The microbial metabolite imidazole propionate dysregulates bone homeostasis by inhibiting AMP-activated protein kinase (AMPK) signaling

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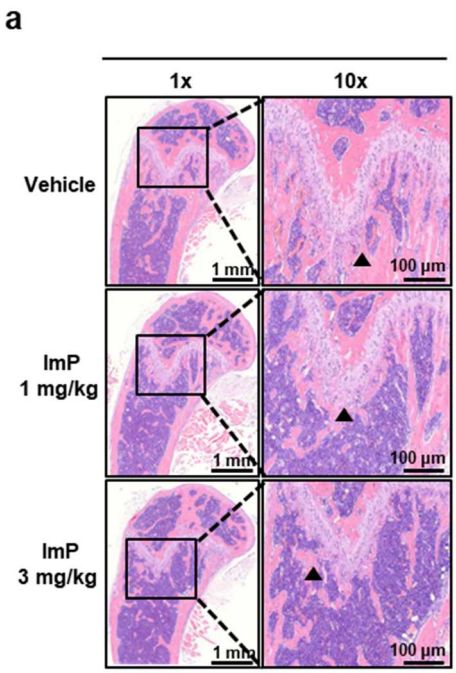
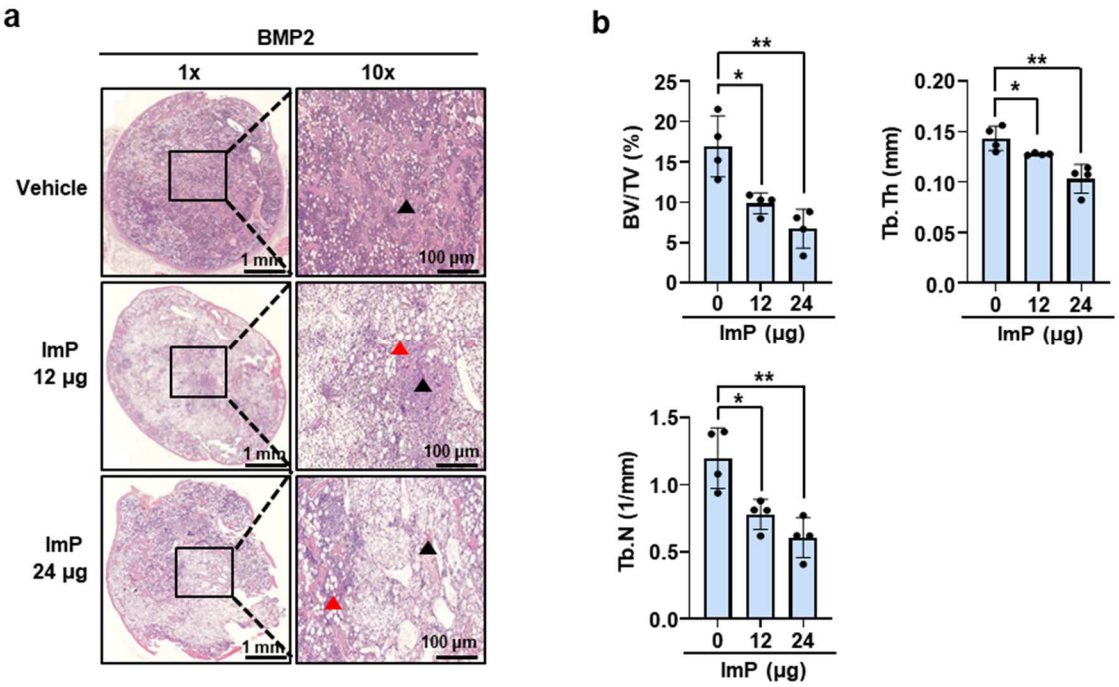
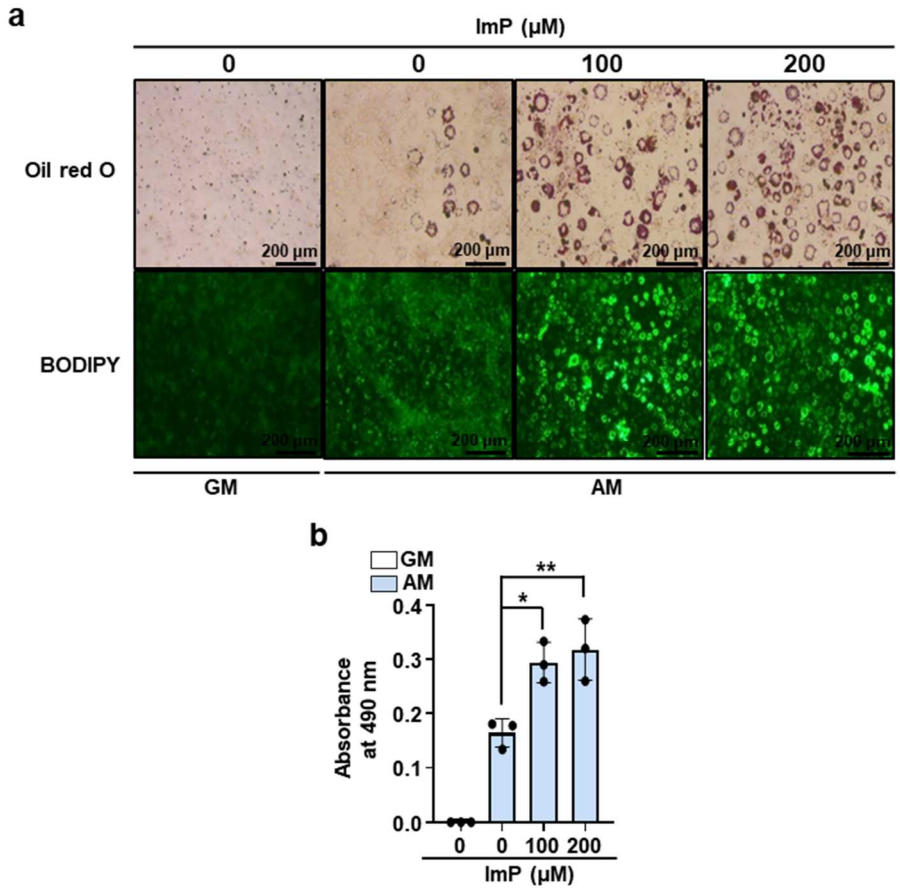
Supplementary Figures



