

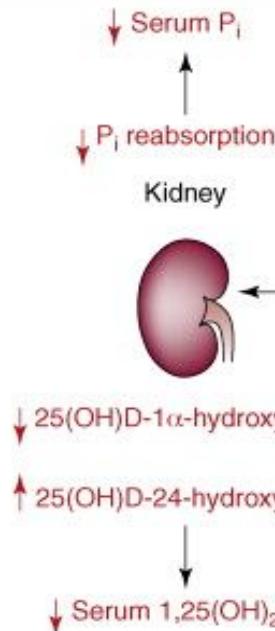
MC4R-dependent suppression of appetite by bone-derived lipocalin 2

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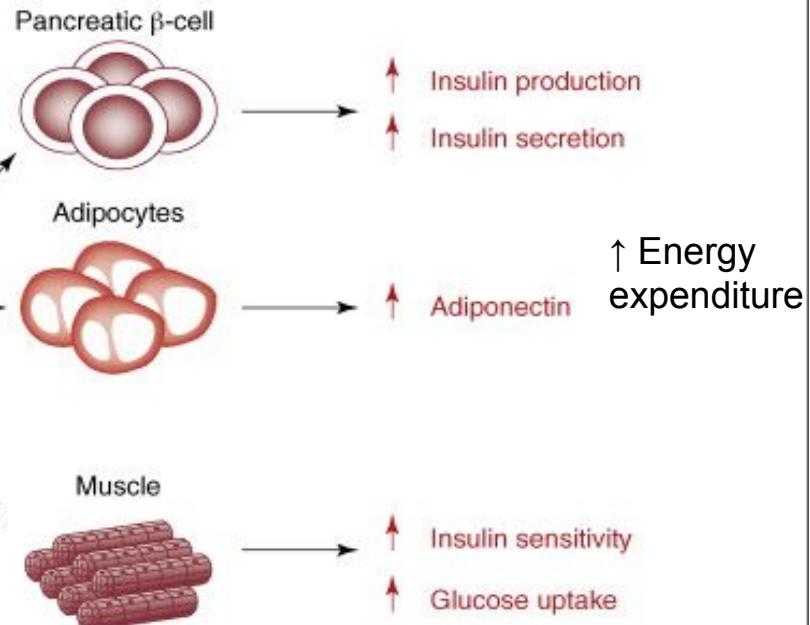
Qiman Gao, Samantha Robinson, Caitlin Anderson, Chia An Lin & Diana Lin
November 20th 2017

Bone as an endocrine organ

Osteocytes and osteoblast



Osteoblast

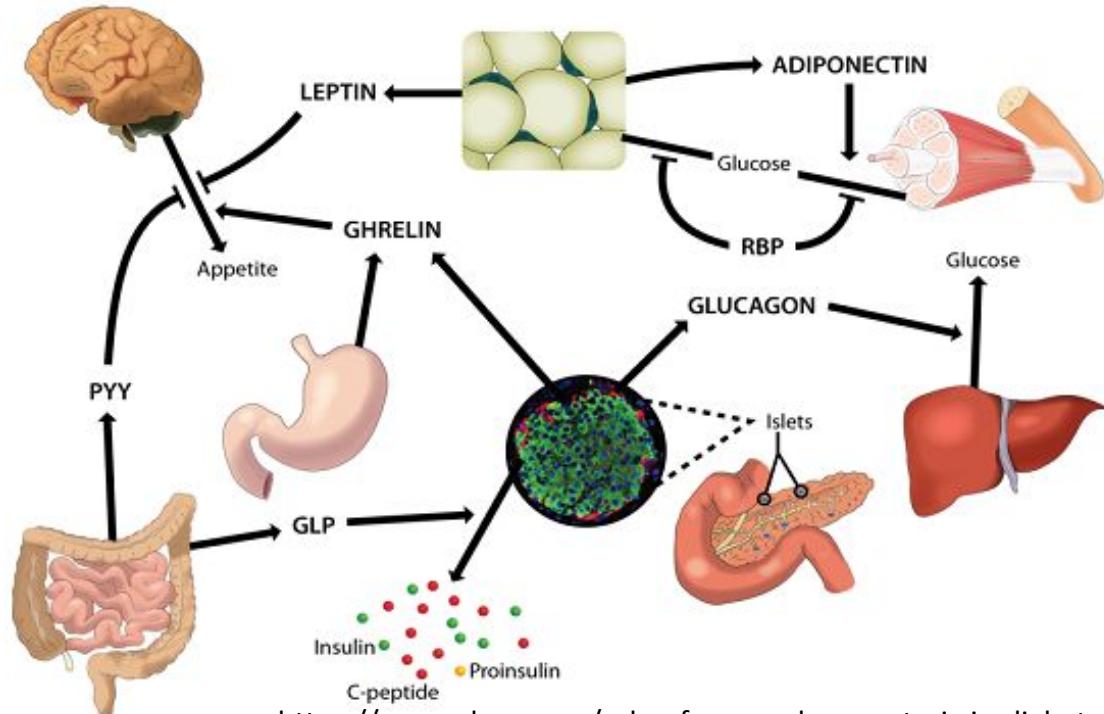


Q: How many other bone-derived hormones exist?

Hunger pathway->Appetite-> Food intake.

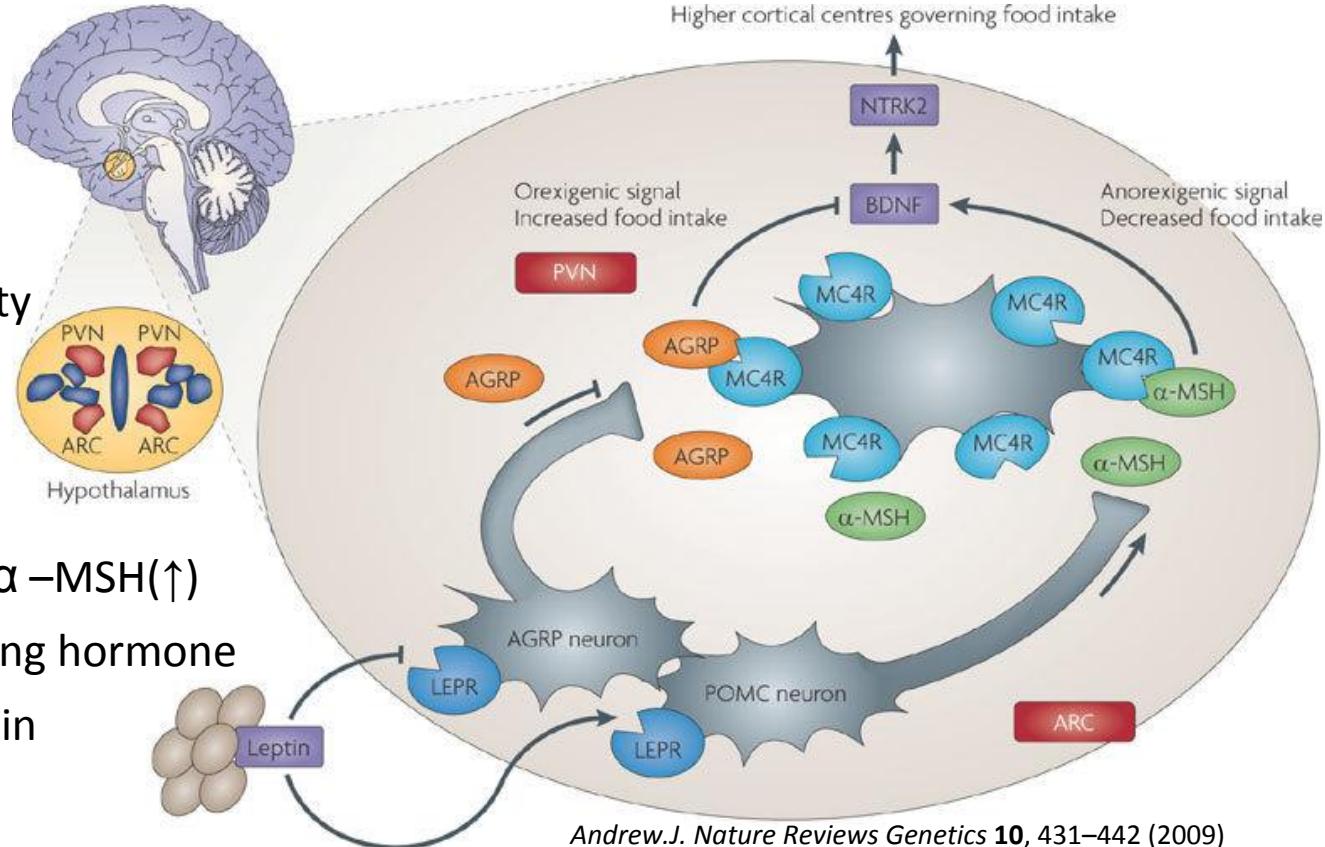
Hypothalamus ↔ Organs

- Pancreas : insulin \downarrow & glucagon \uparrow
- Adipose cells: Leptin (\downarrow appetite)
- Gastrointestinal tract: Ghrelin (\uparrow appetite)

