "ameliorate," when used in reference to a metabolic disorder, means that signs or symptoms associated with the disorder are lessened. Amelioration of the disorder can be identified using any assay generally used by the skilled clinician to monitor the particular metabolic disorder, for example, a glucose tolerance test for monitoring diabetes, or a serum leptin assay for body fat analysis (McPherron and Lee, *supra*, 2002). Amelioration of a metabolic disorder such as obesity or cachexia can be monitored simply by measuring the subject's body weight.

[0083] Heterozygous myostatin knock-out mice have increased skeletal muscle mass, although to a lesser extent than that observed in homozygous mutant mice, indicating that myostatin acts in a dose-dependent manner *in vivo*. Furthermore, overexpression of myostatin in animals has the opposite effect with respect to muscle growth. For example, nude mice carrying myostatin-expressing tumors developed a wasting syndrome characterized by a dramatic loss of muscle and fat weight, and resembling cachexia as occurs in patients with chronic diseases such as cancer or AIDS. In addition, the serum levels of myostatin immunoreactive material have been correlated with the status of patients with respect to muscle wasting (Gonzalez-Kadavid et al., *supra*, 1998). Thus, patients with AIDS, who also showed signs of cachexia as measured by loss of total body weight, had slightly increased serum levels of myostatin immunoreactive material compared to either normal males without AIDS or to AIDS patients that did not have weight loss. Myostatin not only affects muscle mass, but also affects the overall metabolism of an organism. For example, myostatin is expressed in adipose tissue, and myostatin deficient mice have a dramatic reduction in fat accumulation as the animals age. The overall anabolic effect on muscle tissue that results in response to decreased myostatin activity can alter the overall metabolism of the organism and affect the storage of energy in the form of fat, as demonstrated by the introduction of a myostatin mutation into an obese mouse strain (agouti lethal yellow (A^y) mice), which suppressed fat accumulation by five-fold. Abnormal glucose metabolism also was partially suppressed in agouti mice containing the myostatin mutation.

[0084] As such, the agents and methods of the present invention, which reduce or inhibit metalloprotease mediated myostatin activation, can be used to treat or prevent metabolic diseases such as obesity and type 2 diabetes. The methods of the invention are useful, for example, for ameliorating various metabolic disorders, including, for example, the cachexia associated with chronic diseases such as cancer (see Norton et al., *Crit. Rev. Oncol. Hematol.* 7:289-327, 1987), as well as conditions such as type 2 diabetes, obesity, and other metabolic disorders. As used herein, the term "metabolic disorder" refers to a condition that is characterized, at least in part, by an abnormal amount, development or metabolic activity of muscle and/or adipose tissue.

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Such metabolic disorders include, for example, obesity; muscle wasting disorders such as muscular dystrophy, neuromuscular diseases, cachexia, and anorexia; and disorders such as type 2 diabetes, which generally, but not necessarily, is associated with obesity. The term "abnormal," when used in reference to the amount, development or metabolic activity of muscle and/or adipose tissue, is used in a relative sense in comparison to an amount, development or metabolic activity that a skilled clinician or other relevant artisan would recognize as being normal or ideal. Such normal or ideal values are known to the clinician and are based on average values generally observed or desired in a healthy individual in a corresponding population. For example, the clinician would know that obesity is associated with a body weight that is about twenty percent above an "ideal" weight range for a person of a particular height and body type. However, the clinician would recognize that a body builder is not necessarily obese simply by virtue of having a body weight that is twenty percent or more above the weight expected for a person of the same height and body type in an otherwise corresponding population. Similarly, the artisan would know that a patient presenting with what appears to abnormally decreased muscle activity could be identified as having abnormal muscle development, for example, by subjecting the patient to various strength tests and comparing the results with those expected for an average healthy individual in a corresponding population.

[0085] A method for ameliorating a metabolic disorder in a subject can be performed, for example, by administering to the subject an agent that reduces or inhibits the proteolytic activity of a protease that cleaves myostatin pro peptide, thereby preventing activation of latent myostatin in the cell and ameliorating the metabolic disorder. As indicated above, the metabolic disorder can be any disorder associated with increased or undesirably high myostatin activation or activity, including, for example, a muscle wasting disorder such as is associated with muscular dystrophy, cachexia (e.g., associated with a cancer or acquired immunodeficiency disease), or sarcopenia; or a metabolic disorder such as clinical obesity or type 2 diabetes. By way of example, sarcopenia is a metabolic disorder that is characterized by a loss of skeletal muscle mass, quality, and strength, and can lead to frailty in the elderly. Examples of skeletal muscle properties that contribute to its overall quality include contractility, fiber size and type, and glucose uptake and

metabolism. Sarcopenia has important consequences because the loss of lean body mass reduces function, and because a loss of approximately 40% of lean body mass generally is fatal (see, for example, Roubenoff and Castaneda, *J. Amer. Med. Assn.* 286, 2001). A method of the invention provides a means to ameliorate sarcopenia by reducing or inhibiting metalloprotease mediated myostatin activation, thereby allowing increased muscle growth and development in the subject.

[0086] The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1

BMP-1/TLD METALLOPROTEASE FAMILY MEMBERS

CLEAVE MYOSTATIN PRO PEPTIDE

[0087] This example demonstrates that the members of the bone morphogenic protein-1/Tolloid (BMP-1/TLD) family of metalloproteases cleave the myostatin pro peptide.

[0088] Five hundred ng of purified myostatin pro peptide or of purified latent myostatin complex comprising the pro peptide and C-terminal dimer (Lee and McPherron, *supra*, 2001) was incubated overnight at 37°C with 100 ng purified BMP-1, mTLD, mTLL-1, or mTLL-2 (Scott et al., *Devel. Biol.* 213:283-300, 1999).

Reaction products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis using antiserum raised against the myostatin pro peptide (Lee and McPherron, *supra*, 2001).

[0089] A discrete proteolytic cleavage product of the pro peptide was detected in each reaction containing one of the four proteases, but not in control reactions that did not contain a protease. Moreover, each of the proteases cleaved the pro peptide whether it was in a purified form or in a complex with the myostatin C-terminal dimer. These results demonstrate that the BMP-1/TLD metalloproteases cleave the myostatin pro peptide.

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EXAMPLE 2**METALLOPROTEASE CLEAVAGE OF MYOSTATIN PRO PEPTIDE
ACTIVATES LATENT MYOSTATIN**

[0090] This example demonstrates that cleavage of the myostatin pro peptide by a BMP-1/TLD metalloprotease activates latent myostatin.

[0091] Purified myostatin pro peptide and C-terminal dimer complex was incubated with mTLL-1, then examined using a reporter gene assay that specifically detects myostatin activity. A204 rhabdomyosarcoma cells were transfected with the pGL3-(CAGA)₁₂ luciferase reporter gene construct, which comprises the luciferase coding sequence linked to the TGF- β responsive CAGA sequence from the promoter of the TGF- β inducible PAI-1 gene (Thies et al., *supra*, 2001). The transfected cells were contacted with either untreated pro peptide/C-terminal dimer complex or complex that had been pre-incubated with mTLL-1. Incubation of the complex with mTLL-1 dramatically increased the amount of luciferase activity detected in the reporter cell assay, whereas no change was observed in cells treated with mTLL-1 alone or with the myostatin complex alone (Figure 1).

[0092] In order to determine the extent of myostatin activation by mTLL-1, a standard curve was generated using purified myostatin C-terminal dimer in the reporter gene assay (Figure 2), then the amount of luciferase activity in cells treated with the mTLL-1 treated complex was compared to the standard curve. A comparison of the amount of myostatin activity present in the mTLL-1-treated sample and the degree of proteolytic processing of the pro peptide by mTLL-1 in this sample revealed that at least about 50% of the proteolytically-cleaved myostatin complex was active in the reporter assay. These results demonstrate that cleavage of the myostatin pro peptide in a complex of the pro peptide and myostatin C-terminal dimer by the BMP-1/TLD metalloprotease, mTLL-1, activates myostatin.

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EXAMPLE 3**Peptide Substrates for Tolloid Family Members**

[0093] A series of three peptides each of 10, 20, 30, 40, or 50 amino acid residues was synthesized based on the sequence of the myostatin pro peptide, and encompassing the BMP-1/TLD metalloprotease cleavage site (amino acid residues "RD" as shown in bold, below, in wild type peptides; SEQ ID NOS:9, 12, 15, 18, and 21). Peptides in which the arginine residue at the P1 position just upstream of the cleavage site was changed to a glutamine residue (SEQ ID NOS:10, 13, 16, 19, and 22; see bold), and peptides in which the aspartic acid at the P1' position just downstream of the cleavage site was changed to an alanine (SEQ ID NOS:11, 14, 17, 20, and 23; see bold), also were synthesized. The sequences of the peptides are shown below:

[0094] 50-mer

[0095] KDVIRQLLPKAPPLRELIDQYDVQ**R**DDSSDGSLEDDDDYHATTETIITMPT
(SEQ ID NO:9)

[0096] KDVIRQLLPKAPPLRELIDQYDVQ**Q**DDSSDGSLEDDDDYHATTETIITMPT
(SEQ ID NO:10)

[0097] KDVIRQLLPKAPPLRELIDQYDVQ**R**ADSSDGSLEDDDDYHATTETIITMPT
(SEQ ID NO:11)

[0098] 40-mer:

[0099] QLLPKAPPLRELIDQYDVQ**R**DDSSDGSLEDDDDYHATTETI (SEQ ID NO:12);

[0100] QLLPKAPPLRELIDQYDVQ**Q**DDSSDGSLEDDDDYHATTETI (SEQ ID NO:13); and

[0101] QLLPKAPPLRELIDQYDVQ**R**ADSSDGSLEDDDDYHATTETI (SEQ ID NO:14).

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[0102] 30-mer:

[0103] APPLRELIDQYDVQRDDSSDGSLEDDDDYHA (SEQ ID NO:15);

[0104] APPLRELIDQYDVQQDDSSDGSLEDDDDYHA (SEQ ID NO:16); and

[0105] APPLRELIDQYDVQRADSSDGSLEDDDDYHA (SEQ ID NO:17).

[0106] 20-mer:

[0107] ELIDQYDVQRDDSSDGSLED (SEQ ID NO:18);

[0108] ELIDQYDVQQDDSSDGSLED (SEQ ID NO:19); and

[0109] ELIDQYDVQRADSSDGSLED (SEQ ID NO:20).

[0110] 10-mer:

[0111] YDVQRDDSSD (SEQ ID NO:21);

[0112] YDVQQDDSSD (SEQ ID NO:22); and

[0113] YDVQRADSSD (SEQ ID NO:23).

[0114] Peptides were supplied as lyophilized powders and stock solutions of 1.0 mM were prepared in 60% acetonitrile – 0.1%trifluoroacetic acid (TFA) and 40% water. Activity of the enzymes on the peptide substrates was assessed by combining 70 µl of water, 20 µl of either mock conditioned medium or conditioned medium containing the protein of interest, and 10 µl of synthetic peptide. Samples were incubated overnight at either room temperature or 37°C, then reactions were quenched by reducing the pH through the addition of 1.0 µl of 0.1% TFA. Each aliquot was applied to a 2 cm C18 guard column cartridge (Supelco) and peptides were eluted using an acetonitrile gradient (0 – 40% over 20 minutes) in 0.1% TFA. Peaks corresponding to cleaved peptide fragments were identified and confirmed using mass spectrometry. The 40-mer, 30-mer, and 20-mer wild type and R->Q mutant peptides were cleaved by conditioned media containing TLL-2, whereas the peptides containing the D->A mutation at the P1' position

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were not cleaved; the 50-mer was insoluble under the conditions used, and the cleavage products of the 10-mer were difficult to detect due to their small size (i.e., 5-mers).

EXAMPLE 4

ACTIVATION OF LATENT MYOSTATIN BY BMP-1/TOLLOID FAMILY METALLOPROTEASES

[0115] This example demonstrates that BMP-1/TLL family members can cleave and activate latent myostatin.

[0116] **Myostatin purification and analysis.** The generation of CHO cell lines overexpressing myostatin was described previously^{5, 6} (numbered references listed at end of Example 4). Similar strategies were used to generate CHO lines expressing mutant forms of full-length human myostatin and pro peptide/Fc fusion proteins (see U.S. Publ. No. US 2003/0104406 A1). Mutant human full-length myostatin sequences were based on SEQ ID NO:2, and the mutant pro peptide sequences were based on amino acid residues 24 to 266 of SEQ ID NO:2. Myostatin pro peptide/C-terminal dimer complexes were purified from the conditioned medium of CHO expressing cells as described⁵. Pro peptide/Fc fusion proteins were purified using a Protein A-SEPHAROSE gel column. Antibodies directed against bacterially-produced myostatin C-terminal domain and pro peptide were as described^{1, 5}.