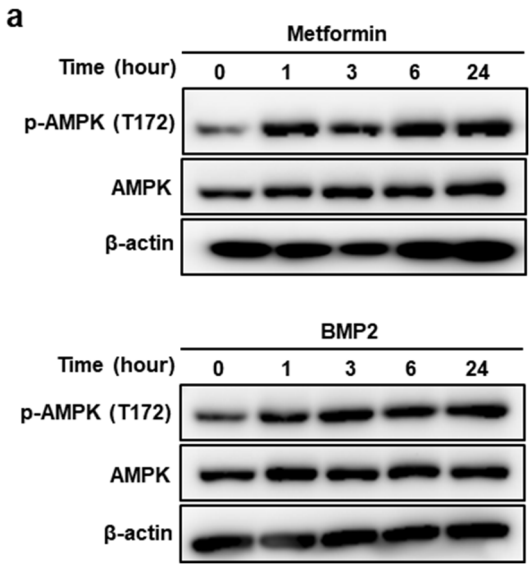
Supplementary Figure 1. Histological staining confirmed that ImP inhibited bone formation in animals. a Mice were treated with ImP (1 mg/kg, 3 mg/kg), harvested 4 weeks later, and H&E staining was performed (n=3). Black triangle, bone; ImP, imidazole propionate; H&E, hematoxylin and eosin.



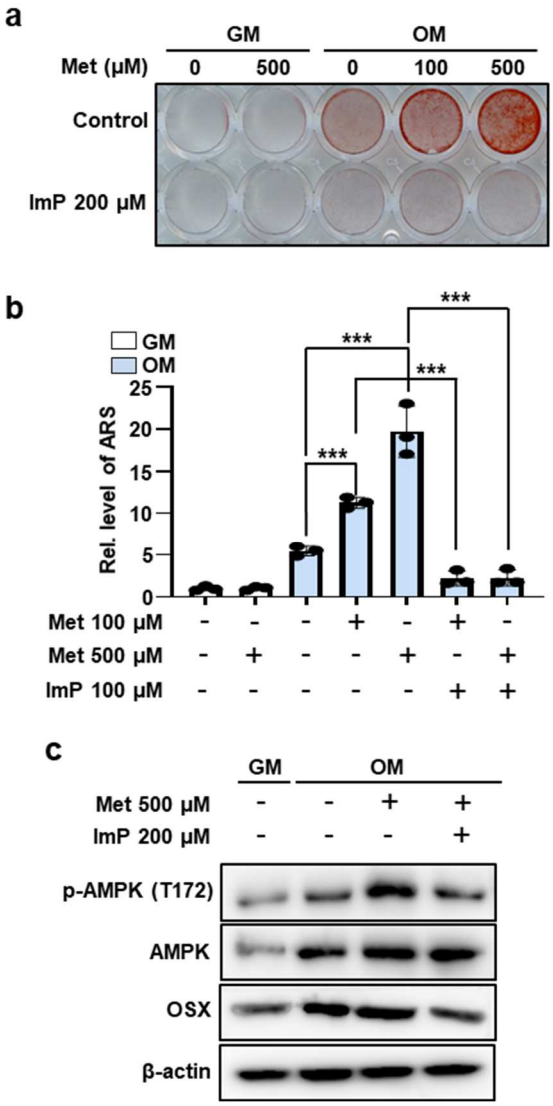
Supplementary Figure 2. The microbial metabolite ImP inhibits bone formation in an ectopic bone formation model. a, b ImP (12 and 24 μg) and BMP2 (3 μg) were administered into subcutaneous space of the back with collagen sponge, and ectopic bones were harvested 4 weeks later (n=4). H&E (**a**) and bone parameter (**b**) analyses were performed. Black triangle, bone; Red triangle, adipocyte; ImP, imidazole propionate; BV/TV, percentage bone volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; H&E, hematoxylin and eosin. Values are presented as the mean ± SD; *, $P < 0.05$; **, $P < 0.01$; compared to the control group.



Supplementary Figure 3. ImP stimulates adipocyte differentiation in 3T3-L1 cells. a, b 3T3-L1 adipocytes were cultured for 6 days and stained with oil red O (upper panel) and BODIPY (lower panel). Quantification of oil red O staining is shown in panel b (n=3). ImP, imidazole propionate; GM, growth media; AM, adipogenic media (1 μg/ml insulin, 2 μM rosiglitazone, and 100 nM dexamethasone). Values are presented as the mean ± SD; *, $P < 0.05$; **, $P < 0.01$; compared to the control group.