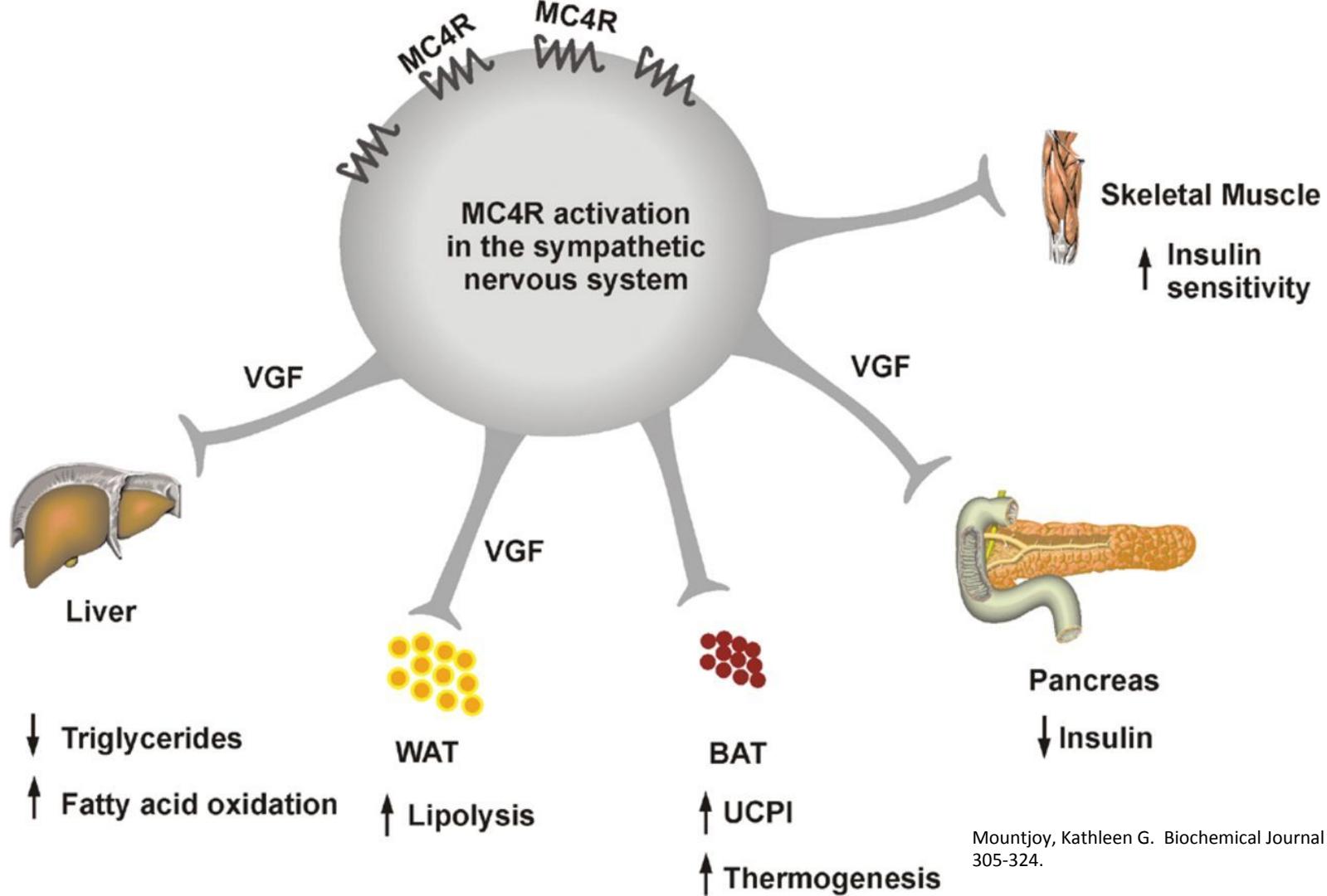


MC4R-The melanocortin 4 receptor

- Paraventricular nucleus(PVN) of hypothalamus
- Regulates (anorexigenic):
 - Food intake
 - Body weight
 - Caloric efficiency
- Mutation of MC4R -> obesity
- Deficiency of Mc4r
-> hyperphagic obesity
- Regulated by AGRP(\downarrow) and α -MSH(\uparrow)
- MSH=melanocyte-stimulating hormone
- AGRP=Agouti-related protein
- Leptin



Andrew J. Nature Reviews Genetics 10, 431–442 (2009)

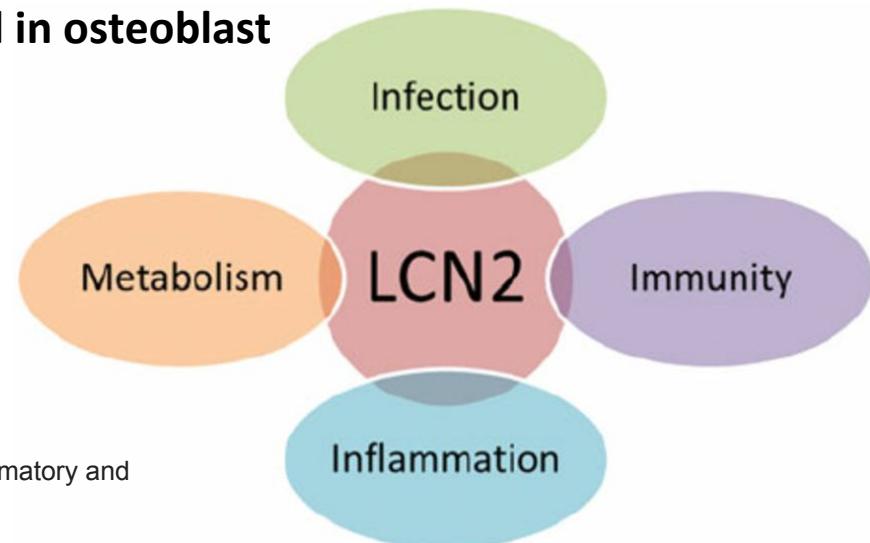


Hypothesis

- The identification of osteocalcin as a regulator of energy metabolism indicates that other hormones synthesized by bone cells may potentially affect additional aspects of energy metabolism
- ->Is there any other bone-derived hormones exist?
- The new founding defensin β 3 as a MC4R ligand reveals that the MC4R-modulating proteins controlling food intake may not be complete
- -> What else ligands for MC4R?

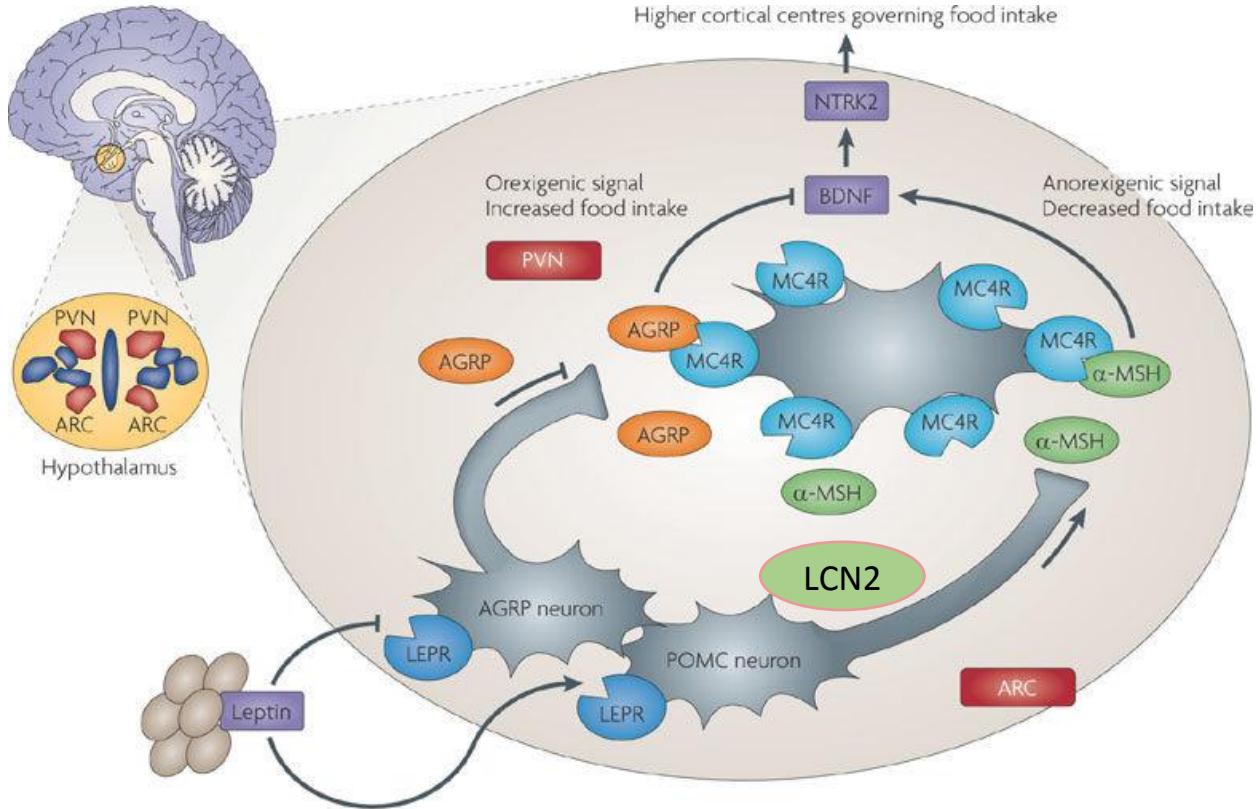
Lipocalin-2 (LCN2)