[0117] **Proteinase and reporter gene assays.** Purified BMP-1, mTLD, mTLL-1, and mTLL-2 proteinases were prepared as described¹⁵. Myostatin activity was measured using the pGL3-(CAGA)₁₂-luciferase reporter assay in A204 rhabdomyosarcoma cells as described⁶. A standard curve using purified myostatin C-terminal dimer was generated for each set of assays in order to quantify myostatin activity.

[0118] **Injection of mice.** Female BALB/c mice (Charles River) weighing 17g to 19g were injected intraperitoneally on days 1, 4, 8, 15, and 22 either with PBS alone or with various proteins diluted in PBS; doses of proteins administered were as follows: pro peptide/Fc fusion proteins - 1 mg/kg or 10 mg/kg; IgG2am (control antibody) - 10 mg/kg; and JA16 (myostatin neutralizing antibody) - 60 mg/kg. Mice were sacrificed

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on day 29 for muscle analysis. Muscles from both sides of each animal were dissected and weighed; the average weight was used for each muscle.

[0119] The generation of Chinese hamster ovary (CHO) cells overexpressing myostatin has been described^{5, 6}. Like other TGF- β family members, myostatin produced by CHO cells is cleaved at a dibasic site to generate an N-terminal pro peptide and a disulfide-linked dimer of C-terminal fragments. In the course of characterizing the secretion of myostatin by these cells, the presence was noted of a discrete cleavage product of the pro peptide (as detected by western blot analysis using antibodies specific for the pro peptide). This cleavage product was detected in the conditioned medium of CHO cells transfected with expression constructs containing either the full-length myostatin precursor protein (not shown) or the myostatin pro peptide alone in the absence of the C-terminal domain (Figure 3A). Because the myostatin pro peptide can maintain the C-terminal dimer in a latent state both *in vitro*^{5, 6} and *in vivo*^{7, 8}, and because proteolytic cleavage of the TGF- β pro peptide is believed to be one mechanism for activating latent TGF- β ¹⁰⁻¹⁴, a role for cleavage of the myostatin pro peptide in regulating myostatin latency was investigated.

[0120] N-terminal sequencing revealed that the pro peptide degradation product detected in CHO cell conditioned medium resulted from proteolytic cleavage between arginine 75 and aspartate 76. In order to determine whether either of these amino acid residues is essential for proteolytic cleavage, CHO cell lines expressing mutant versions of the pro peptide, in which either the arginine or aspartate residue was changed to glutamine or alanine, respectively, were generated. To enhance stability of these proteins for *in vivo* studies (see below), the mutant pro peptides were fused with an Fc domain. Although changing the arginine to glutamine had no effect on proteolytic cleavage, no degradation product could be detected in conditioned medium prepared from CHO cells expressing the aspartate to alanine mutant pro peptide/Fc fusion protein (Figure 3B; see, also, Example 3). The requirement for aspartate at the cleavage site suggested that members of the BMP-1/TLD family of metalloproteinases were responsible for generating this degradation product. A number of substrates have been identified for mammalian members of the BMP-1/TLD family, and in nearly every case, proteolytic cleavage has

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been shown to occur immediately N-terminal to an aspartate residue^{15,16}. Furthermore, mutagenesis studies have documented the importance of the aspartate residue in rendering these sites susceptible to proteolytic cleavage¹⁷. As there were no apparent reports of other proteinases with a similar specificity or requirement for an aspartate residue just C-terminal to the scissile bond in protein substrates, the ability of members of the BMP-1/TLD family to cleave the myostatin pro peptide *in vitro* was investigated.

[0121] Myostatin was purified from the conditioned medium of overproducing CHO cells⁵. After successive fractionation on hydroxyapatite, lentil lectin SEPHAROSE gel, DEAE agarose, and heparin SEPHAROSE gel, a purified preparation of the myostatin latent complex was obtained that consisted of the N-terminal pro peptide bound non-covalently to the C-terminal dimer (Figure 3C). As shown in Figure 3D, incubation of the purified latent complex with purified BMP-1 resulted in complete cleavage of the pro peptide to generate a single product with an electrophoretic mobility identical to that detected in conditioned medium prepared from CHO cells engineered to overproduce myostatin. N-terminal sequencing of BMP-1-treated pro peptide confirmed that cleavage occurred immediately N-terminal to aspartate 76.

[0122] The ability of the other mammalian members of the BMP-1/TLD family, including mTLD, mTLL-1, and mTLL-2, to cleave the pro peptide also was tested. For these experiments, enzyme concentrations were used that resulted in only partial cleavage, thus allowing a comparison of the relative activities of the four enzymes. As shown in Figure 3E, incubation of the latent complex with each of the four proteinases resulted in cleavage of the pro peptide. Three of the proteinases, BMP-1, mTLL-1, and mTLL-2, were approximately equally effective in cleaving the pro peptide, while mTLD was consistently less active than the other three, even though the same mTLD preparation was fully active against known substrates such as procollagen. All four of these proteinases also cleaved pro peptide that had been purified away from the C-terminal dimer.

[0123] In order to determine the effect of proteolytic cleavage of the pro peptide on myostatin latency, myostatin biological activity was measured in latent complexes treated with each of the four proteinases. For this purpose, a reporter gene assay was used in

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which A204 rhabdomyosarcoma cells were transfected with the pGL3-(CAGA)₁₂-luciferase construct and incubated with myostatin⁶. As described previously, the addition of purified myostatin C-terminal dimer to these cells resulted in an increase in luciferase activity above basal levels (Figure 4A). In contrast, purified myostatin latent complex was inactive in this assay, but could be activated by incubation at 80°C for 5 minutes (Figure 4B). As shown in Figure 4C, the latent complex was also activated by pretreatment with BMP-1. Based on quantification of myostatin activity relative to a standard curve, cleavage of the pro peptide by BMP-1 was approximately as effective as heat treatment in activating the latent complex. The latent complex was also activated by pretreatment with the other proteinases, and the extent of activation correlated roughly with the extent of proteolytic cleavage by these enzymes (Figure 4D).

[0124] The requirement for aspartate at the cleavage site also was examined. A CHO cell line expressing high levels of a mutant form of myostatin, in which aspartate 76 was changed to alanine, was generated and the latent complex was purified from the conditioned medium of these cells. As shown in Figure 3C, the mutation had no effect on the ability of the pro peptide to bind to the C-terminal dimer; the mutant pro peptide and C-terminal dimer remained tightly associated throughout the purification. Moreover, the mutant pro peptide maintained the complex in a latent form that could be activated by heating, as assessed by the luciferase reporter assay (Figure 4B). However, the mutant pro peptide in the latent complex was completely resistant to proteolysis by each of the four proteinases, BMP-1, mTLD, mTLL-1, and mTLL-2 (Figures 3D and E), and was resistant to activation by these proteinases (Figures 4C and 4D).

[0125] Finally, the role of proteolytic cleavage of the pro peptide *in vivo* was investigated by examining the effect of injecting wild type and mutant versions of the pro peptide into mice. As determined in previous experiments, the half-life of wild type pro peptide after intraperitoneal injections into mice could be increased from approximately 2 hours to 5 to 7 days by fusing the pro peptide to an Fc domain. For this reason, CHO cell lines expressing wild type or mutant (aspartate 76 to alanine) pro peptide fused to an Fc domain were generated, and the fusion proteins were purified using a Protein A SEPHAROSE gel column. The aspartate to alanine mutation did not affect the

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activity of the pro peptide *in vitro*, as the purified wild type and mutant pro peptide/Fc fusion proteins were equally effective in inhibiting the activity of the purified myostatin C-terminal dimer in the reporter gene assay (Figure 5).

[0126] In order to assess the activities of these proteins *in vivo*, adult mice were given weekly injections of purified wild type or mutant pro peptide/Fc fusion proteins and sacrificed after four weeks for muscle analysis. For comparison, a set of mice also was injected with the JA16 myostatin neutralizing monoclonal antibody, which causes an approximately 25-30% increase in muscle mass after 12 weeks of treatment¹⁸. As shown in Table 1 (below), injection of wild type pro peptide/Fc fusion protein had no effect on muscle mass at doses of 1 and 10 mg/kg/week. Similarly, little or no effect was seen following injection of the aspartate to alanine mutant pro peptide/Fc fusion protein at a dose of 1 mg/kg/week. However, injection of the mutant pro peptide/Fc fusion protein at 10 mg/kg/week led to a statistically significant ($p < 0.0001$) increase of 18-27% in the weight of each skeletal muscle examined. This magnitude of increase in muscle weights observed at the higher dose of the mutant pro peptide/Fc fusion protein was approximately twice that seen following injection of the JA16 myostatin neutralizing monoclonal antibody, which resulted in muscle weight increases of 10-16%.

[0127] These results demonstrate that members of the BMP-1/TLD family of metalloproteinases cleave myostatin pro peptide bound to the C-terminal dimer and activate the latent complex. Furthermore, a mutant form of the pro peptide that was resistant to cleavage by BMP-1/TLD proteinases caused increases in muscle mass when injected into adult mice, presumably by forming latent complexes incapable of being activated by this group of proteinases. This general mechanism for regulating the activity of the C-terminal dimer has been described for certain other TGF- β family members. In the case of TGF- β , proteolytic cleavage of its associated pro peptide by plasmin^{10, 11} or by matrix metalloproteinases¹²⁻¹⁴ is believed to be one mechanism for activating latency *in vivo*. In the case of the BMPs, members of the BMP-1/TLD family appear to play an important role in regulating the activity of the C-terminal dimer by cleaving and inactivating the BMP antagonist chordin^{15,19-22}.

[0128] All four mammalian proteinases in the BMP-1/TLD family can cleave the myostatin pro peptide *in vitro*, and one or more can be involved in regulating myostatin activity *in vivo*. In this regard, mTLL-2, unlike the other three proteinases, is expressed specifically in skeletal muscle during embryonic development¹⁵. The identification of the specific proteinase or proteinases involved in regulating myostatin latency will provide targets for identifying agents useful for modulating muscle mass, and will allow targeting of these enzymes for the development of novel muscle enhancing agents for both human therapeutic and agricultural applications.

Table 1

	pectoralis	triceps	quadriceps	gastrocnemius	tibialis
PBS (n=10)	82.8 ± 2.8	85.5 ± 1.6	142.0 ± 2.6	95.5 ± 1.5	32.6 ± 0.8
IgG2am (10 mg/kg, n=10)	87.7 ± 1.9	87.8 ± 1.6	148.4 ± 2.3	98.7 ± 2.1	33.8 ± 0.9
wild type (1 mg/kg, n=10)	84.3 ± 1.6	85.3 ± 1.8	145.7 ± 2.2	96.0 ± 1.4	33.2 ± 0.4
D76A (1 mg/kg, n=9)	89.4 ± 3.5	90.0 ± 2.0	150.7 ± 2.9 ^a	97.7 ± 2.2	34.1 ± 0.6
wild type (10 mg/kg, n=10)	87.5 ± 3.4	88.5 ± 2.7	147.1 ± 4.0	98.2 ± 2.2	33.3 ± 0.8
D76A (10 mg/kg, n=10)	105.1 ± 1.2 ^{b, c}	102.1 ± 1.2 ^{b, d}	175.6 ± 1.2 ^{b, e}	112.6 ± 1.1 ^{b, d}	40.3 ± 1.2 ^{b, d}
JA16 (60 mg/kg, n=10)	96.0 ± 1.2 ^f	94.8 ± 1.1 ^f	160.3 ± 1.1 ^b	104.9 ± 1.1 ^f	37.5 ± 1.1 ^f

^a*p* < 0.05 (vs. PBS)

^b*p* < 0.0001 (vs. PBS)

^c*p* < 0.05 (vs. JA16)

^d*p* < 0.01 (vs. JA16)

^e*p* < 0.001 (vs. JA16)

^f*p* < 0.001 (vs. PBS)

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[0129] The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