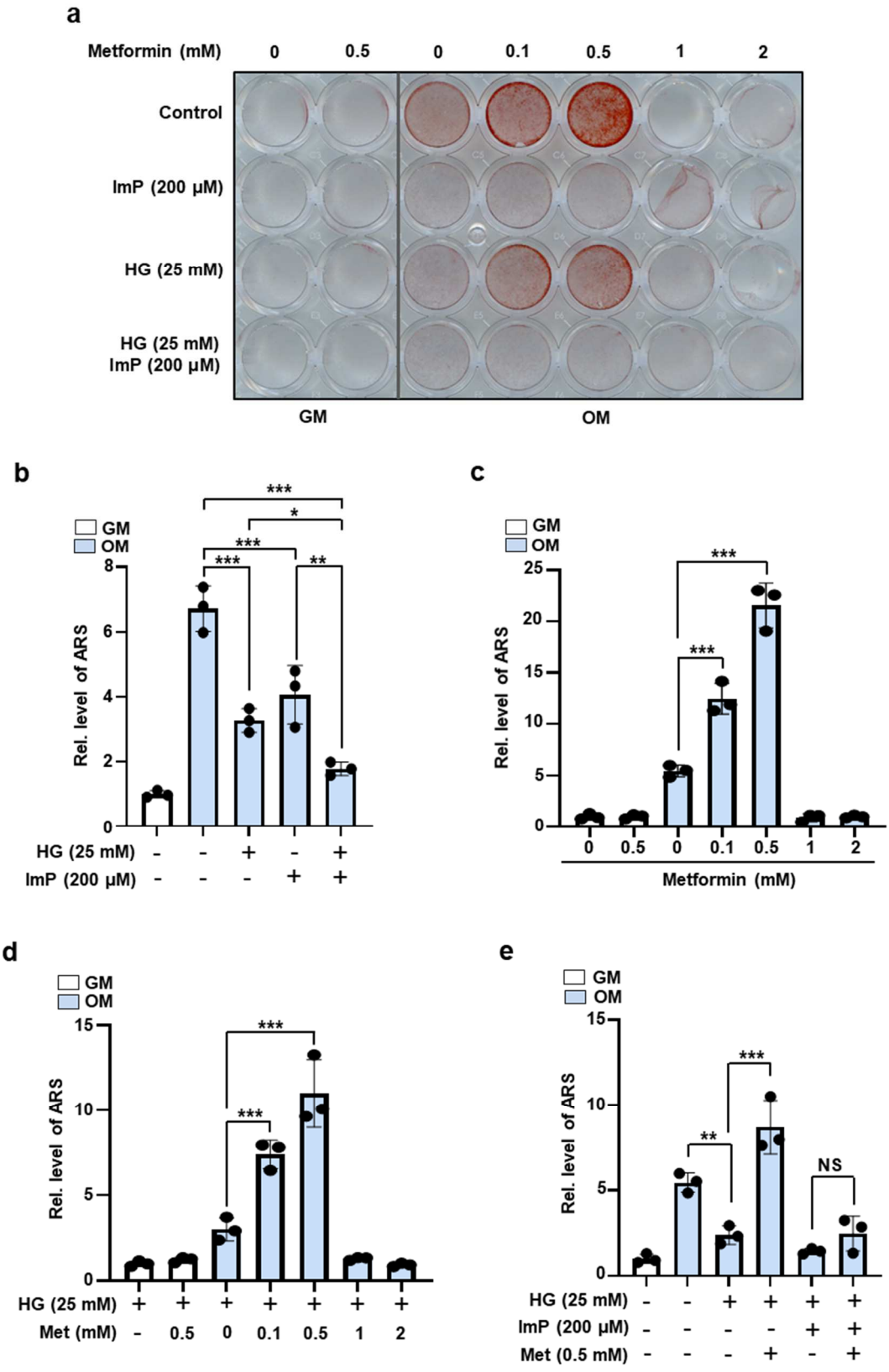


Supplementary Figure 4. Metformin and BMP2 promote phosphorylation of AMPK. a, b BMSCs were treated with metformin (100 μM) or BMP2 (100 ng/ml) for 0, 1, 3, 6, and 24 h. Western blot analysis was performed with antibodies against phosphorylated form of AMPK (T172) and non-phosphorylated AMPK (n=3).



Supplementary Figure 5. ImP inhibits metformin-induced matrix calcification in BMSCs.

a–c BMSCs were cultured with metformin in osteogenic medium for 14 days. **a** Alizarin red S staining was performed to assess calcification (n=3). **b** The staining was quantified using 10% cetylpyridinium chloride (n=3). **c** BMSCs were cultured with metformin and ImP in osteogenic medium for 4 days, and western blot analysis was performed with antibodies against OSX, and p-AMPK (n=3). ImP, imidazole propionate; BMSC, bone marrow stromal cell; OM, osteogenic media (50 μg/ml ascorbic acid and 5 mM β-glycerophosphate). Values are presented as the mean ± SD; ***, $P < 0.001$; compared to the control group.


a
Metformin (mM)
0
0.5
0
0.1
0.5
1
2
Control
ImP (200 μM)
HG (25 mM)
HG (25 mM)
ImP (200 μM)
GM
OM
b
GM
OM
Rel. level of ARS
HG (25 mM)
ImP (200 μM)
c
GM
OM
Rel. level of ARS
Metformin (mM)
d
GM
OM
Rel. level of ARS
HG (25 mM)
Met (mM)
e
GM
OM
Rel. level of ARS
NS
HG (25 mM)
ImP (200 μM)
Met (0.5 mM)

Supplementary Figure 6. Changes in calcium deposition in BMSCs cultured under high glucose conditions with metformin and ImP treatments. a BMSCs were cultured under high glucose conditions (25 mM) with each drug, and calcium deposition was assessed using Alizarin Red staining (n=3). b Calcium deposition decreased under high glucose and ImP-treated conditions (n=3). c Metformin increased calcium deposition (n=3). d Under high glucose conditions, metformin also increased calcium deposition (n=3). e ImP treatment further reduced calcium deposition in the presence of both high glucose and metformin (n=3). ImP, imidazole propionate; BMSC, bone marrow stromal cell; HG, High glucose; Met, Metformin; GM, Growth medium; OM, Osteogenic medium. Values are presented as the mean ± SD; NS, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; compared to the control group. ***, $P < 0.001$; compared to the control group.