- Lipocalin-2 (LCN2), is a secreted glycoprotein that belongs to a group of transporters of small lipophilic molecules in circulation.
- First found in neutrophils
- LCN2 has been recently characterized as an adipose-derived cytokine
- LCN2 is an attractive biomarker of: immunity, infection, metabolism, inflammation.
- **The receptors for LCN2 are not fully clear**
- **In this paper: LCN2 is found highly expressed in osteoblast**



Objective

- To identify anorexigenic signals that originate from osteoblast:LCN2
- LCN2 binds to MC4R similar to α -MSH

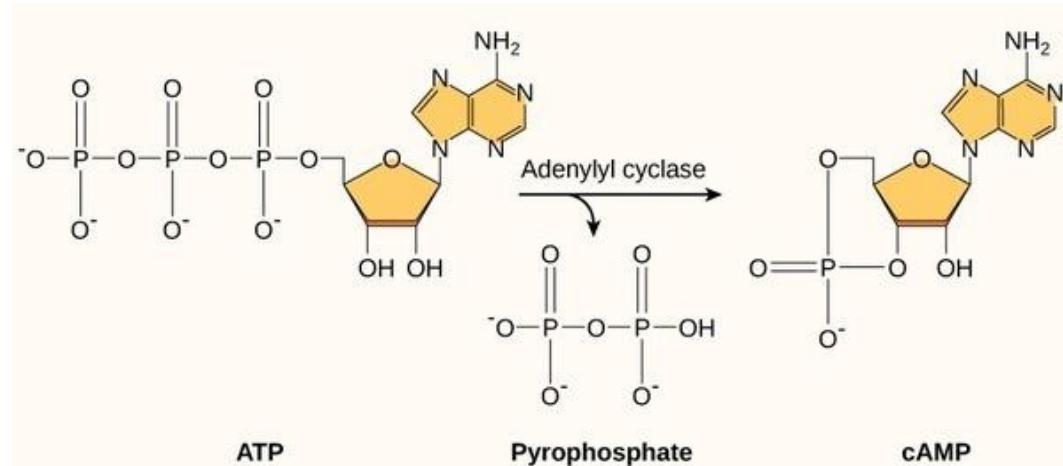


Foxo1 gene

- Encode **Forkhead box protein O1**
- FOXO1 is a transcription factor that plays important roles in regulation of gluconeogenesis and glycogenolysis by insulin signaling, and is also central to the decision for a preadipocyte to commit to adipogenesis [Nakae J, 2003]
- Mice with osteoblast-specific knockdown of Foxo1 (**Foxo1_{osb} ^{-/-}**) show **improved energy metabolism** through regulation of osteocalcin [Rached, M 2010]
- **Gene that encoded LCN2** was one of the **most highly upregulated** in osteoblast bone and serum of Foxo1_{osb} ^{-/-} mice.

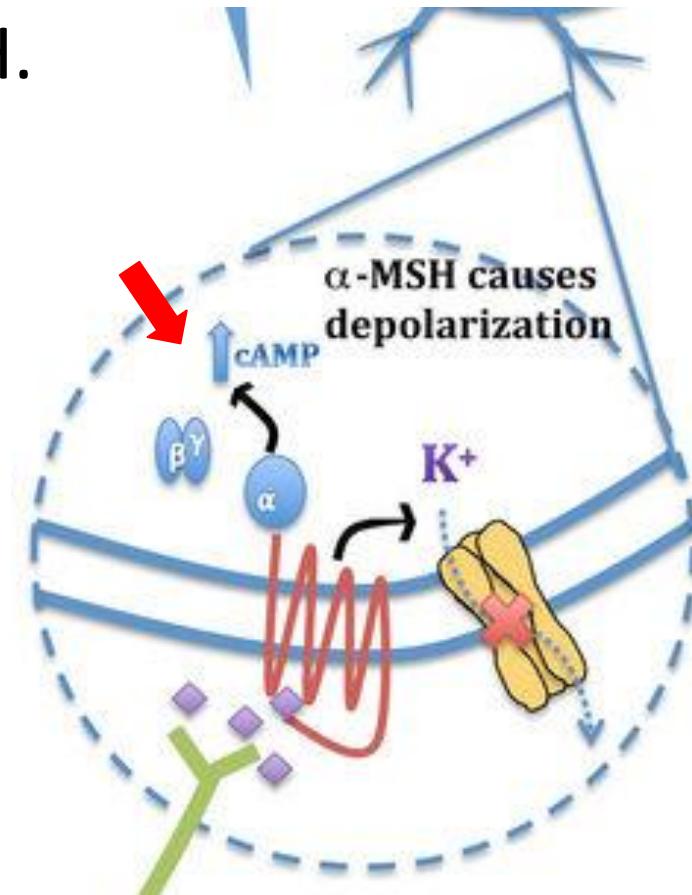
Background knowledge: cAMP

- Cyclic adenosine monophosphate (cAMP), is a derivative of adenosine triphosphate (ATP)
- cAMP is a second messenger for intracellular signal transduction in many different organisms.
- conveying the cAMP-dependent pathway.



cAMP is activated by α -MSH.

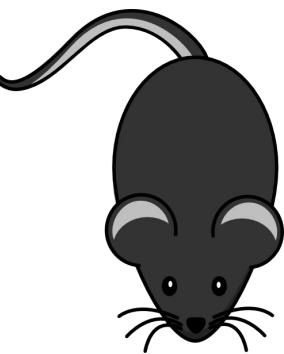
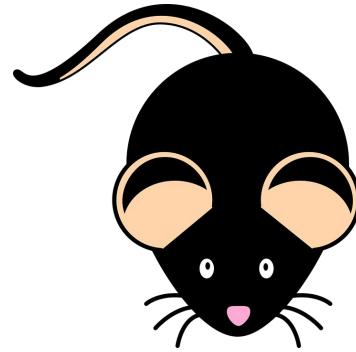
- There is another MC4R signalling pathway, involving cAMP/PKA-dependent activation of KATP channels and α -MSH-induced hyperpolarization, has been demonstrated in MC4R neurons in the dorsal motor nucleus of the vagus in the brainstem
- [Doronin, et al. Journal of Biological Chemistry 279.46 (2004): 48231-48237.]



A model for α -MSH and AgRP signalling at PVN MC4R neurons [GL Masoud, 2015. Nature 520.7545: 94-98.]

Background knowledge: Cre-recombinant

- Cre= Cyclization recombination
- Catalyze a recombination between 2 loxP sites
- loxP= locus of crossing ([x-ing]-over) Bacteriophage P1
- Used for gene translocation, inversion
- Gene Deletion = floxed
- Promoter-regulated Cre: The promoter region defines the areas in which Cre will be expressed. eg: Mouse alpha1(I)-collagen promoter can drive efficient Cre recombinase expression specifically in osteoblast.



To generate mice with:

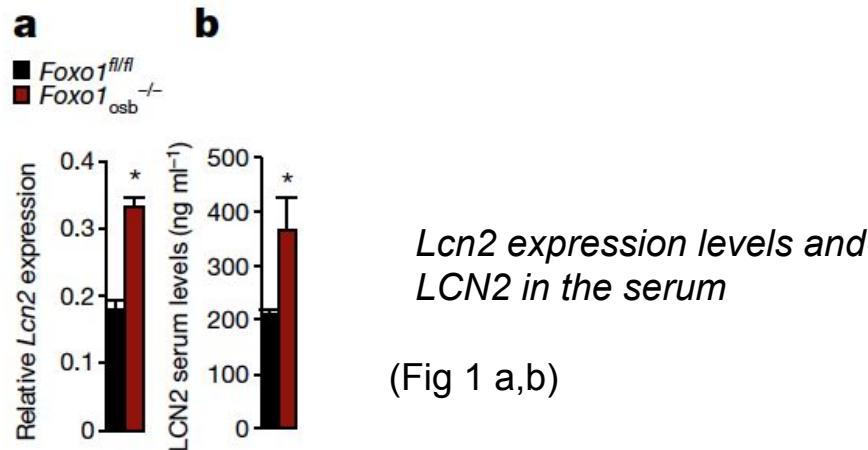
Osteoblast-specific
Lcn2osb+/-
Lcn2(OC)osb+/-

Adipocyte-specific
Lcn2fat+/-

Global deletion of
Lcn2Lcn2+/-

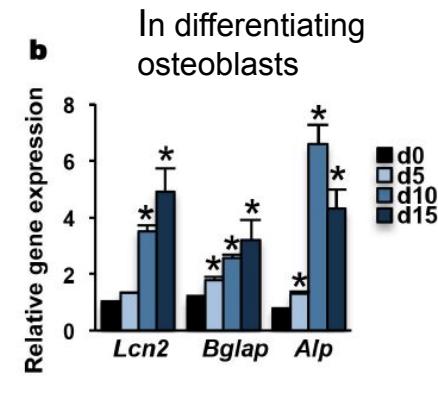
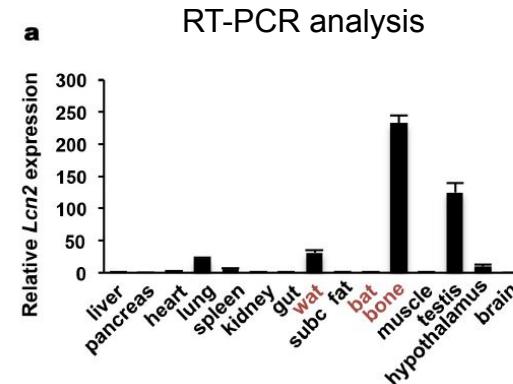
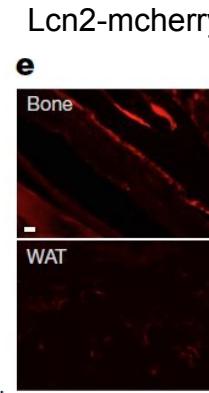
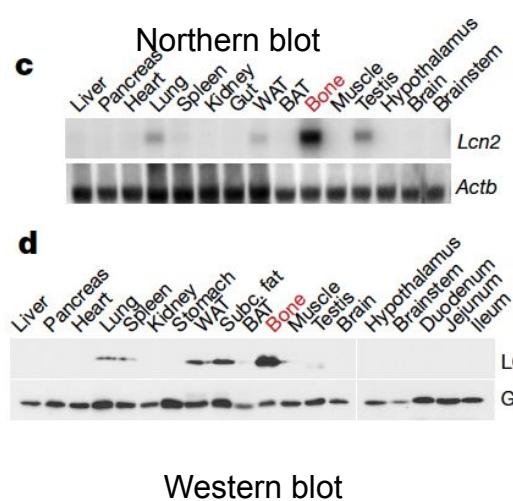
LCN2 is an osteoblast-enriched hormone