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SEQUENCE LISTING

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WISCONSIN ALUMNI RESEARCH FOUNDATION
WYETH

<120> METALLOPROTEASE ACTIVATION OF MYOSTATIN, AND METHODS OF MODULATING MYOSTATIN ACTIVITY

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Met	Gln	Val	Asp	Gly	Lys	Pro	Lys	Cys	Cys	Phe	Phe	Lys	Phe	Ser	Ser
	130					135					140				
Lys	Ile	Gln	Tyr	Asn	Lys	Val	Val	Lys	Ala	Gln	Leu	Trp	Ile	Tyr	Leu
145					150					155					160
Arg	Pro	Val	Glu	Thr	Pro	Thr	Thr	Val	Phe	Val	Gln	Ile	Leu	Arg	Leu
				165					170					175	
Ile	Lys	Pro	Met	Lys	Asp	Gly	Thr	Arg	Tyr	Thr	Gly	Ile	Arg	Ser	Leu
			180					185					190		
Lys	Leu	Asp	Met	Asn	Pro	Gly	Thr	Gly	Ile	Trp	Gln	Ser	Ile	Asp	Val
		195					200					205			
Lys	Thr	Val	Leu	Gln	Asn	Trp	Leu	Lys	Gln	Pro	Glu	Ser	Asn	Leu	Gly
	210					215					220				
Ile	Glu	Ile	Lys	Ala	Leu	Asp	Glu	Asn	Gly	His	Asp	Leu	Ala	Val	Thr
225					230					235					240
Phe	Pro	Gly	Pro	Gly	Glu	Asp	Gly	Leu	Asn	Pro	Phe	Leu	Glu	Val	Lys
				245					250					255	
Val	Thr	Asp	Thr	Pro	Lys	Arg	Ser	Arg	Arg	Asp	Phe	Gly	Leu	Asp	Cys
			260					265					270		
Asp	Glu	His	Ser	Thr	Glu	Ser	Arg	Cys	Cys	Arg	Tyr	Pro	Leu	Thr	Val
		275					280					285			
Asp	Phe	Glu	Ala	Phe	Gly	Trp	Asp	Trp	Ile	Ile	Ala	Pro	Lys	Arg	Tyr
	290					295					300				
Lys	Ala	Asn	Tyr	Cys	Ser	Gly	Glu	Cys	Glu	Phe	Val	Phe	Leu	Gln	Lys
305					310					315					320

agg cct gtc aag act cct gcg aca gtg ttt gtg caa atc ctg aqa ctc 528

Arg	Pro	Val	Lys	Thr 165	Pro	Ala	Thr	Val	51/6 Phe 170	Val	Gln	Ile	Leu	Arg 175	Leu	
atc	aaa	ccc	atg	aaa	gac	ggt	aca	agg	tat	act	gga	atc	cga	tct	ctg	576
Ile	Lys	Pro	Met 180	Lys	Asp	Gly	Thr	Arg 185	Tyr	Thr	Gly	Ile	Arg 190	Ser	Leu	
aaa	ctt	gac	atg	aac	cca	ggc	act	ggg	att	tgg	cag	agc	att	gat	gtg	624
Lys	Leu	Asp 195	Met	Asn	Pro	Gly	Thr 200	Gly	Ile	Trp	Gln	Ser 205	Ile	Asp	Val	
aag	aca	gtg	ttg	cag	aac	tgg	ctc	aaa	caa	cct	gaa	tcc	aac	tta	ggc	672
Lys	Thr 210	Val	Leu	Gln	Asn	Trp 215	Leu	Lys	Gln	Pro	Glu 220	Ser	Asn	Leu	Gly	
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Ile 225	Glu	Ile	Lys	Ala 230	Leu	Asp	Glu	Asn	Gly	His 235	Asp	Leu	Ala	Val	Thr 240	
ttc	cca	gaa	cca	gga	gaa	gat	gga	ctg	act	ccc	ttt	tta	gaa	gtc	aag	768
Phe	Pro	Glu	Pro	Gly 245	Glu	Asp	Gly	Leu	Thr 250	Pro	Phe	Leu	Glu	Val 255	Lys	
gta	aca	gac	aca	cca	aaa	aga	tct	agg	aga	gat	ttt	ggg	ctt	gat	tgt	816
Val	Thr	Asp	Thr 260	Pro	Lys	Arg	Ser	Arg 265	Arg	Asp	Phe	Gly	Leu 270	Asp	Cys	
gat	gaa	cac	tcc	aca	gaa	tct	cga	tgc	tgt	cgt	tac	cct	cta	act	gtg	864
Asp	Glu	His 275	Ser	Thr	Glu	Ser	Arg 280	Cys	Cys	Arg	Tyr	Pro 285	Leu	Thr	Val	
gat	ttt	gaa	gct	ttt	gga	tgg	gat	tgg	att	att	gca	cct	aaa	aga	tat	912
Asp	Phe 290	Glu	Ala	Phe	Gly	Trp 295	Asp	Trp	Ile	Ile	Ala 300	Pro	Lys	Arg	Tyr	
aag	gcc	aat	tac	tgc	tct	gga	gaa	tgt	gaa	ttt	gta	ttt	ttg	caa	aag	960
Lys 305	Ala	Asn	Tyr	Cys	Ser 310	Gly	Glu	Cys	Glu	Phe 315	Val	Phe	Leu	Gln	Lys 320	
tat	cct	cat	acc	cat	ctt	gtg	cac	caa	gca	aac	ccc	aga	ggg	tca	gcc	1008
Tyr	Pro	His	Thr	His 325	Leu	Val	His	Gln	Ala 330	Asn	Pro	Arg	Gly	Ser 335	Ala	
ggc	ccc	tgc	tgt	act	cct	aca	aag	atg	tct	cca	att	aat	atg	cta	tat	1056
Gly	Pro	Cys	Cys 340	Thr	Pro	Thr	Lys	Met 345	Ser	Pro	Ile	Asn	Met 350	Leu	Tyr	
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Phe	Asn	Gly 355	Glu	Gly	Gln	Ile	Ile 360	Tyr	Gly	Lys	Ile	Pro 365	Ala	Met	Val	
gta	gat	cgc	tgt	ggg	tgt	tca	tga									1128
Val	Asp 370	Arg	Cys	Gly	Cys	Ser 375										

<210> 4
 <211> 375
 <212> PRT
 <213> Bovine

<400> 4

Met	Gln	Lys	Leu	Gln	Ile	Ser	Val	Tyr	Ile	Tyr	Leu	Phe	Met	Leu	Ile	
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			20					25					30			

51/7

Val Glu Lys Glu Gly Leu Cys Asn Ala Cys Leu Trp Arg Glu Asn Thr
 35 40 45
 Thr Ser Ser Arg Leu Glu Ala Ile Lys Ile Gln Ile Leu Ser Lys Leu
 50 55 60
 Arg Leu Glu Thr Ala Pro Asn Ile Ser Lys Asp Ala Ile Arg Gln Leu
 65 70 75 80
 Leu Pro Lys Ala Pro Pro Leu Leu Glu Leu Ile Asp Gln Phe Asp Val
 85 90 95
 Gln Arg Asp Ala Ser Ser Asp Gly Ser Leu Glu Asp Asp Asp Tyr His
 100 105 110
 Ala Arg Thr Glu Thr Val Ile Thr Met Pro Thr Glu Ser Asp Leu Leu
 115 120 125
 Thr Gln Val Glu Gly Lys Pro Lys Cys Cys Phe Phe Lys Phe Ser Ser
 130 135 140
 Lys Ile Gln Tyr Asn Lys Leu Val Lys Ala Gln Leu Trp Ile Tyr Leu
 145 150 155 160
 Arg Pro Val Lys Thr Pro Ala Thr Val Phe Val Gln Ile Leu Arg Leu
 165 170 175
 Ile Lys Pro Met Lys Asp Gly Thr Arg Tyr Thr Gly Ile Arg Ser Leu
 180 185 190
 Lys Leu Asp Met Asn Pro Gly Thr Gly Ile Trp Gln Ser Ile Asp Val
 195 200 205
 Lys Thr Val Leu Gln Asn Trp Leu Lys Gln Pro Glu Ser Asn Leu Gly
 210 215 220
 Ile Glu Ile Lys Ala Leu Asp Glu Asn Gly His Asp Leu Ala Val Thr
 225 230 235 240
 Phe Pro Glu Pro Gly Glu Asp Gly Leu Thr Pro Phe Leu Glu Val Lys
 245 250 255
 Val Thr Asp Thr Pro Lys Arg Ser Arg Arg Asp Phe Gly Leu Asp Cys
 260 265 270
 Asp Glu His Ser Thr Glu Ser Arg Cys Cys Arg Tyr Pro Leu Thr Val
 275 280 285
 Asp Phe Glu Ala Phe Gly Trp Asp Trp Ile Ile Ala Pro Lys Arg Tyr
 290 295 300

51/8

Lys Ala Asn Tyr Cys Ser Gly Glu Cys Glu Phe Val Phe Leu Gln Lys
305 310 315 320

Tyr Pro His Thr His Leu Val His Gln Ala Asn Pro Arg Gly Ser Ala
325 330 335

Gly Pro Cys Cys Thr Pro Thr Lys Met Ser Pro Ile Asn Met Leu Tyr
340 345 350

Phe Asn Gly Glu Gly Gln Ile Ile Tyr Gly Lys Ile Pro Ala Met Val
355 360 365

Val Asp Arg Cys Gly Cys Ser
370 375

<210> 5
<211> 1128
<212> DNA
<213> Gallus gallus

<220>
<221> CDS
<222> (1)..(1125)

<400> 5

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gcg gtt gat ccg gtg gct ctg gat ggc agt agt cag ccc aca gag aac Ala Val Asp Pro Val Ala Leu Asp Gly Ser Ser Gln Pro Thr Glu Asn 20 25 30	96
gct gaa aaa gac gga ctg tgc aat gct tgt acg tgg aga cag aat aca Ala Glu Lys Asp Gly Leu Cys Asn Ala Cys Thr Trp Arg Gln Asn Thr 35 40 45	144
aaa tcc tcc aga ata gaa gcc ata aaa att caa atc ctc agc aaa ctg Lys Ser Ser Arg Ile Glu Ala Ile Lys Ile Gln Ile Leu Ser Lys Leu 50 55 60	192
cgc ctg gaa caa gca cct aac att agc agg gac gtt att aag cag ctt Arg Leu Glu Gln Ala Pro Asn Ile Ser Arg Asp Val Ile Lys Gln Leu 65 70 75 80	240
tta ccc aaa gct cct cca ctg cag gaa ctg att gat cag tat gat gtc Leu Pro Lys Ala Pro Pro Leu Gln Glu Leu Ile Asp Gln Tyr Asp Val 85 90 95	288
cag agg gac gac agt agc gat ggc tct ttg gaa gac gat gac tat cat Gln Arg Asp Asp Ser Ser Asp Gly Ser Leu Glu Asp Asp Asp Tyr His 100 105 110	336
gcc aca acc gag acg att atc aca atg cct acg gag tct gat ttt ctt Ala Thr Thr Glu Thr Ile Ile Thr Met Pro Thr Glu Ser Asp Phe Leu 115 120 125	384
gta caa atg gag gga aaa cca aaa tgt tgc ttc ttt aag ttt agc tct Val Gln Met Glu Gly Lys Pro Lys Cys Cys Phe Phe Lys Phe Ser Ser 130 135 140	432
aaa ata caa tat aac aaa gta gta aag gca caa tta tgg ata tac ttg Lys Ile Gln Tyr Asn Lys Val Val Lys Ala Gln Leu Trp Ile Tyr Leu 145 150 155 160 165 170 175 180	480

145									51/9									155									160	
agg	caa	gtc	caa	aaa	cct	aca	acg	gtg	ttt	gtg	cag	atc	ctg	aga	ctc		528											
Arg	Gln	Val	Gln	Lys	Pro	Thr	Thr	Val	Phe	Val	Gln	Ile	Leu	Arg	Leu													
				165					170					175														
att	aag	ccc	atg	aaa	gac	ggg	aca	aga	tat	act	gga	att	cga	tct	ttg		576											
Ile	Lys	Pro	Met	Lys	Asp	Gly	Thr	Arg	Tyr	Thr	Gly	Ile	Arg	Ser	Leu													
			180					185					190															
aaa	ctt	gac	atg	aac	cca	ggc	act	ggg	atc	tgg	cag	agt	att	gat	gtg		624											
Lys	Leu	Asp	Met	Asn	Pro	Gly	Thr	Gly	Ile	Trp	Gln	Ser	Ile	Asp	Val													
		195				200						205																
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Lys	Thr	Val	Leu	Gln	Asn	Trp	Leu	Lys	Gln	Pro	Glu	Ser	Asn	Leu	Gly													
	210					215					220																	
atc	gaa	ata	aaa	gct	ttt	gat	gag	act	gga	cga	gat	ctt	gct	gtc	aca		720											
Ile	Glu	Ile	Lys	Ala	Phe	Asp	Glu	Thr	Gly	Arg	Asp	Leu	Ala	Val	Thr													
	225				230					235					240													
ttc	cca	gga	cca	gga	gaa	gat	gga	ttg	aac	cca	ttt	tta	gag	gtc	aga		768											
Phe	Pro	Gly	Pro	Gly	Glu	Asp	Gly	Leu	Asn	Pro	Phe	Leu	Glu	Val	Arg													
				245					250					255														
gtt	aca	gac	aca	ccg	aaa	cgg	tcc	cgc	aga	gat	ttt	ggc	ctt	gac	tgt		816											
Val	Thr	Asp	Thr	Pro	Lys	Arg	Ser	Arg	Arg	Asp	Phe	Gly	Leu	Asp	Cys													
			260					265					270															
gat	gag	cac	tca	acg	gaa	tcc	cga	tgt	tgt	cgc	tac	ccg	ctg	aca	gtg		864											
Asp	Glu	His	Ser	Thr	Glu	Ser	Arg	Cys	Cys	Arg	Tyr	Pro	Leu	Thr	Val													
		275				280						285																
gat	ttc	gaa	gct	ttt	gga	tgg	gac	tgg	att	ata	gca	cct	aaa	aga	tac		912											
Asp	Phe	Glu	Ala	Phe	Gly	Trp	Asp	Trp	Ile	Ile	Ala	Pro	Lys	Arg	Tyr													
	290					295					300																	
aaa	gcc	aat	tac	tgc	tcc	gga	gaa	tgc	gaa	ttt	gtg	ttt	cta	cag	aaa		960											
Lys	Ala	Asn	Tyr	Cys	Ser	Gly	Glu	Cys	Glu	Phe	Val	Phe	Leu	Gln	Lys													
	305				310					315					320													
tac	ccg	cac	act	cac	ctg	gta	cac	caa	gca	aat	ccc	aga	ggc	tca	gca		1008											
Tyr	Pro	His	Thr	His	Leu	Val	His	Gln	Ala	Asn	Pro	Arg	Gly	Ser	Ala													
				325					330					335														
ggc	cct	tgc	tgc	aca	ccc	acc	aag	atg	tcc	cct	ata	aac	atg	ctg	tat		1056											
Gly	Pro	Cys	Cys	Thr	Pro	Thr	Lys	Met	Ser	Pro	Ile	Asn	Met	Leu	Tyr													
			340					345					350															
ttc	aat	gga	aaa	gaa	caa	ata	ata	tat	gga	aag	ata	cca	gcc	atg	gtt		1104											
Phe	Asn	Gly	Lys	Glu	Gln	Ile	Ile	Tyr	Gly	Lys	Ile	Pro	Ala	Met	Val													
		355				360						365																
gta	gat	cgt	tgc	ggg	tgc	tca	tga										1128											
Val	Asp	Arg	Cys	Gly	Cys	Ser																						
	370					375																						