Supplementary Figure 7. Uncropped and unedited blot/gel images

Figure 2

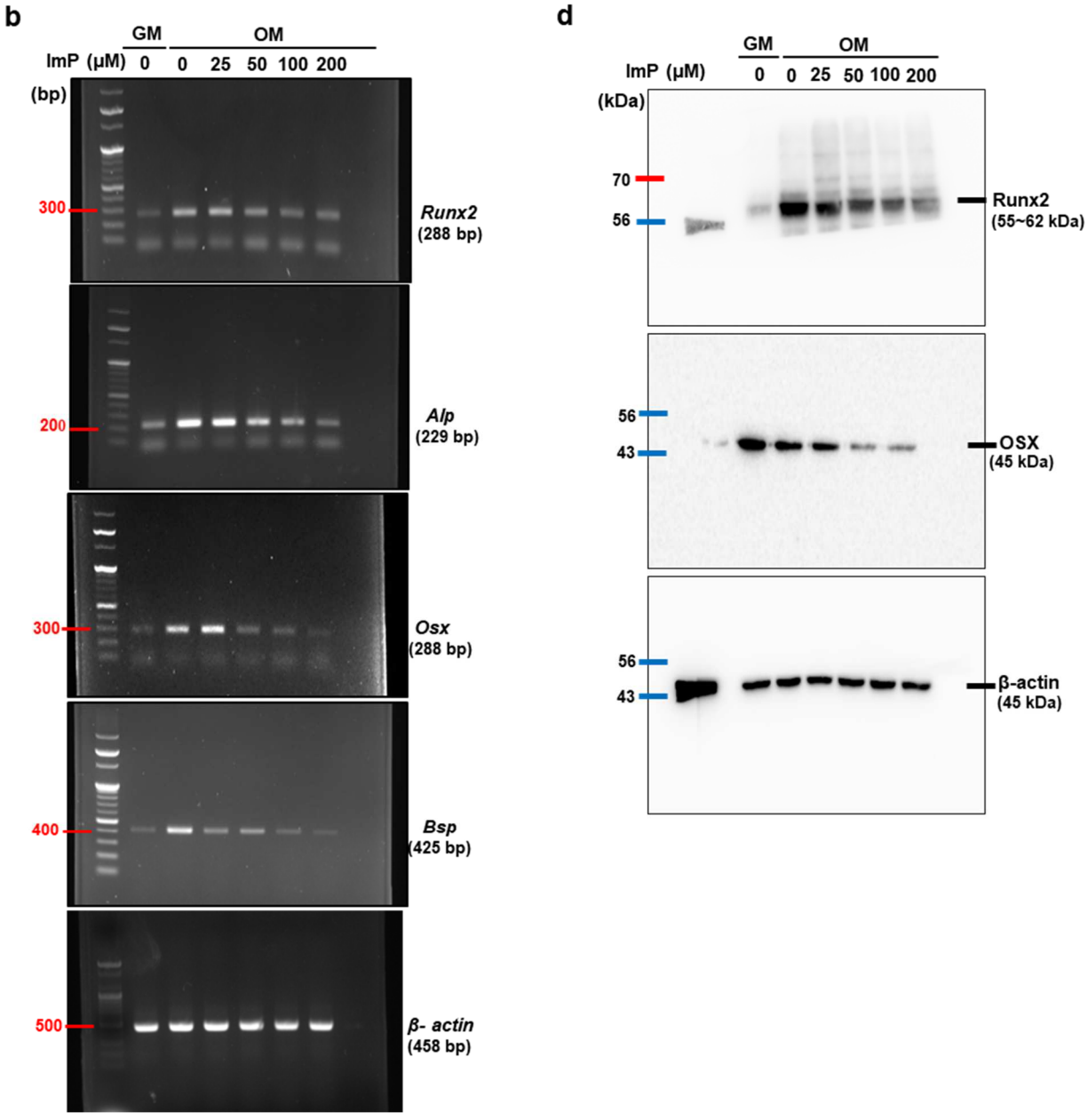


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Figure 3