- Osteoblast-specific knockdown of $Foxo1_{osb}^{-/-}$ (key transcription factor)
 - Improved energy metabolism (in part because of osteocalcin activation)
 - Researchers searched for osteoblast secreted molecules that regulate energy homeostasis downstream of FOXO1
 - Among them: LCN2 was the most highly upregulated in osteoblast cells, bone and serum of $Foxo1_{osb}^{-/-}$



What was the confusion?

- LCN2 was previously considered an adipokine that is associated with obesity
- Analyzed LCN2 expression in all tissues
 - Bone is the predominant organ where *Lcn2* is expressed (fig 1 c-e & ext 1a)
 - LCN2 expression increases with osteoblast differentiation → similar to osteocalcin and alkaline phosphatase (ext 1b)
 - Conclusion: LCN2 is an osteoblast enriched secreted protein that is upregulated in *Foxo1_{osb}*^{-/-}

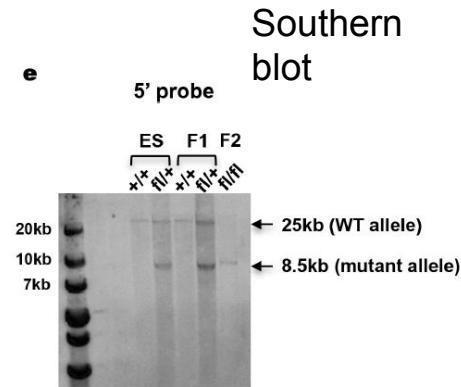
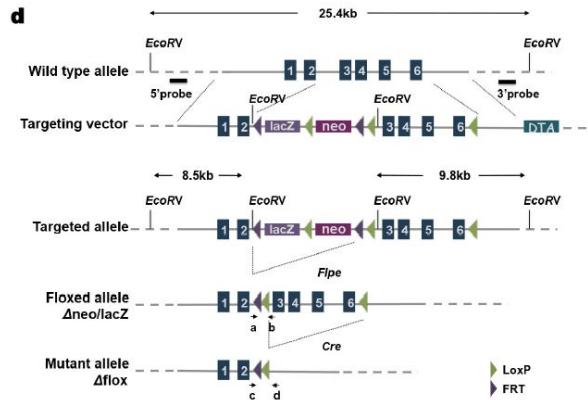


Ext. 1 a,b

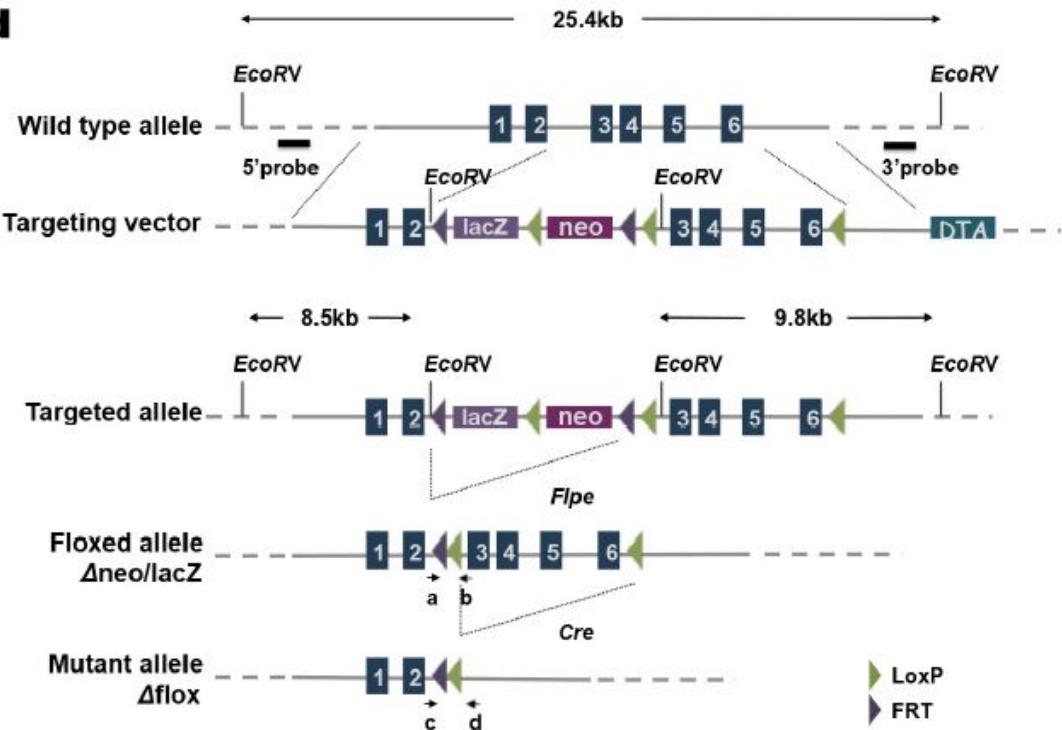
Fig 1 c-e

The cellular origin of LCN2: adipocytes or osteoblasts?

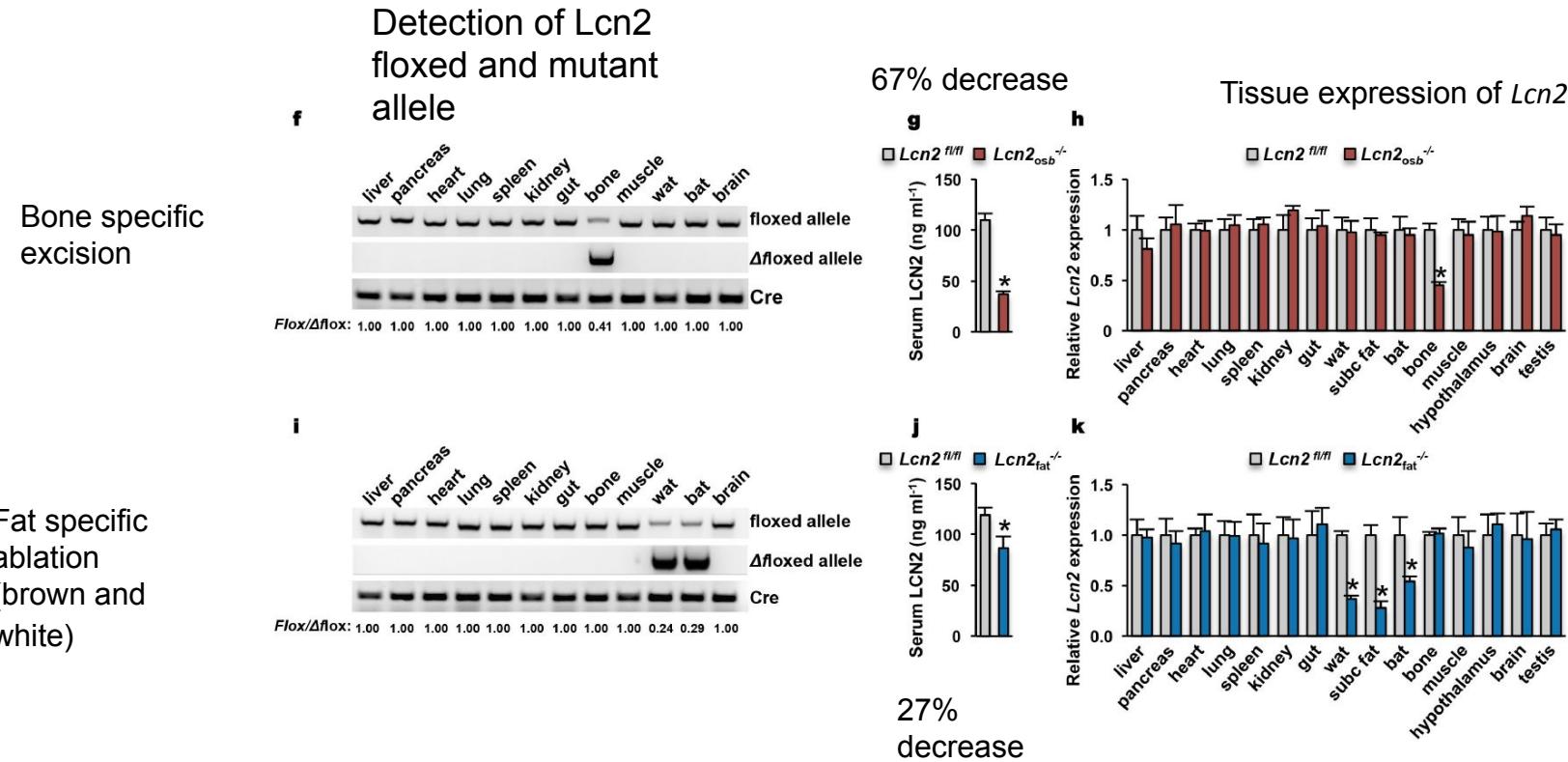
- Determine the cellular origin of LCN2
 - What cells are most contributing
- Generated mice that lacked:
 - LCN2 in osteoblasts ($Lcn2_{osb}^{-/-}$) and adipocytes ($Lcn2_{fat}^{-/-}$) (ext fig 1- d-e) using Cre-LoxP system
- Measured the decrease in serum levels of LCN2