<210> 6
 <211> 375
 <212> PRT
 <213> Gallus gallus

<400> 6

Met Gln Lys Leu Ala Val Tyr Val Tyr Ile Tyr Leu Phe Met Gln Ile
 1 5 10 15

51/10

Ala Val Asp Pro Val Ala Leu Asp Gly Ser Ser Gln Pro Thr Glu Asn
20 25 30

Ala Glu Lys Asp Gly Leu Cys Asn Ala Cys Thr Trp Arg Gln Asn Thr
35 40 45

Lys Ser Ser Arg Ile Glu Ala Ile Lys Ile Gln Ile Leu Ser Lys Leu
50 55 60

Arg Leu Glu Gln Ala Pro Asn Ile Ser Arg Asp Val Ile Lys Gln Leu
65 70 75 80

Leu Pro Lys Ala Pro Pro Leu Gln Glu Leu Ile Asp Gln Tyr Asp Val
85 90 95

Gln Arg Asp Asp Ser Ser Asp Gly Ser Leu Glu Asp Asp Asp Tyr His
100 105 110

Ala Thr Thr Glu Thr Ile Ile Thr Met Pro Thr Glu Ser Asp Phe Leu
115 120 125

Val Gln Met Glu Gly Lys Pro Lys Cys Cys Phe Phe Lys Phe Ser Ser
130 135 140

Lys Ile Gln Tyr Asn Lys Val Val Lys Ala Gln Leu Trp Ile Tyr Leu
145 150 155 160

Arg Gln Val Gln Lys Pro Thr Thr Val Phe Val Gln Ile Leu Arg Leu
165 170 175

Ile Lys Pro Met Lys Asp Gly Thr Arg Tyr Thr Gly Ile Arg Ser Leu
180 185 190

Lys Leu Asp Met Asn Pro Gly Thr Gly Ile Trp Gln Ser Ile Asp Val
195 200 205

Lys Thr Val Leu Gln Asn Trp Leu Lys Gln Pro Glu Ser Asn Leu Gly
210 215 220

Ile Glu Ile Lys Ala Phe Asp Gly Thr Gly Arg Asp Leu Ala Val Thr
225 230 235 240

Phe Pro Gly Pro Gly Glu Asp Gly Leu Asn Pro Phe Leu Glu Val Arg
245 250 255

Val Thr Asp Thr Pro Lys Arg Ser Arg Arg Asp Phe Gly Leu Asp Cys
260 265 270

Asp Glu His Ser Thr Glu Ser Arg Cys Cys Arg Tyr Pro Leu Thr Val
275 280 285

51/11

Asp Phe Glu Ala Phe Gly Trp Asp Trp Ile Ile Ala Pro Lys Arg Tyr
 290 295 300

Lys Ala Asn Tyr Cys Ser Gly Glu Cys Glu Phe Val Phe Leu Gln Lys
 305 310 315 320

Tyr Pro His Thr His Leu Val His Gln Ala Asn Pro Arg Gly Ser Ala
 325 330 335

Gly Pro Cys Cys Thr Pro Thr Lys Met Ser Pro Ile Asn Met Leu Tyr
 340 345 350

Phe Asn Gly Lys Glu Gln Ile Ile Tyr Gly Lys Ile Pro Ala Met Val
 355 360 365

Val Asp Arg Cys Gly Cys Ser
 370 375

<210> 7
 <211> 1125
 <212> DNA
 <213> Danio rerio

<220>
 <221> CDS
 <222> (1)..(1122)

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 1 5 10 15

ggt cca gtg ggt tat gga gat ata acg gcg cac cag cag cct tcc aca 96
 Gly Pro Val Gly Tyr Gly Asp Ile Thr Ala His Gln Gln Pro Ser Thr
 20 25 30

gcc acg gag gaa agc gag ctg tgt tcc aca tgt gag ttc aga caa cac 144
 Ala Thr Glu Glu Ser Glu Leu Cys Ser Thr Cys Glu Phe Arg Gln His
 35 40 45

agc aag ctg atg aga ctg cat gcc atc aag tcc caa att ctt agc aaa 192
 Ser Lys Leu Met Arg Leu His Ala Ile Lys Ser Gln Ile Leu Ser Lys
 50 55 60

ctc cga ctc aag cag gct cca aac atc agc cgg gac gtg gtc aag cag 240
 Leu Arg Leu Lys Gln Ala Pro Asn Ile Ser Arg Asp Val Val Lys Gln
 65 70 75 80

ctg tta ccc aaa gca ccg cct ttg caa caa ctt ctg gat cag tac gat 288
 Leu Leu Pro Lys Ala Pro Pro Leu Gln Gln Leu Leu Asp Gln Tyr Asp
 85 90 95

gtt tta gga gat gac agt aag gat gga gct gtg gaa gag gac gat gaa 336
 Val Leu Gly Asp Asp Ser Lys Asp Gly Ala Val Glu Glu Asp Asp Glu
 100 105 110

cat gcc acc aca gag acc atc atg acc atg gcc aca gaa cct gac ccc 384
 His Ala Thr Thr Glu Thr Ile Met Thr Met Ala Thr Glu Pro Asp Pro
 115 120 125

att gtt caa gta gat cgg aaa ccg aag tgt tgc ttt ttc tcc ttc agt 432
 Ile Val Gln Val Asp Arg Lys Pro Lys Cys Cys Phe Phe Ser Phe Ser
 130 135 140

51/12

ccg Pro 145	aag Lys	atc Ile	caa Gln	gcg Ala	aac Asn 150	cgg Arg	atc Ile	gta Val	aga Arg	gcg Ala 155	cag Gln	ctc Leu	tgg Trp	gtt Val	cat His 160	480
ctg Leu	aga Arg	ccg Pro	gcg Ala	gag Glu 165	gag Glu	gcg Ala	acc Thr	acc Thr	gtc Val 170	ttc Phe	tta Leu	cag Gln	ata Ile	tct Ser 175	cgg Arg	528
ctg Leu	atg Met	ccc Pro	gtt Val 180	aag Lys	gac Asp	gga Gly	gga Gly	aga Arg 185	cac His	cga Arg	ata Ile	cga Arg	tcc Ser 190	ctg Leu	aaa Lys	576
atc Ile	gac Asp	gtg Val 195	aac Asn	gca Ala	gga Gly	gtc Val	acg Thr 200	tct Ser	tgg Trp	cag Gln	agt Ser	ata Ile 205	gac Asp	gta Val	aag Lys	624
cag Gln	gtg Val 210	ctc Leu	acg Thr	gtg Val	tgg Trp	tta Leu 215	aaa Lys	caa Gln	ccg Pro	gag Glu	acc Thr 220	aac Asn	cga Arg	ggc Gly	atc Ile	672
gag Glu 225	att Ile	aac Asn	gca Ala	tat Tyr	gac Asp 230	gcg Ala	aag Lys	gga Gly	aac Asn	gac Asp 235	ttg Leu	gcc Ala	gtc Val	act Thr	tca Ser 240	720
acc Thr	gag Glu	act Thr	ggg Gly	gag Glu 245	gat Asp	gga Gly	ctg Leu	ctc Leu	ccc Pro 250	ttt Phe	atg Met	gag Glu	gtg Val	aaa Lys 255	ata Ile	768
tca Ser	gag Glu	ggc Gly	cca Pro 260	aaa Lys	cga Arg	atc Ile	cgg Arg	agg Arg 265	gac Asp	tcc Ser	gga Gly	ctg Leu	gac Asp 270	tgc Cys	gat Asp	816
gag Glu	aat Asn	tcc Ser 275	tca Ser	gag Glu	tct Ser	cgc Arg	tgc Cys 280	tgc Cys	agg Arg	tac Tyr	cct Pro	ctc Leu 285	act Thr	gtg Val	gac Asp	864
ttc Phe	gag Glu 290	gac Asp	ttt Phe	ggc Gly	tgg Trp	gac Asp 295	tgg Trp	att Ile	att Ile	gct Ala	cca Pro 300	aaa Lys	cgc Arg	tat Tyr	aag Lys	912
gcg Ala 305	aat Asn	tac Tyr	tgt Cys	tca Ser	gga Gly 310	gaa Glu	tgc Cys	gac Asp	tac Tyr	atg Met 315	tac Tyr	ctg Leu	cag Gln	aag Lys	tat Tyr 320	960
ccc Pro	cac His	acc Thr	cat His	ctg Leu 325	gtg Val	aac Asn	aag Lys	gcc Ala	agt Ser 330	ccg Pro	aga Arg	gga Gly	acg Thr	gct Ala 335	ggg Gly	1008
ccc Pro	tgc Cys	tgc Cys	act Thr 340	ccc Pro	acc Thr	aag Lys	atg Met	tct Ser 345	ccc Pro	atc Ile	aac Asn	atg Met	ctt Leu 350	tac Tyr	ttt Phe	1056
aac Asn	ggc Gly	aaa Lys 355	gag Glu	cag Gln	atc Ile	atc Ile	tac Tyr 360	ggc Gly	aag Lys	atc Ile	cct Pro	tcg Ser 365	atg Met	gta Val	gta Val	1104
gac Asp	cgc Arg 370	tgt Cys	ggc Gly	tgc Cys	tca Ser	tga										1125

<210> 8
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 <212> PRT
 <213> Danio rerio
 <400> 8

51/13
 Met His Phe Thr Gln Val Leu Ile Ser Leu Ser Val Leu Ile Ala Cys
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 Gly Pro Val Gly Tyr Gly Asp Ile Thr Ala His Gln Gln Pro Ser Thr
 20 25 30
 Ala Thr Glu Glu Ser Glu Leu Cys Ser Thr Cys Glu Phe Arg Gln His
 35 40 45
 Ser Lys Leu Met Arg Leu His Ala Ile Lys Ser Gln Ile Leu Ser Lys
 50 55 60
 Leu Arg Leu Lys Gln Ala Pro Asn Ile Ser Arg Asp Val Val Lys Gln
 65 70 75 80
 Leu Leu Pro Lys Ala Pro Pro Leu Gln Gln Leu Leu Asp Gln Tyr Asp
 85 90 95
 Val Leu Gly Asp Asp Ser Lys Asp Gly Ala Val Glu Glu Asp Asp Glu
 100 105 110
 His Ala Thr Thr Glu Thr Ile Met Thr Met Ala Thr Glu Pro Asp Pro
 115 120 125
 Ile Val Gln Val Asp Arg Lys Pro Lys Cys Cys Phe Phe Ser Phe Ser
 130 135 140
 Pro Lys Ile Gln Ala Asn Arg Ile Val Arg Ala Gln Leu Trp Val His
 145 150 155 160
 Leu Arg Pro Ala Glu Glu Ala Thr Thr Val Phe Leu Gln Ile Ser Arg
 165 170 175
 Leu Met Pro Val Lys Asp Gly Gly Arg His Arg Ile Arg Ser Leu Lys
 180 185 190
 Ile Asp Val Asn Ala Gly Val Thr Ser Trp Gln Ser Ile Asp Val Lys
 195 200 205
 Gln Val Leu Thr Val Trp Leu Lys Gln Pro Glu Thr Asn Arg Gly Ile
 210 215 220
 Glu Ile Asn Ala Tyr Asp Ala Lys Gly Asn Asp Leu Ala Val Thr Ser
 225 230 235 240
 Thr Glu Thr Gly Glu Asp Gly Leu Leu Pro Phe Met Glu Val Lys Ile
 245 250 255
 Ser Glu Gly Pro Lys Arg Ile Arg Arg Asp Ser Gly Leu Asp Cys Asp
 260 265 270
 Glu Asn Ser Ser Glu Ser Arg Cys Cys Arg Tyr Pro Leu Thr Val Asp