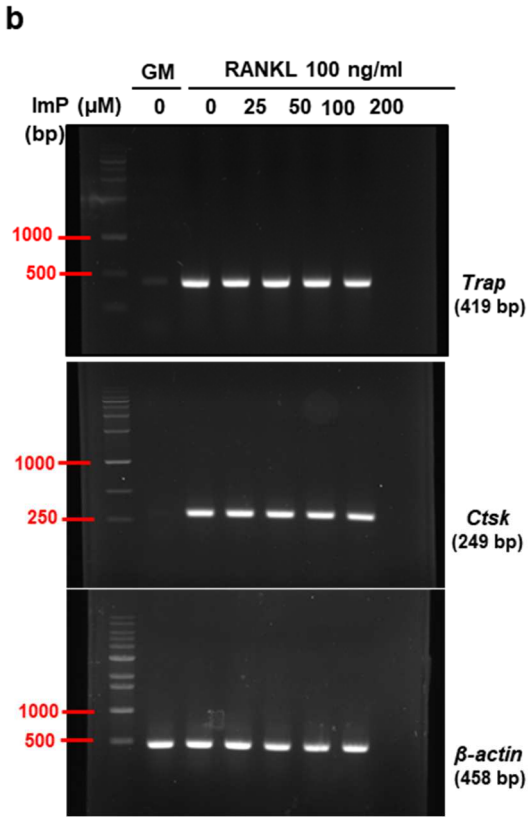

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Figure 4

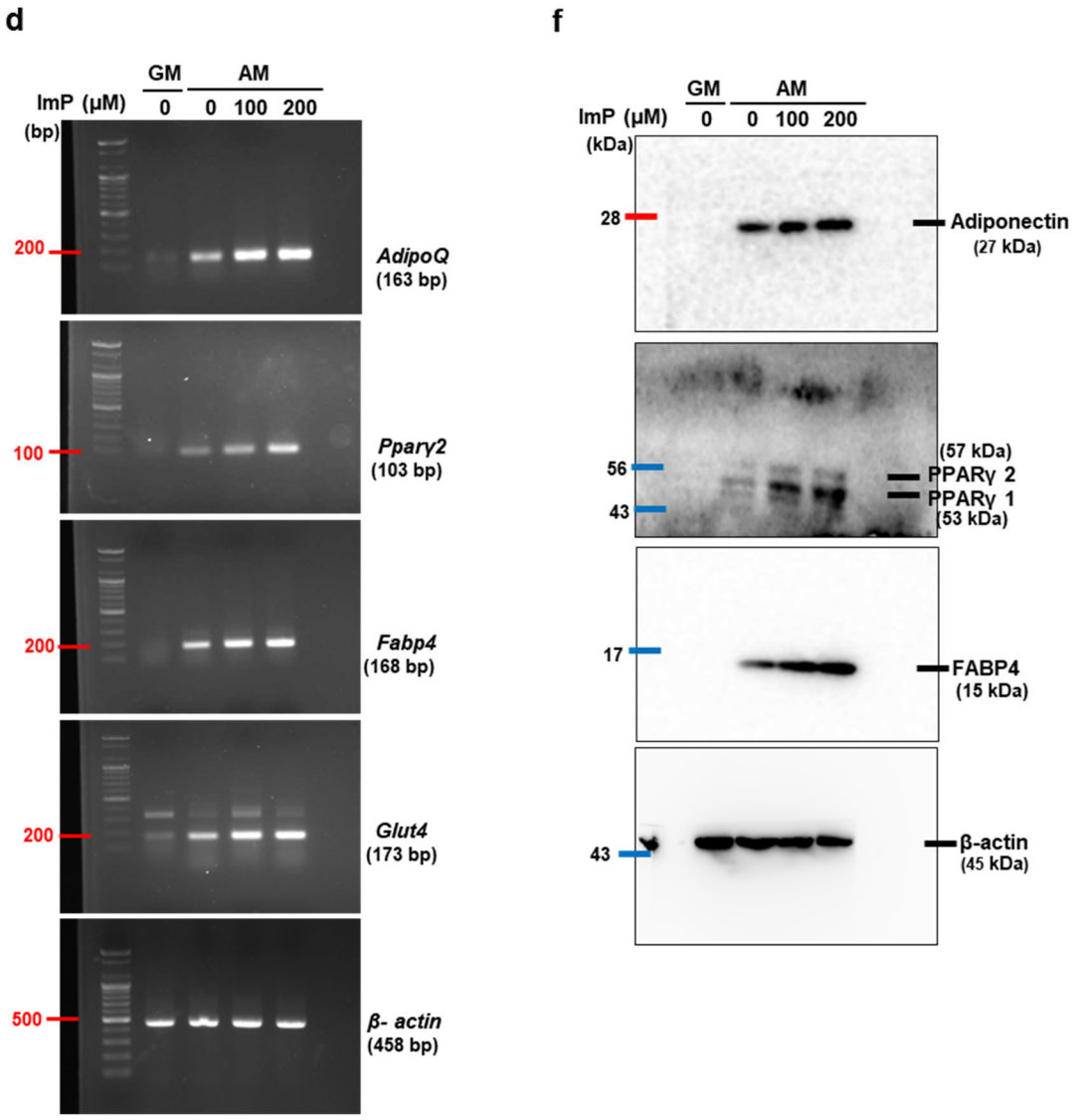

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