ext fig 1- d-e

d

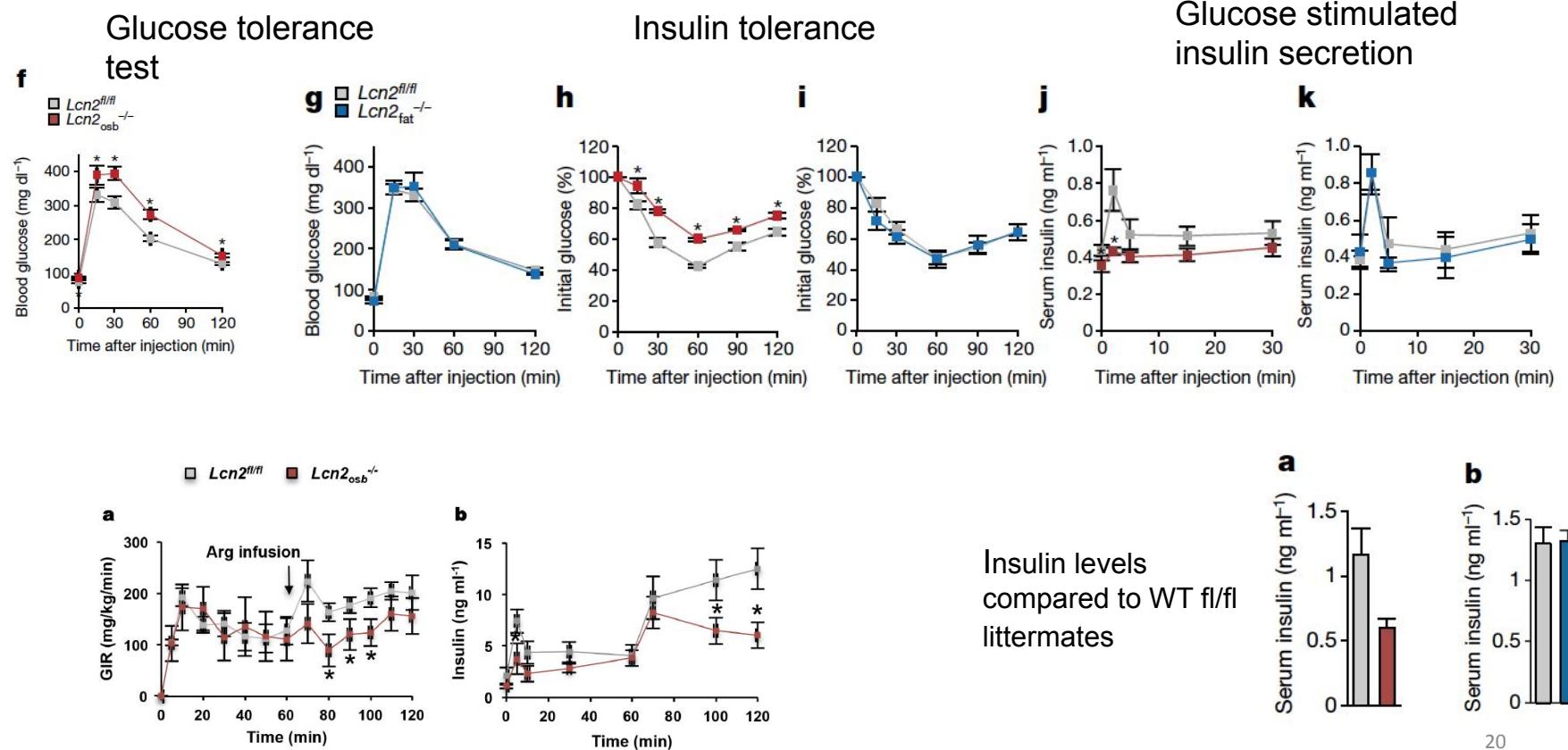
Tissue expression of *Lcn2* and decrease in serum LCN2 levels as compared to fl/fl littermates



Phenotypes: $Lcn2_{osb}^{-/-}$ and $Lcn2_{fat}^{-/-}$: glucose metabolism

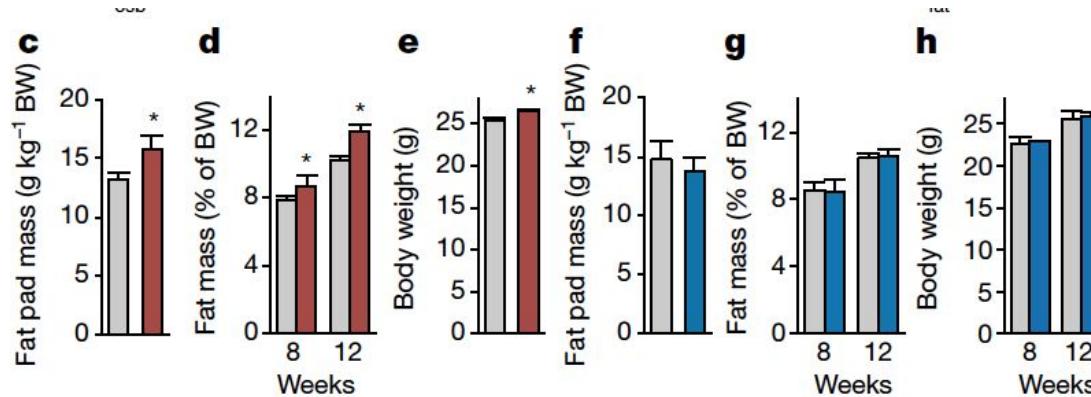
- **Purpose:** examine glucose metabolism in $Lcn2_{osb}^{-/-}$ and $Lcn2_{fat}^{-/-}$
- $Lcn2_{osb}^{-/-}$
 - Compromised glucose metabolism
 - Decreased glucose tolerance and insulin sensitivity
 - A lack of insulin secretion after glucose or arginine challenge
 - 50% reduction in insulin levels
 - Islet numbers and size, beta-cell mass and beta-cell proliferation were decreased
- $Lcn2_{fat}^{-/-}$
 - None of these parameters were affected

Glucose metabolism analysis



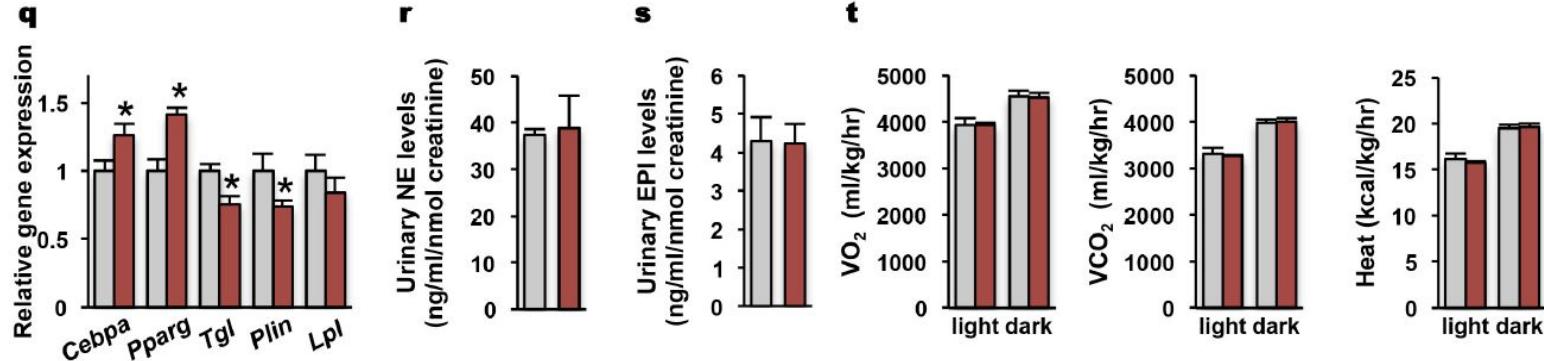
Body weight in $Lcn2_{osb}^{-/-}$ and $Lcn2_{fat}^{-/-}$

- $Lcn2_{osb}^{-/-}$
 - Increased gonadal fat weight (16.5%)
 - Total fat mass (19.6%)
 - Body weight (5%)
 - Adipogenic factors increased and lipolytic factors decreased
- $Lcn2_{fat}^{-/-}$
 - no observed effects



$Lcn2_{osb}^{-/-}$ mice

■ $Lcn2^{fl/fl}$ ad lib ■ $Lcn2_{osb}^{-/-}$



- Expression of adipogenic factors increased and lipolytic factors decreased
- Energy expenditure and sympathetic nervous system activity were comparable to WT littermates