<210>	9
<211>	50
<212>	PRT
<213>	Homo sapiens

<400> 9

Lys Asp Val Ile Arg Gln Leu Leu Pro Lys Ala Pro Pro Leu Arg Glu
1 5 10 15

Leu Ile Asp Gln Tyr Asp Val Gln Arg Asp Asp Ser Ser Asp Gly Ser
20 25 30

Leu Glu Asp Asp Asp Tyr His Ala Thr Thr Glu Thr Ile Ile Thr Met
35 40 45

Pro Thr
50

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<210> 10
<211> 50
<212> PRT
<213> Artificial sequence
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<220>
<223> Mutant peptide portion of human myostatin

<400> 10

Lys Asp Val Ile Arg Gln Leu Leu Pro Lys Ala Pro Pro Leu Arg Glu
1 5 10 15

Leu Ile Asp Gln Tyr Asp Val Gln Gln Asp Asp Ser Ser Asp Gly Ser
20 25 30

Leu Glu Asp Asp Asp Tyr His Ala Thr Thr Glu Thr Ile Ile Thr Met

35 40 51/15 45

Pro Thr
50

<210> 11
<211> 50
<212> PRT
<213> Artificial sequence

<220>
<223> Mutant peptide portion of human myostatin

<400> 11

Lys Asp Val Ile Arg Gln Leu Leu Pro Lys Ala Pro Pro Leu Arg Glu
1 5 10 15

Leu Ile Asp Gln Tyr Asp Val Gln Arg Ala Asp Ser Ser Asp Gly Ser
20 25 30

Leu Glu Asp Asp Asp Tyr His Ala Thr Thr Glu Thr Ile Ile Thr Met
35 40 45

Pro Thr
50

<210> 12
<211> 40
<212> PRT
<213> Homo sapiens

<400> 12

Gln Leu Leu Pro Lys Ala Pro Pro Leu Arg Glu Leu Ile Asp Gln Tyr
1 5 10 15

Asp Val Gln Arg Asp Asp Ser Ser Asp Gly Ser Leu Glu Asp Asp Asp
20 25 30

Tyr His Ala Thr Thr Glu Thr Ile
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Tyr His Ala Thr Thr Glu Thr Ile
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The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. Use of a latent myostatin complex comprising a myostatin pro-peptide and a myostatin C-terminal fragment, and a metalloprotease that can cleave the myostatin pro-peptide, and an agent that increases or decreases proteolytic cleavage of the pro-peptide by the metalloprotease in comparison to the proteolytic cleavage when the agent is absent, for modulating myostatin activation in a subject,

wherein the metalloprotease is mammalian bone morphogenic protein-1 (BMP-1),

wherein the myostatin complex comprises SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8, and

wherein the agent is a peptide comprising an amino acid sequence selected from SEQ ID NOs: 9-23.

2. The use of claim 1, which comprises increasing myostatin activation, said method comprising contacting the latent myostatin complex and metalloprotease with an agent that increases proteolytic cleavage of the pro-peptide by the metalloprotease, for increasing myostatin activation.

3. The use of claim 1, wherein the agent decreases proteolytic cleavage of the pro-peptide by the metalloprotease, for reducing or inhibiting myostatin activation.

4. The use of claim 3, wherein, in the subject, muscle mass is increased, fat content is decreased, or a combination thereof, in comparison to the subject not being contacted with the agent.

5. The use of claim 4, wherein the subject is an animal raised as a food source.

6. The use of claim 5, wherein the animal is a mammalian species, an avian species, or a piscine species.

7. The use of claim 6, wherein mammalian species is an ovine species, a porcine species, or a bovine species.
8. The use of claim 6, wherein the avian species is a chicken or a turkey.
9. The use of claim 1, wherein the subject is a human.
10. An *in vitro* method of modulating myostatin activation in a sample, comprising contacting a latent myostatin complex comprising a myostatin pro-peptide and a myostatin C-terminal fragment, and a metalloprotease that can cleave the myostatin pro-peptide, and an agent that increases or decreases proteolytic cleavage of the pro-peptide by the metalloprotease in comparison to the proteolytic cleavage when the agent is absent, thereby modulating myostatin activation,
wherein the metalloprotease is mammalian bone morphogenic protein-1 (BMP-1),
wherein the myostatin complex comprises SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8, and
wherein the agent is a peptide comprising an amino acid sequence selected from SEQ ID NOs: 9-23.
11. The method of claim 10, which comprises increasing myostatin activation, said method comprising contacting the latent myostatin complex and metalloprotease with an agent that increases proteolytic cleavage of the pro-peptide by the metalloprotease, thereby increasing myostatin activation.
12. The method of claim 10, wherein the sample comprises a cell sample, a tissue sample, or a biological fluid sample.
13. The method of claim 10, wherein the agent decreases proteolytic cleavage of the pro-peptide by the metalloprotease, thereby reducing or inhibiting myostatin activation.
14. A method of increasing muscle mass in an animal raised as a food source comprising administering to said animal a latent myostatin complex comprising a myostatin

pro-peptide and a myostatin C-terminal fragment, and a metalloprotease that can cleave the myostatin pro-peptide, with an agent that reduces or inhibits proteolytic cleavage of the pro-peptide by the metalloprotease in comparison to the proteolytic cleavage when the agent is absent, for increasing the muscle mass in said animal,

wherein the metalloprotease is mammalian bone morphogenic protein-1 (BMP-1),

wherein the myostatin complex comprises SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO:8, and

wherein the agent is a peptide comprising an amino acid sequence selected from SEQ ID NOs: 9-23.

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Reporter assay for MSTN activity

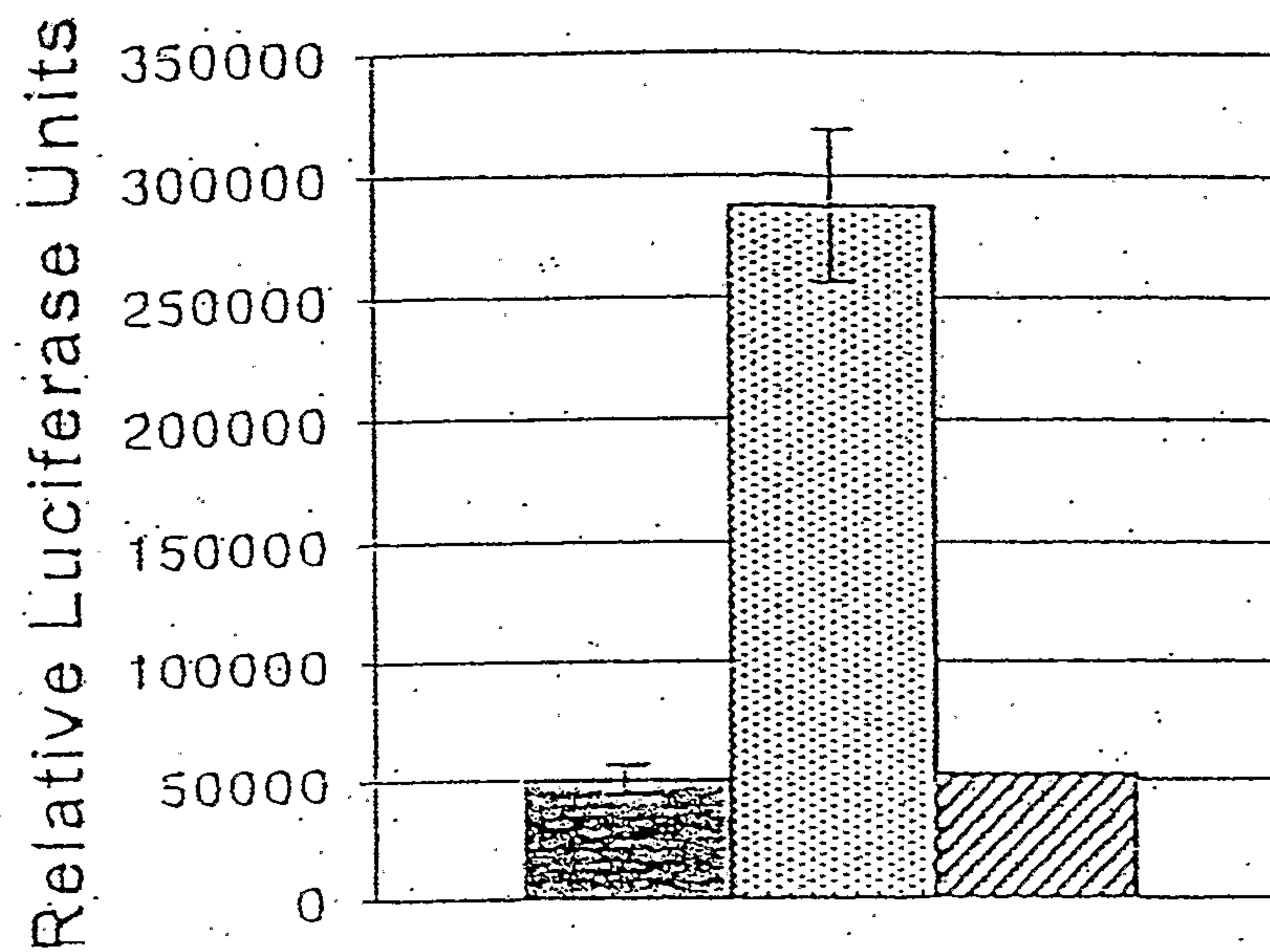


Figure 1

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Myostatin (MSTN) standard curve