Figure 5

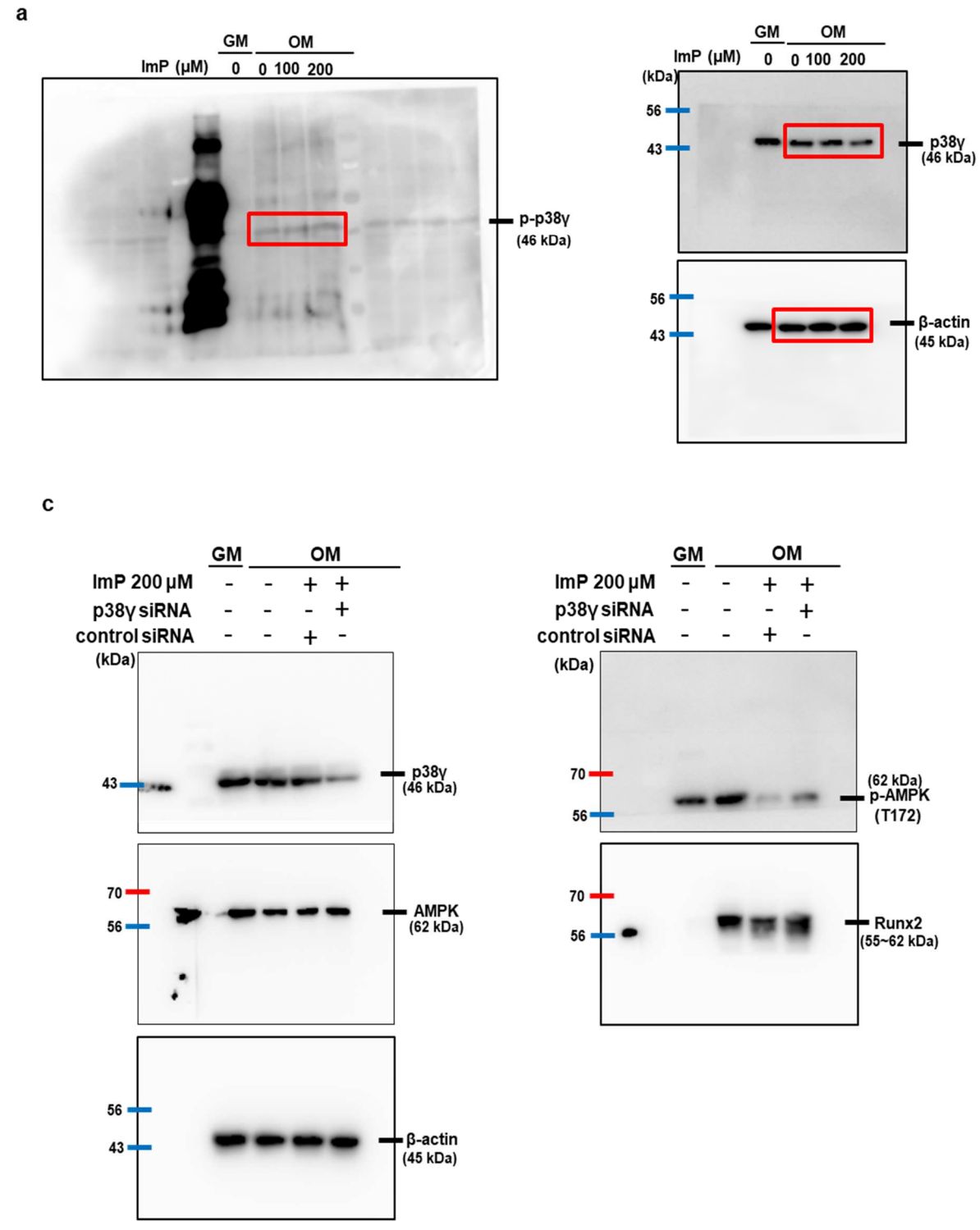

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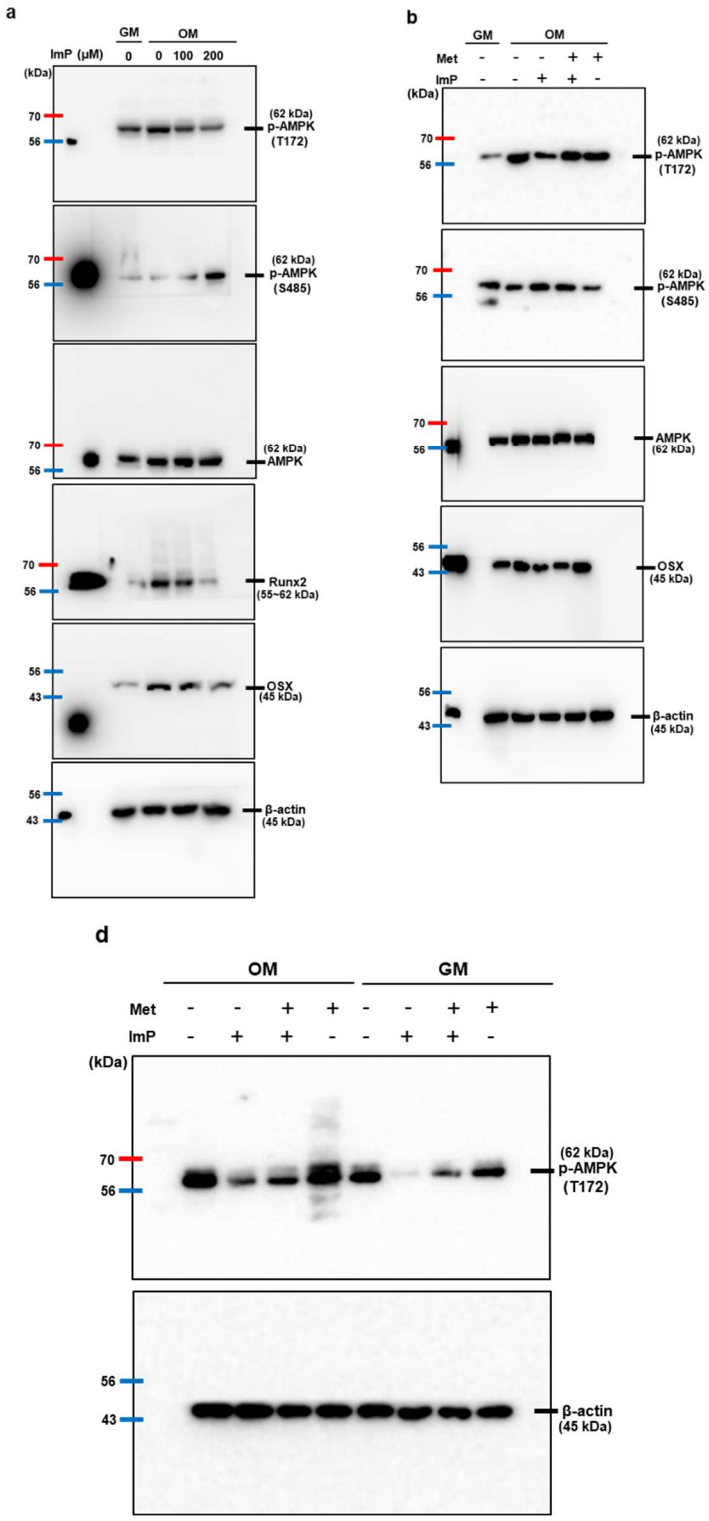
Figure 6