Appetite/ food intake in $Lcn2_{osb}^{-/-}$ mice

Purpose: to see the effect of LCN2 on appetite

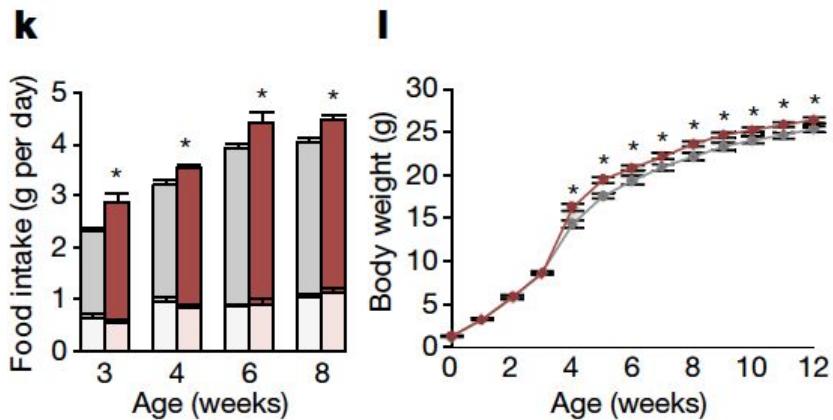


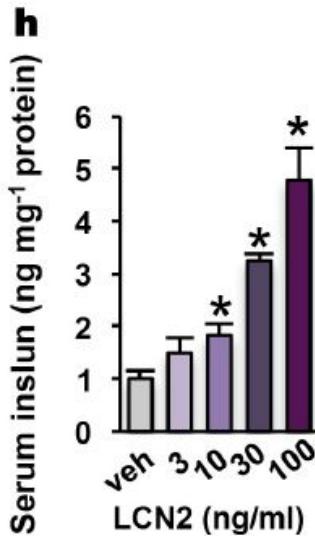
Fig 2 k,l

- 16.4% increase in food intake at 3 months
- Food intake was not affected in $Lcn2_{fat}^{-/-}$
- During growth, food intake was increased by 23.7% in 3-week old osteoblast knockout mice
- Preceded the increase in body weight that manifested at 4 weeks of age
- Conclusion: Neither bone marrow or white fat contribute to the LCN2 in circulation that is able to regulate appetite and glucose metabolism.

Pair feeding $Lcn2_{osb}^{-/-}$ mice with WT littermates

- Feed them the same amount as WT mice
- Body weight, fat mass and insulin sensitivity were normalized
- Insulin levels in the serum and insulin secretion after glucose load remained compromised
- Is LCN2 signalling in beta cells directly?

Does LCN2 signal in beta cells directly?



- Monitored expression of insulin secretion in pancreatic islets treated with increasing doses of LCN2
- Purpose: does LCN2 stimulate insulin secretion directly in primary pancreatic islets?
- Result: yes it does
- LCN2's ability to improve glucose tolerance probably reflects direct action on pancreatic islet cells

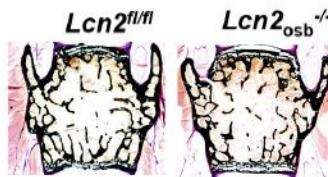
Verifying the phenotype: using another Cre driver (Bglap-Cre)

- These mice reproduce the phenotype seen in the $Lcn2_{osb}^{-/-}$
- Bone mass and osteocalcin expression & activity → not affected
- **They wanted to validate in a driver that expressed cre recombinase at later points but was also osteoblast specific to corroborate results**
 - Data came to the same conclusions

Is metabolic phenotype caused by bone defect?

- Bone mass as well as osteocalcin expression and activity were not affected in $Lcn2_{osb}^{-/-}$ mice
- Metabolic phenotype is not caused by a bone defect or changes in osteocalcin activity

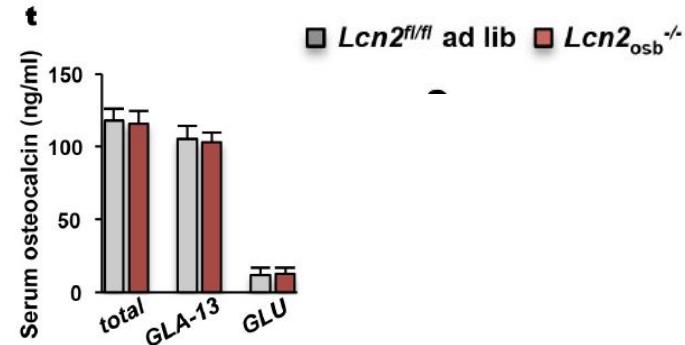
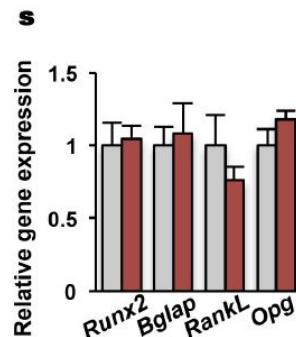
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BV/TV (%) 14.57 ± 0.38 14.13 ± 1.01

Ob.N/T.Ar (#/mm²) 104.29 ± 6.82 101.90 ± 13.08

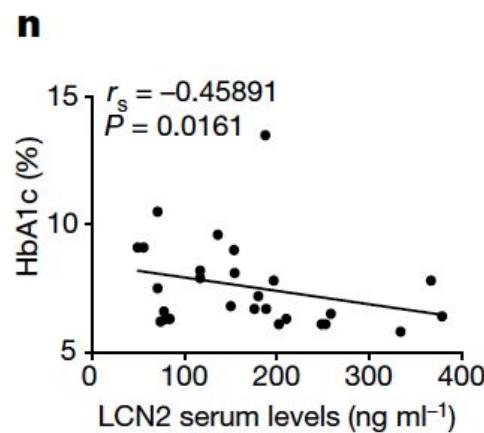
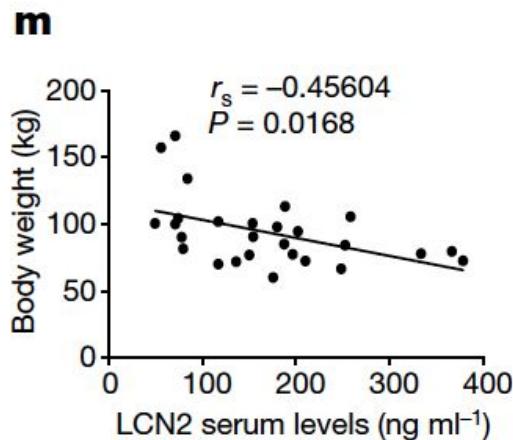
OcS/BS (%) 11.23 ± 1.87 11.22 ± 1.93



Ext. data Fig 4 r-t

Validating these results in humans

- Serum levels of LCN2 inversely correlates with body weight and glycated haemoglobin in patients with type 2 diabetes mellitus (which is usually increased in people with uncontrolled type II diabetes)

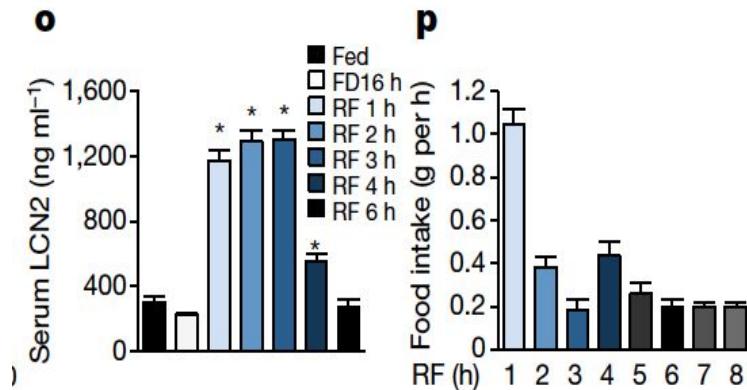


Physiological role for LCN2 in feeding regulation:

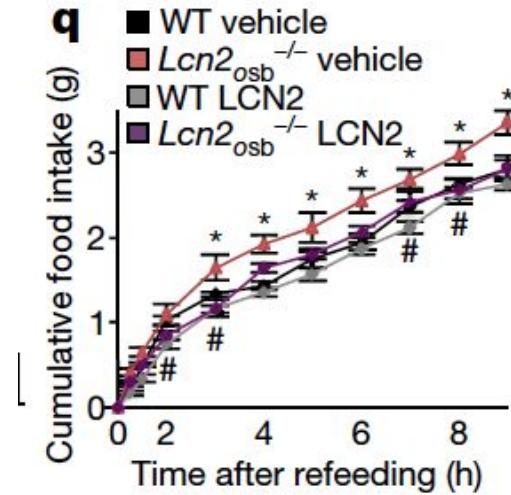
- 3x increase in serum LCN2 1-3 hrs after feeding WT mice after overnight fasting
 - Correlated with a 1.6 fold increase in *Lcn2* expression in bone
 - Not in other tissues
- Similarly, food intake was suppressed 1-3 hours after feeding these WT mice (the time points match)
- *Lcn2*_{osb}^{-/-} had higher food intake at all of the time points after refeeding
 - Twofold higher rate at 2h after feeding
- Intraperitoneal administration of LCN2 to fasted *Lcn2*_{osb}^{-/-} mice immediately after refeeding suppressed food intake with 1 hr. as efficiently as in WT mice