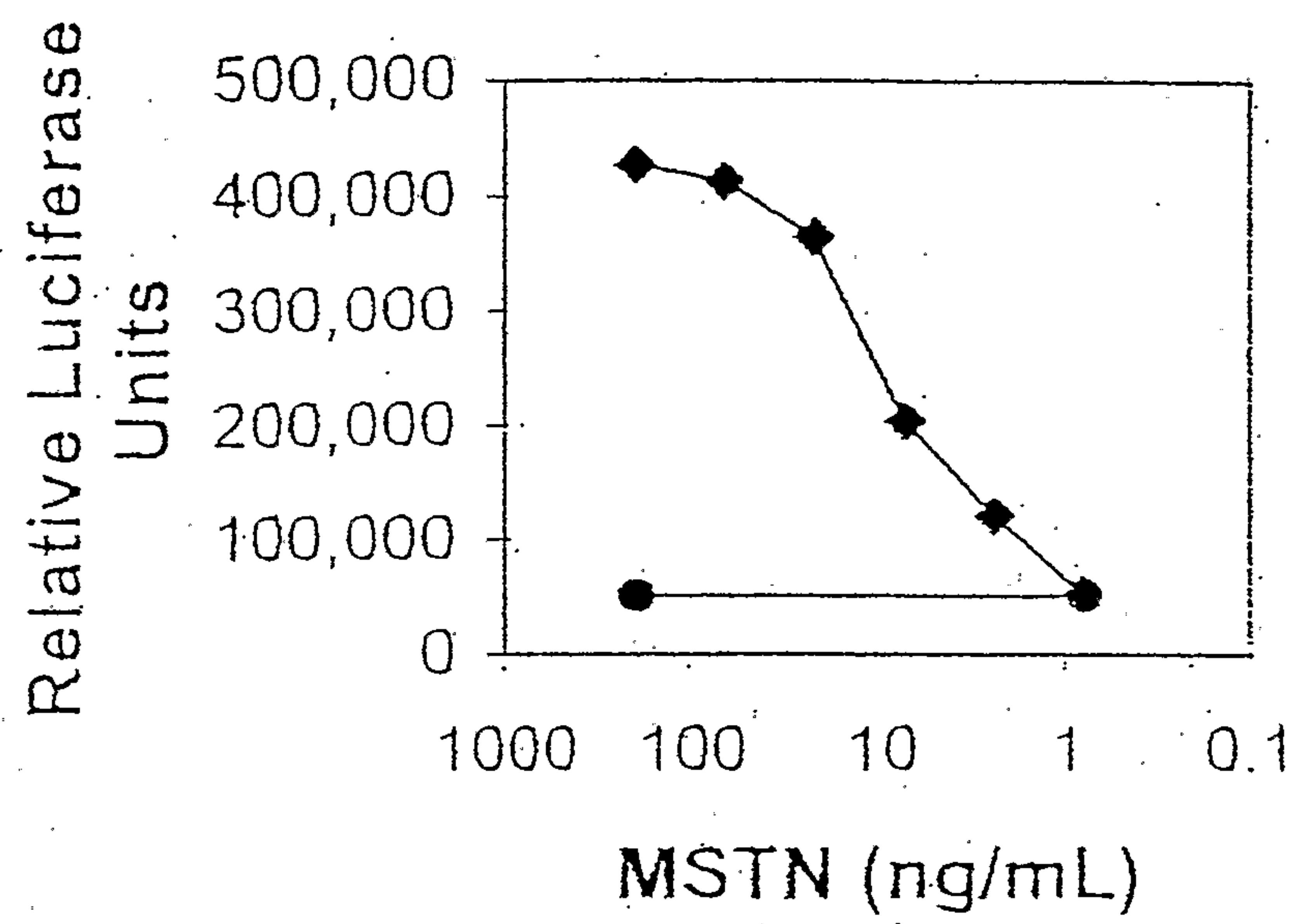


Figure 2

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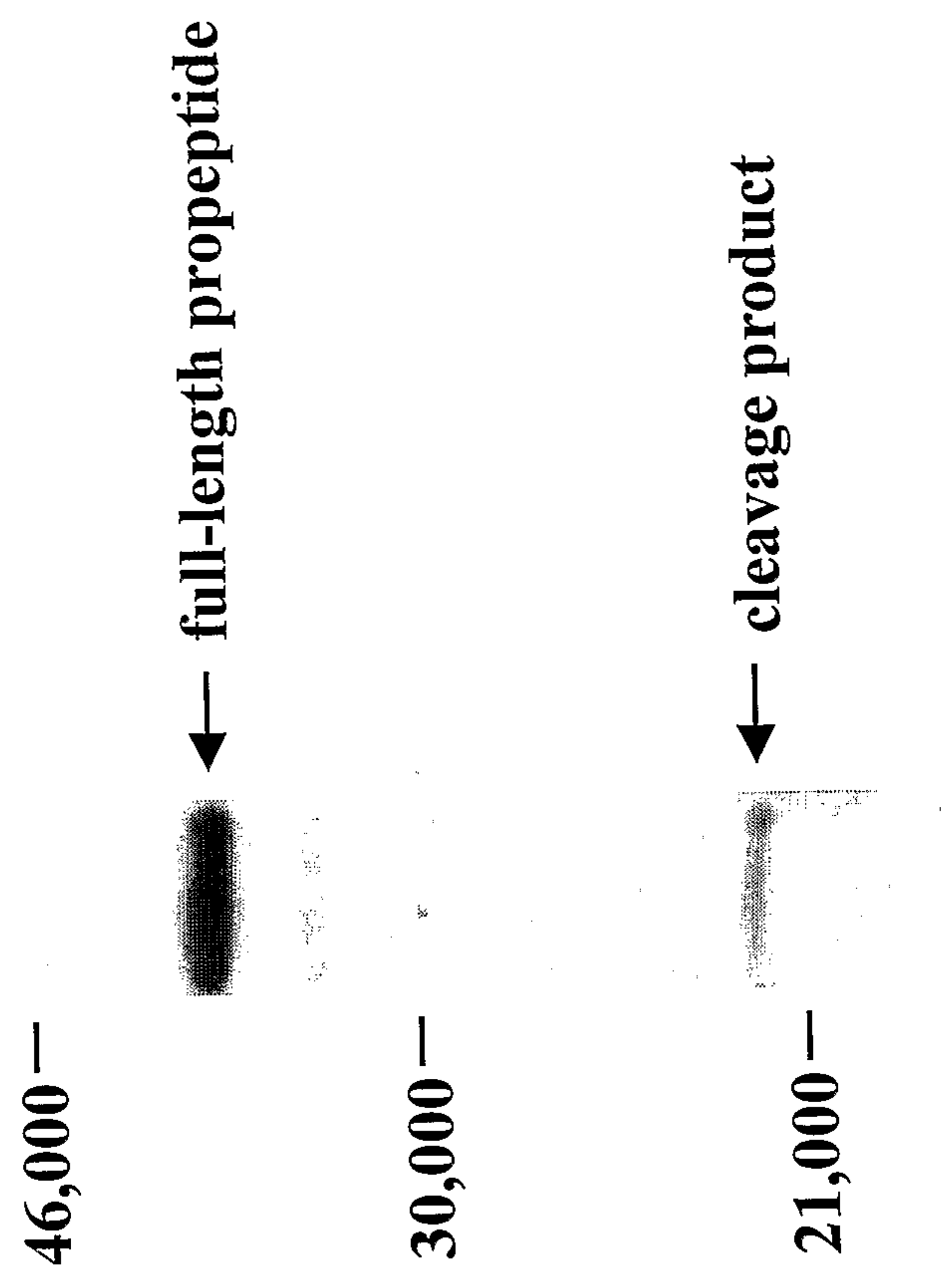


Figure 3A

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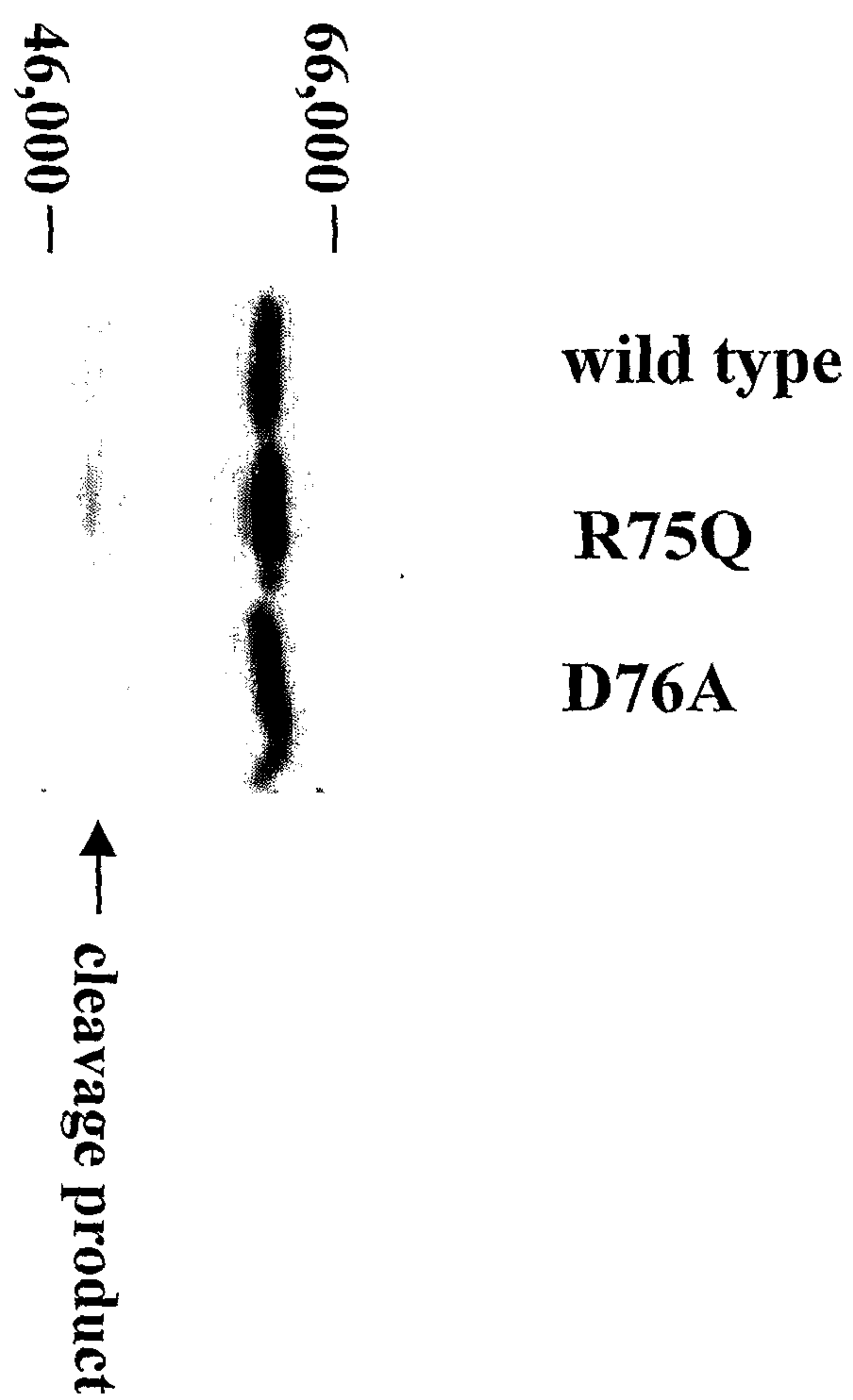


Figure 3B

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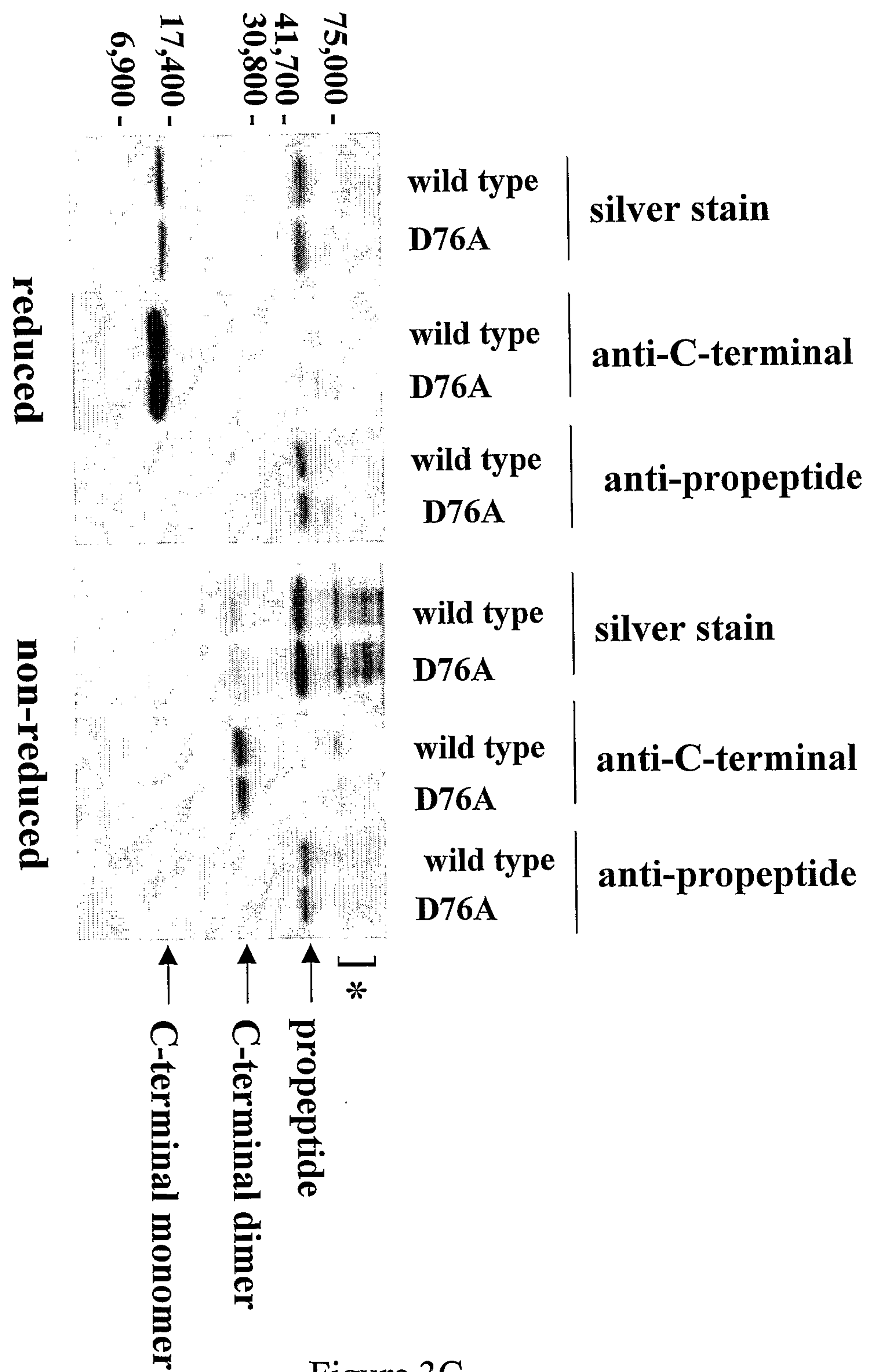