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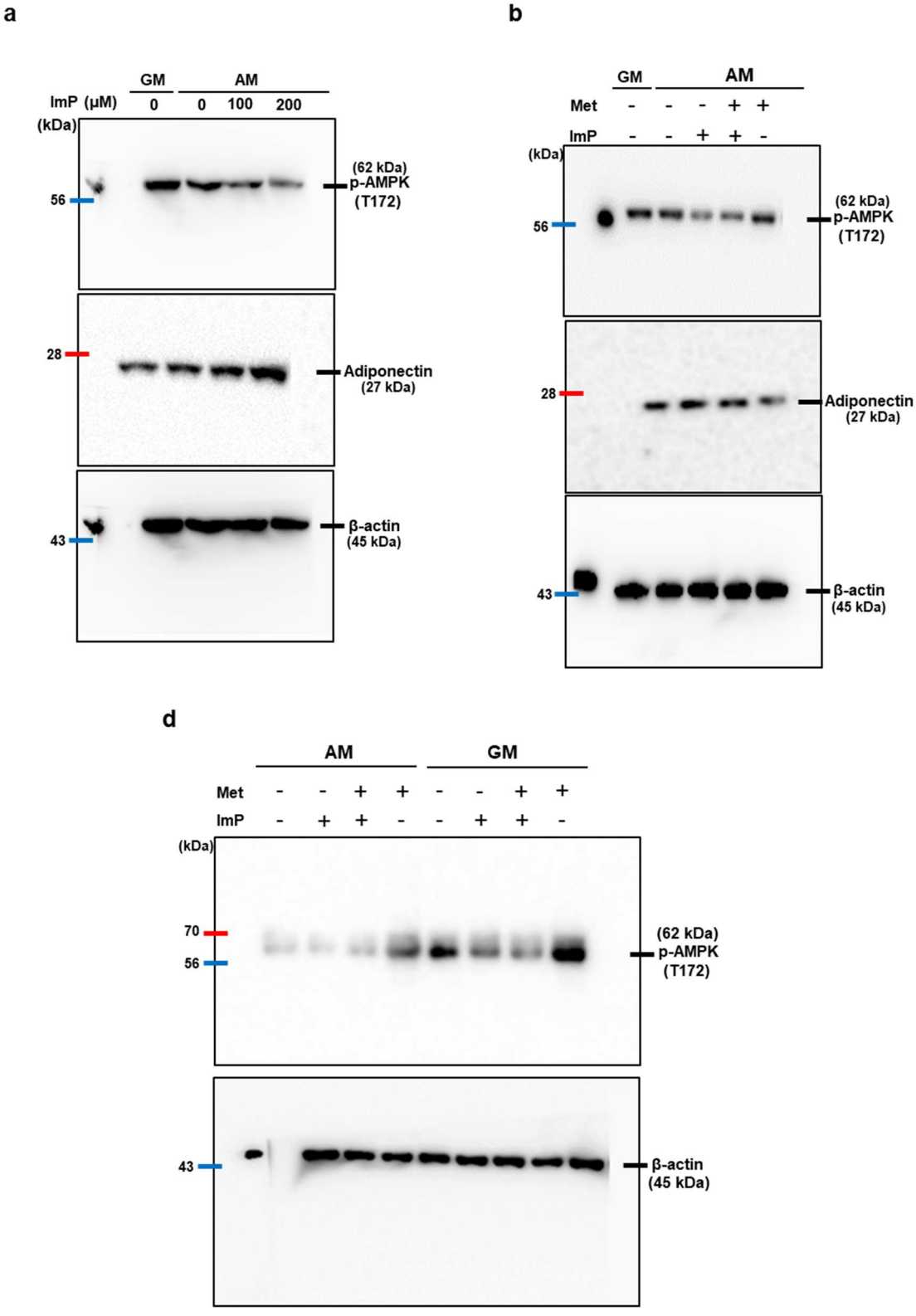

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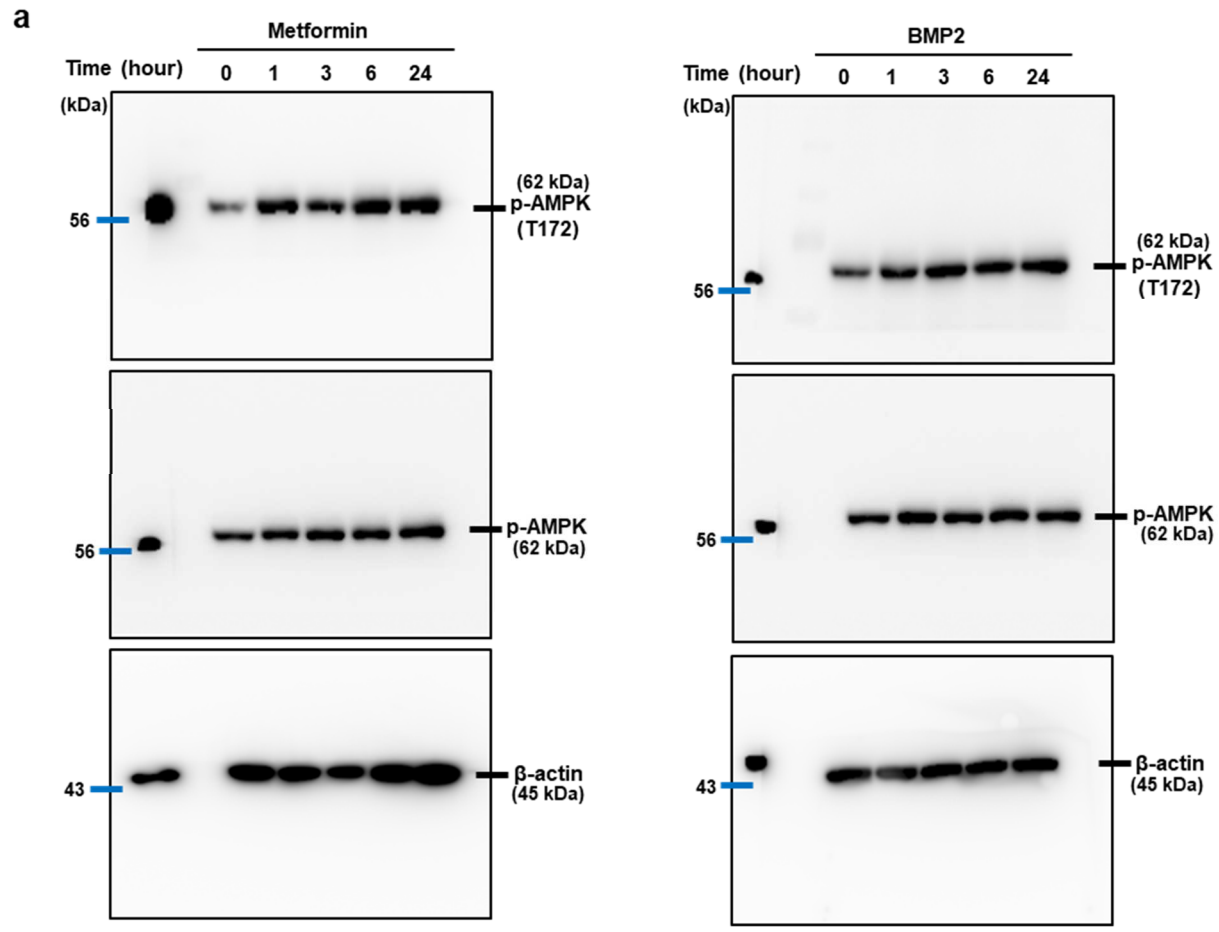
Supplementary Figure 4