LCN2 in feeding regulation

In WT mice after
refeeding w/ overnight
fast

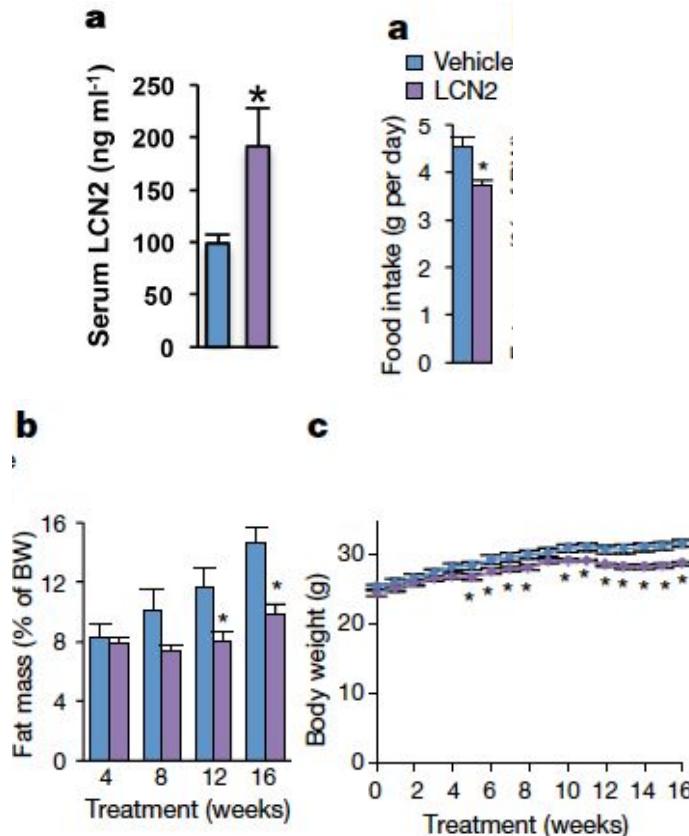


An increase in LCN2 in the serum by 3 hours correlated with a decrease in food intake



Lcn2^{osb} had higher food intake at all of the time points after refeeding

Effects of exogenous LCN2 treatment



Aim: Does exogenous LCN2 exert a sustained anorexigenic effect?

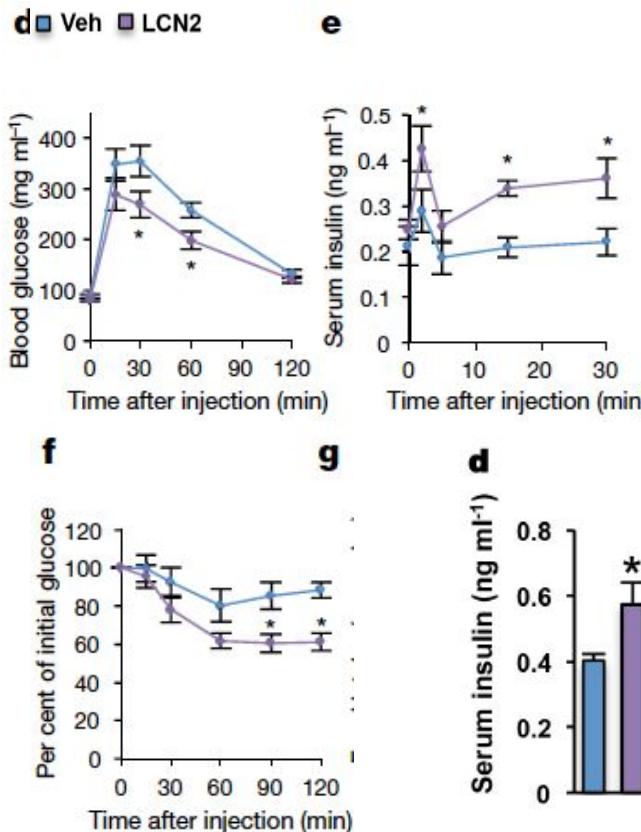
Treatment: LCN2 (150ng g⁻¹ per day) or vehicle

Time: 16 weeks, daily injections into body cavity

Effects:

- 2 fold increase in LCN2 serum levels
- 18% decrease in food intake,
- Decreases in:
 - Fat mass (32%)
 - Body weight (9.4%)
 - body-weight gain (34%)

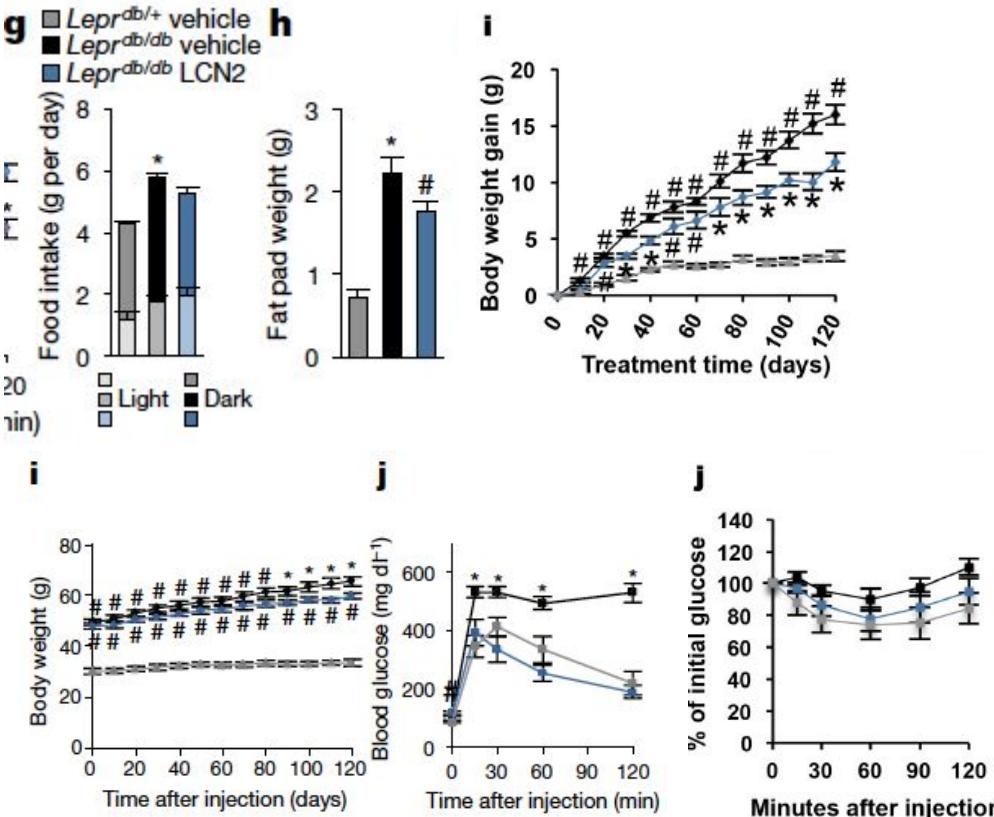
Effects of exogenous LCN2 treatment



Effects of exogenous LCN2 on Glucose and Insulin:

- Increased:
 - circulating insulin levels (d2)
 - glucose tolerance (d1)
 - insulin secretion (e)
 - insulin sensitivity (f)
 - energy expenditure

LCN2 suppresses food intake in obese mice



Lepr^{db/db} mice have an inactive leptin receptor
Used as a model of obesity and diabetes

Effects:

Suppressed:

- food intake (16.5%)
- gonadal fat (22%)
- body-weight gain (26%)

Glucose/Insulin:

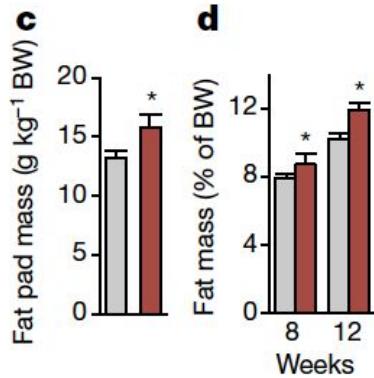
- Improved Glucose tolerance
- Improved Insulin sensitivity
- Improved energy expenditure

Conclusion:

- 1) LCN2 can counteract the effects of decreased leptin signalling on appetite and energy expenditure.
- 2) Supports observation that *Lcn2* expression is upregulated in obesity to counteract adiposity, inflammation and insulin resistance.

Can LCN2 cross the blood-brain barrier?