Figure 3C

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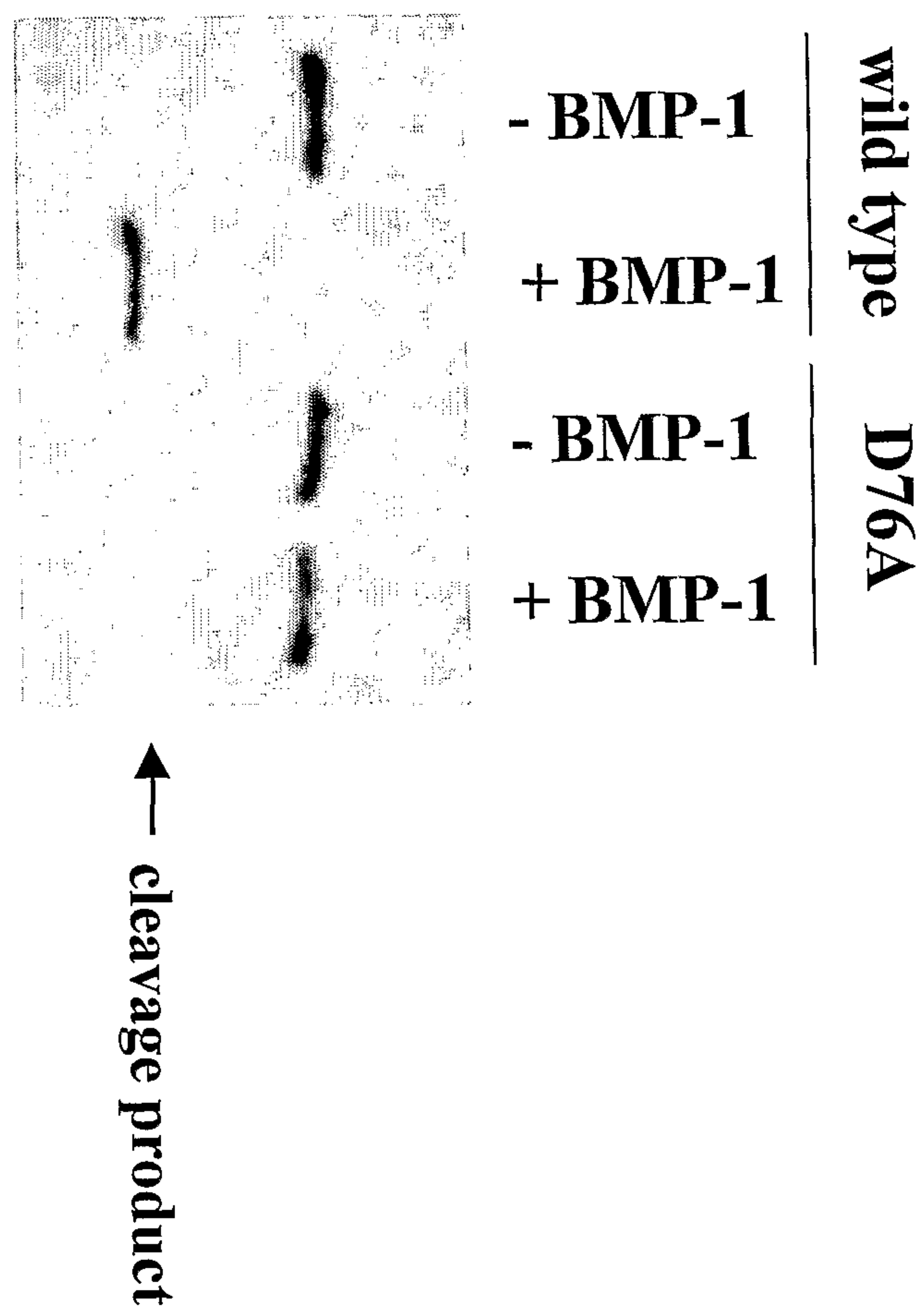


Figure 3D

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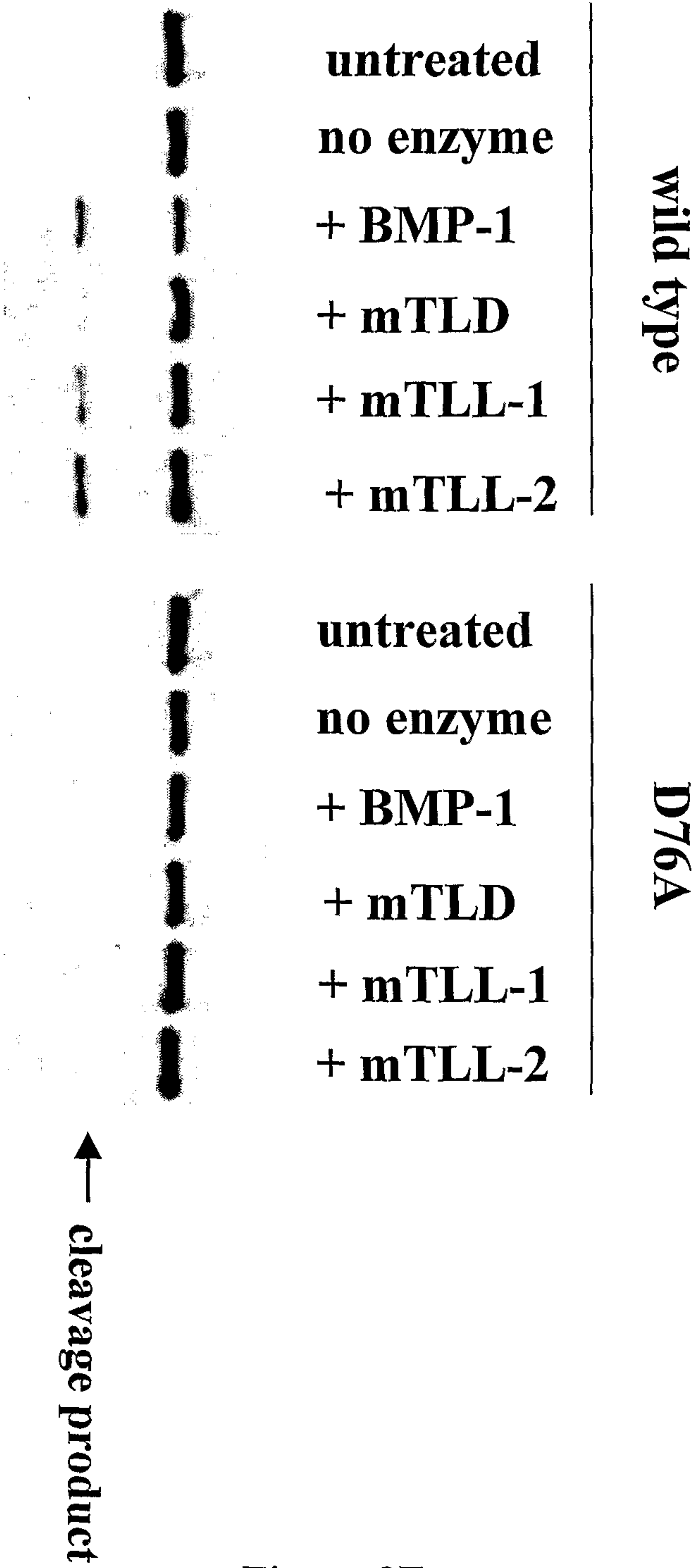


Figure 3E

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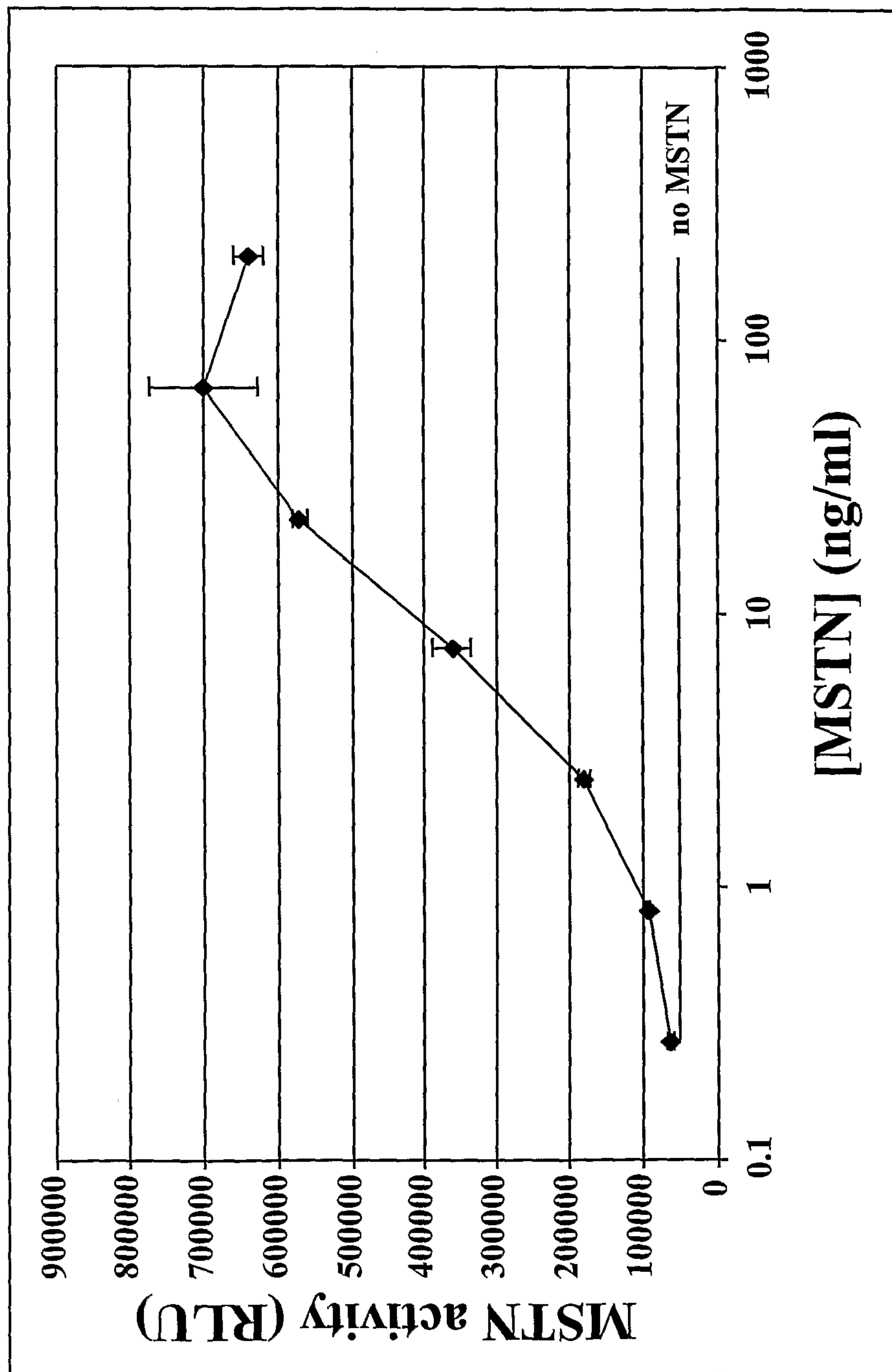


Figure 4A

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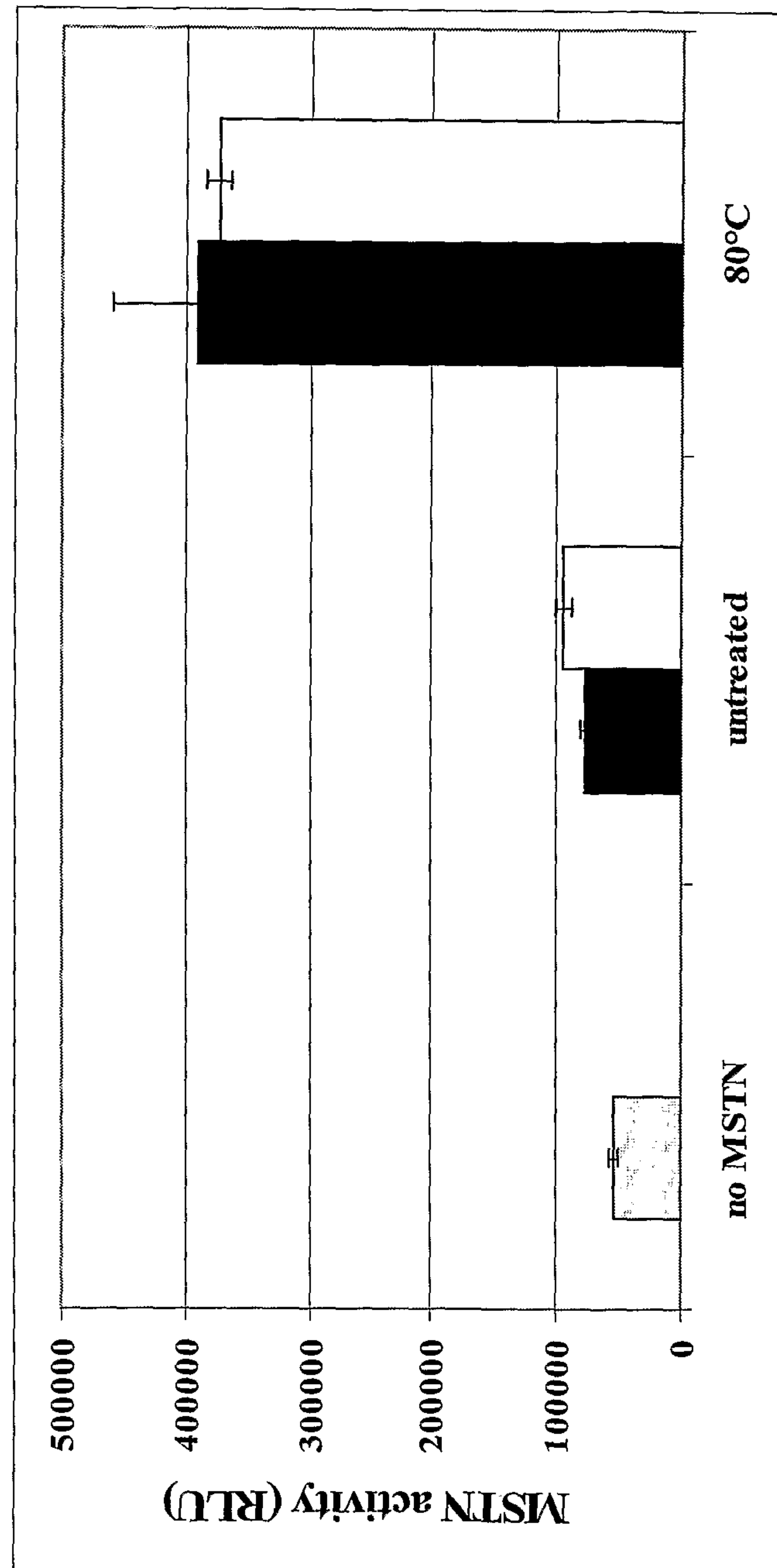