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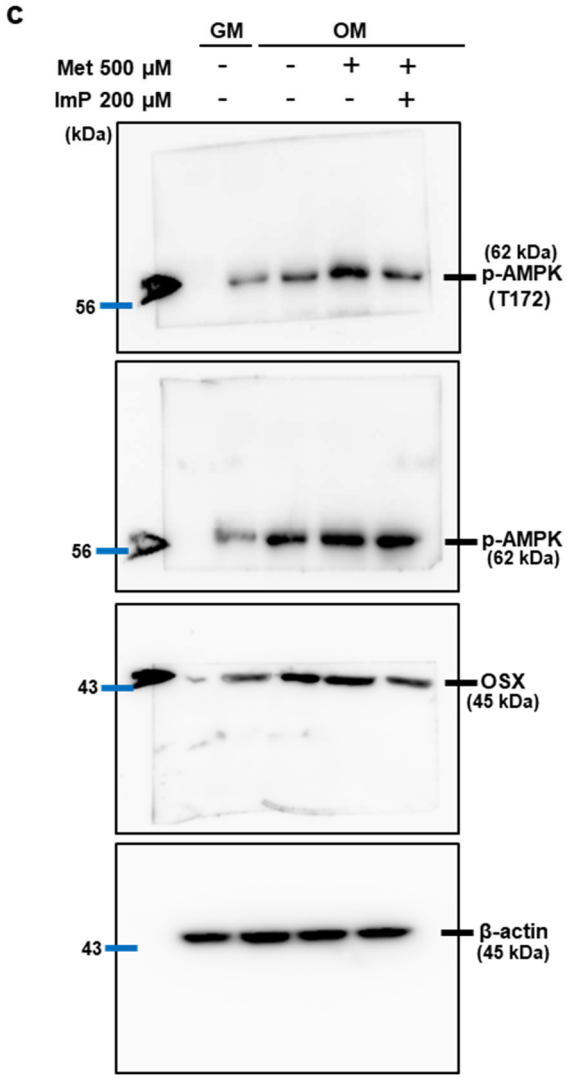
Supplementary Figure 5



Supplementary Table 1. List of Primers

Alp (229 bp)	(F) 5'-TACATTCCCCATGTGATGGC-3'
	(R) 5'-ACCTCTCCCTTGAGTGTGGG-3'
Bsp (425 bp)	(F) 5'-ACACTTACCGAGCTTATGAG-3'
	(R) 5'-AGGTTCCCCGTTCTCACTTT-3'
Oc (147 bp)	(F) 5'-GTTTGTAGGCGGTCTTCAAGC-3'
	(R) 5'-GCAATAAGGTAGTGAACAGAC-3'
Osx (288 bp)	(F) 5′-TGAGGAAGAAGCCCATTCAC- 3′
	(R) 5′-ACTTCTTCTCCCGGGTGTG-3′
Runx2 (288 bp)	(F) 5′-TCTCCAACCCACGAATGCACTA-3′
	(R) 5′-ATAGCGTGCTGCCATTCGAGGT-3′
AdipoQ (163 bp)	(F) 5′-CCTGGAGAAGCCGCTTATGT-3′
	(R) 5′-AGAGTCCCGGAATGTTGCAG-3′
Pparγ2 (103 bp)	(F) 5′-TCGCTGATGCACTGCCTATG-3′
	(R) 5′-GAGAGGTCCACAGAGCTGATT-3′
Glut4 (173 bp)	(F) 5′-AATGTCCTTGCTCCAGCTCC-3′
	(R) 5′-CAGCTCCTATGGTGGCGTAG-3′
Fabp4 (168 bp)	(F) 5′-TACATGAAAGAAGTGGGAGTG-3′
	(R) 5′-GGTGATTTCATCGAATTCCAC-3′
CtsK (249 bp)	(F) 5′-TACCCATATGTGGGCCAGGA-3′
	(R) 5′-ATAGCCCACCACCAACACTG-3′
Trap (419 bp)	(F) 5′-TCCGTGCTCGGCGATGGACCAGA-3′
	(R) 5′-CTGGAGTGCACGATGCCAGCGACA-3′
β-actin (458 bp)	(F) 5′-TTCTTTGCAGCTCCTTCGTTGCCG-3′
	(R) 5′-TGGATGGCTACGTACATGGCTGGG-3′
p38γ (100 bp)	(F) 5′-CAGAGTGCAGAGGCCAAGAA-3′
	(R) 5′-GATTCACAGCCTGAGGGCTT-3′
18S rRNA	(F) 5′-GGCCGTTCTTAGTTGGTGGA-3′
	(R) 5′-CCCGACATCTAAGGGCATC-3′