Figure 4B

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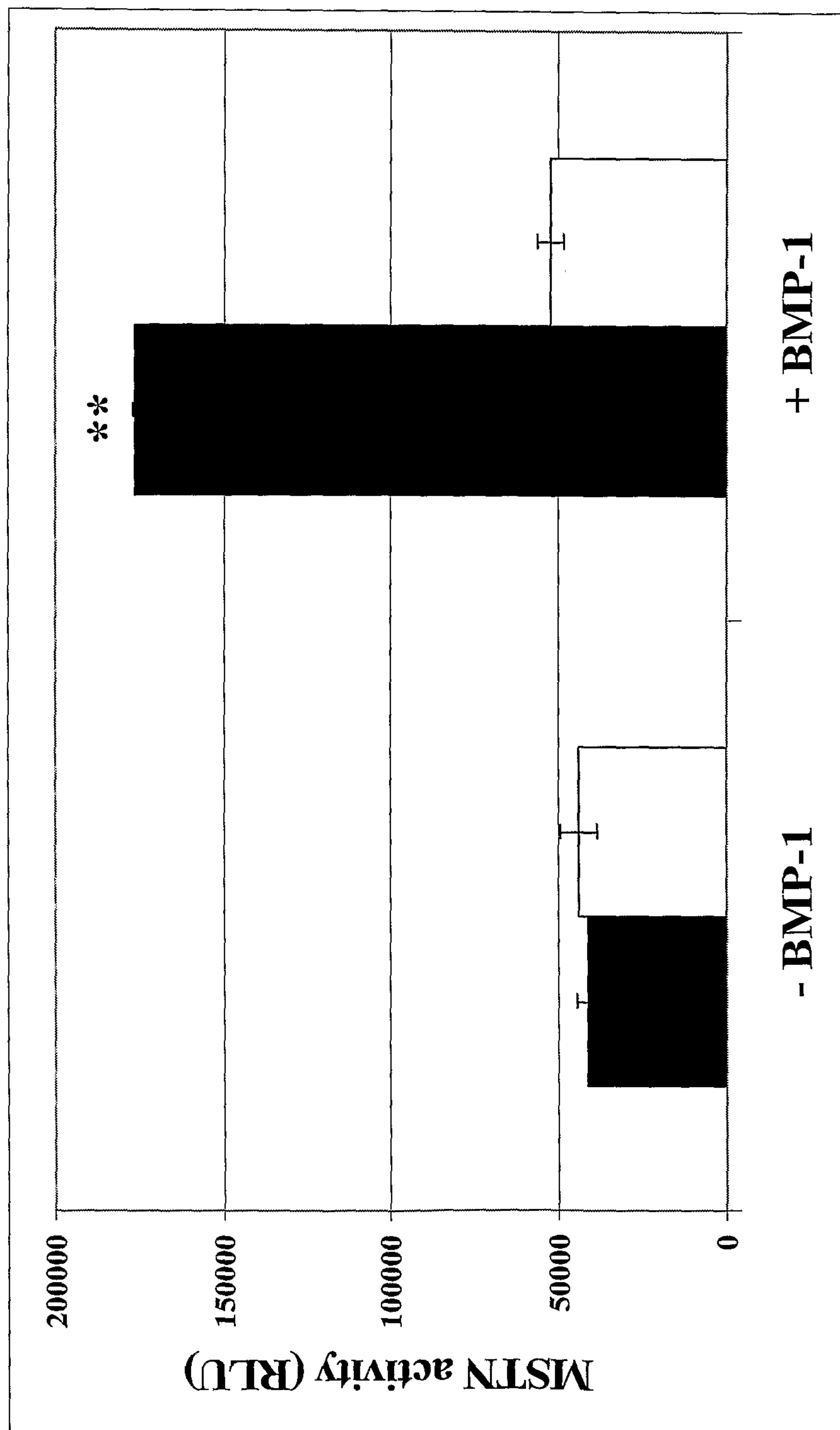


Figure 4C

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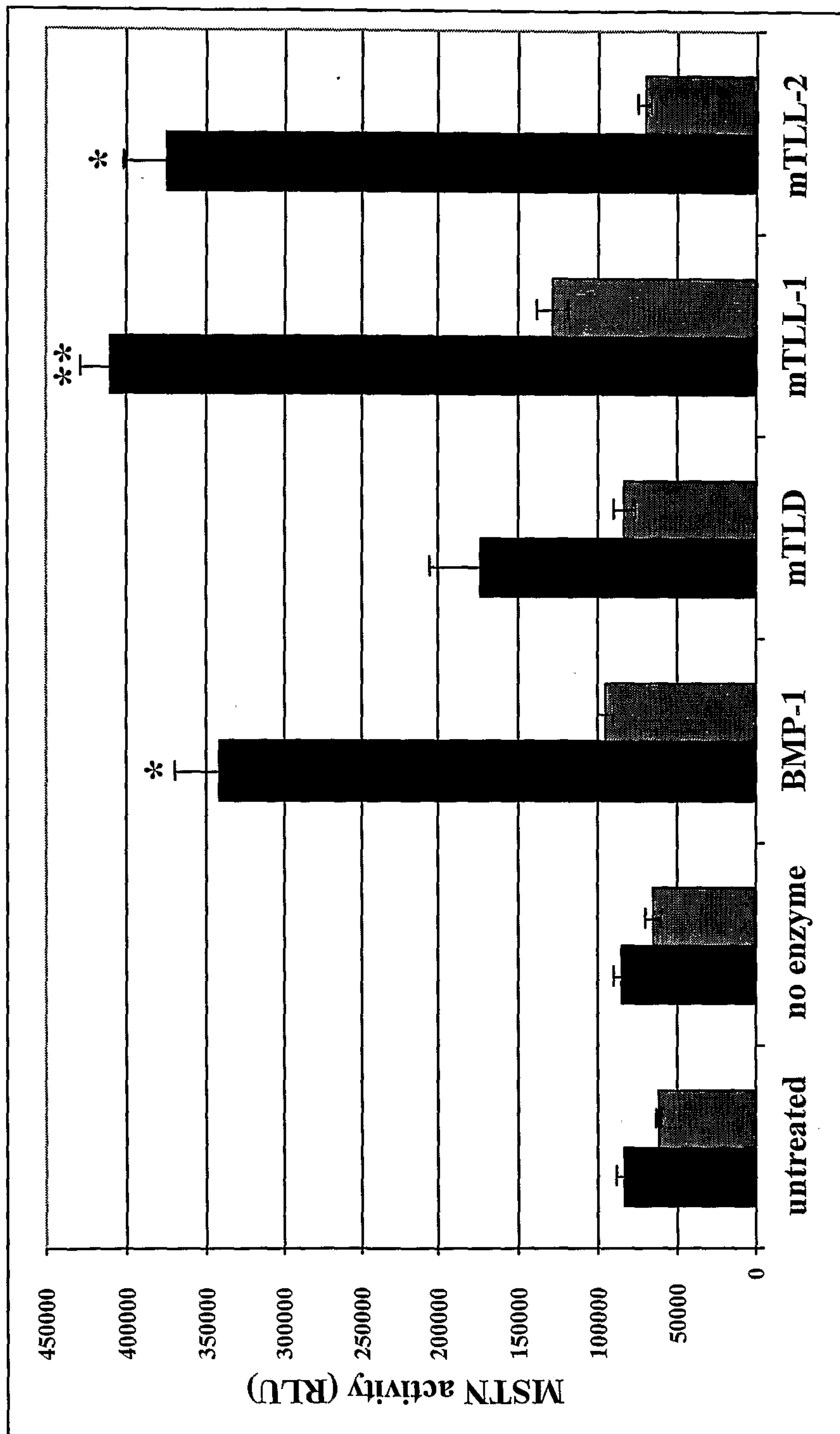


Figure 4D

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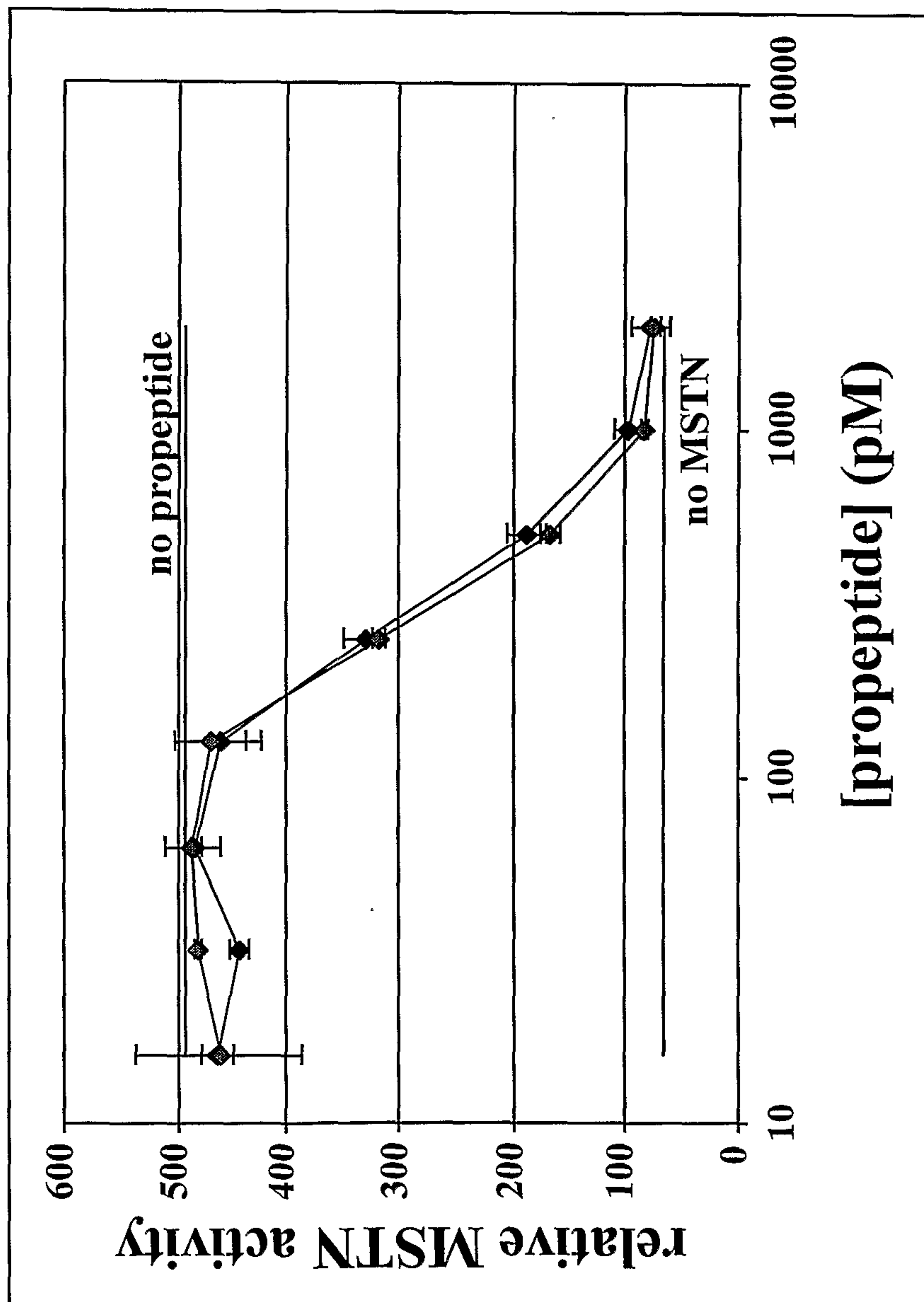


Figure 5