□ $Lcn2^{fl/fl}$ ■ $Lcn2_{obs}^{-/-}$



Creation of a global knockout

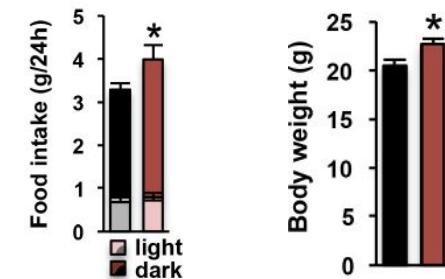
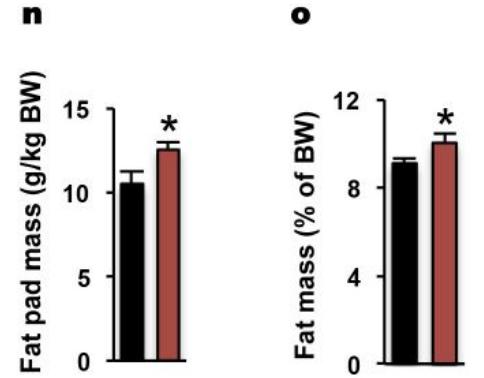
Reasoning:

- Lcn2_{obs}^{-/-} mice did not show changes in the expression of adipose or gut derived hormones that affect food intake
- LCN2 is not expressed in the hypothalamus

Similarities between the mice:

- Hyperphagia
- Entire metabolic phenotype
- Lack of changes in bone mass

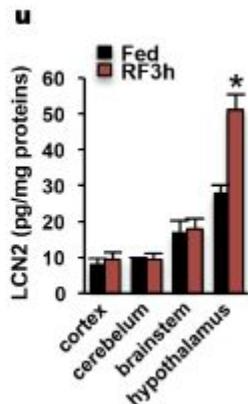
■ $Lcn2^{+/+}$ □ $Lcn2^{-/-}$



Can LCN2 cross the blood-brain barrier?

t

	LCN2 (pg/mg proteins)		
	LCN2 ^{-/-}	LCN2 ^{+/+}	LCN2 ^{+/+}
	veh	LCN2	veh
serum (ng/ml)	0.06 ± 0.04	107.05 ± 6.46	138.39 ± 22.64
cortex	0.00	5.67 ± 0.50	6.76 ± 0.56
cerebellum	0.00	8.44 ± 0.63	7.25 ± 1.53
thalamus	0.00	8.93 ± 1.13	6.32 ± 0.47
brainstem	0.00	16.96 ± 1.18	14.73 ± 0.55
hypothalamus	0.00	27.52 ± 0.60	28.16 ± 1.13



Treatment: Administered a restorative dose of LCN2 (107 ng ml⁻¹)

Observations: Within 2 hours of treatment LCN2 accumulated in:

- Brainstem
- Thalamus
- Hypothalamus

Similar to levels seen in WT

Treatment: Fasting and refeeding of WT mice

Observations: A twofold increase in LCN2 levels in the hypothalamus

LCN2 acts on the hypothalamus to suppress appetite

Treatment: Intracerebroventricular (ICV) injections of LCN2 at a restorative dose

Observations:

***Lcn2*^{-/-}**

- Normalized appetite
- Decreased body-weight gain (5%)

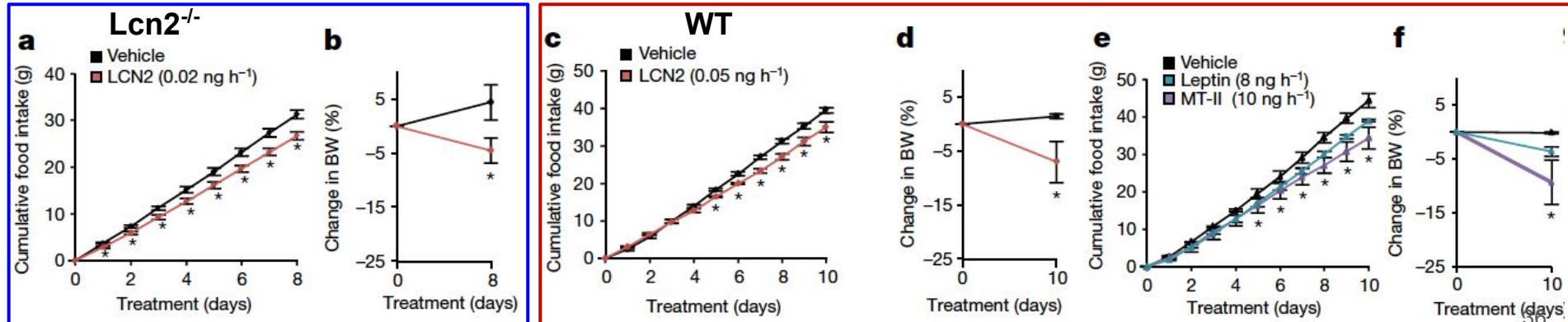
WT:

- Decreased appetite
- Decreased body-weight gain

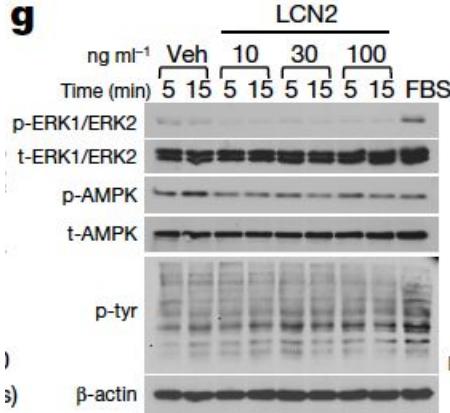
WT:

- Trends similar to ICV injections of other anorexigens :
 - Leptin
 - Melanotan II (α -MSH)

Conclusion: LCN2 suppresses appetite in the mouse by signalling directly in the brain



LCN2 activates cAMP signalling in the hypothalamus



Aim: How does LCN2 suppress food intake after signalling in the hypothalamus

Materials: GT1-7 hypothalamic cells

Observations:

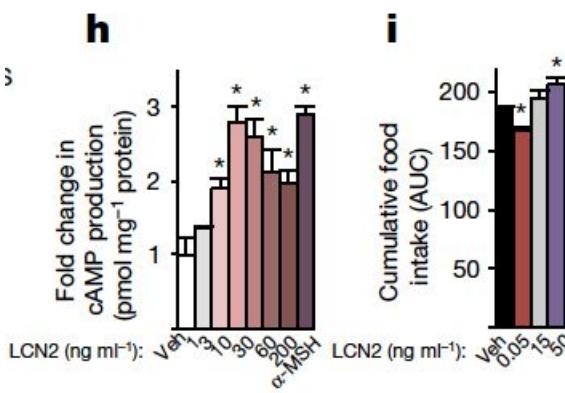
- Did not induce phosphorylation of:
 - AMPK
 - ERK1
 - ERK2
 - Tyrosine kinase
- Did activate cAMP
 - Activated with the same efficiency as α-MSH

Treatment: ICV infusion of increasing concentrations of LCN2 in WT mice

Observations:

- Same bell shaped response curve seen in appetite regulation

Conclusion: suggests receptor desensitization as seen in the case of signalling that is mediated by G-protein-coupled receptors



LCN2 activates MC4R signalling in the hypothalamus

Extended Figure 7

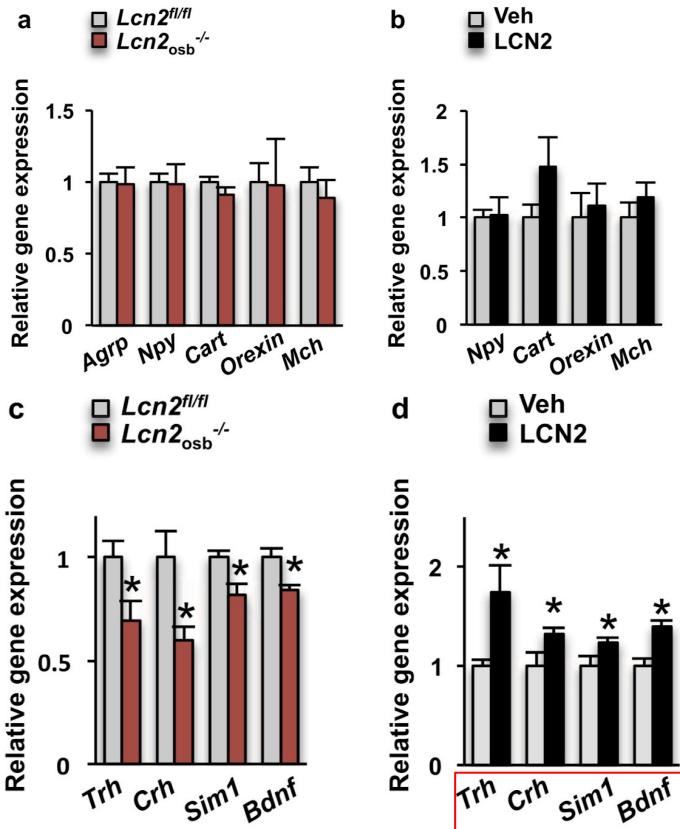
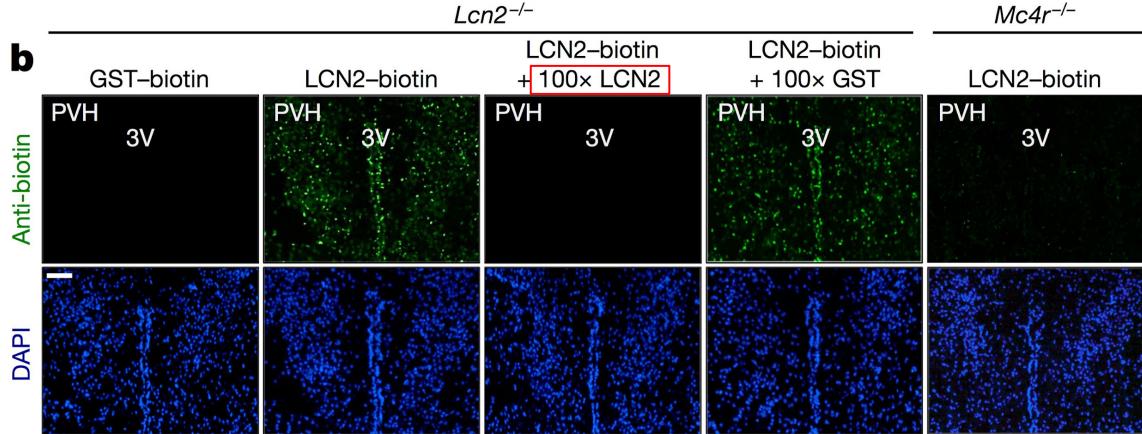


Figure 5



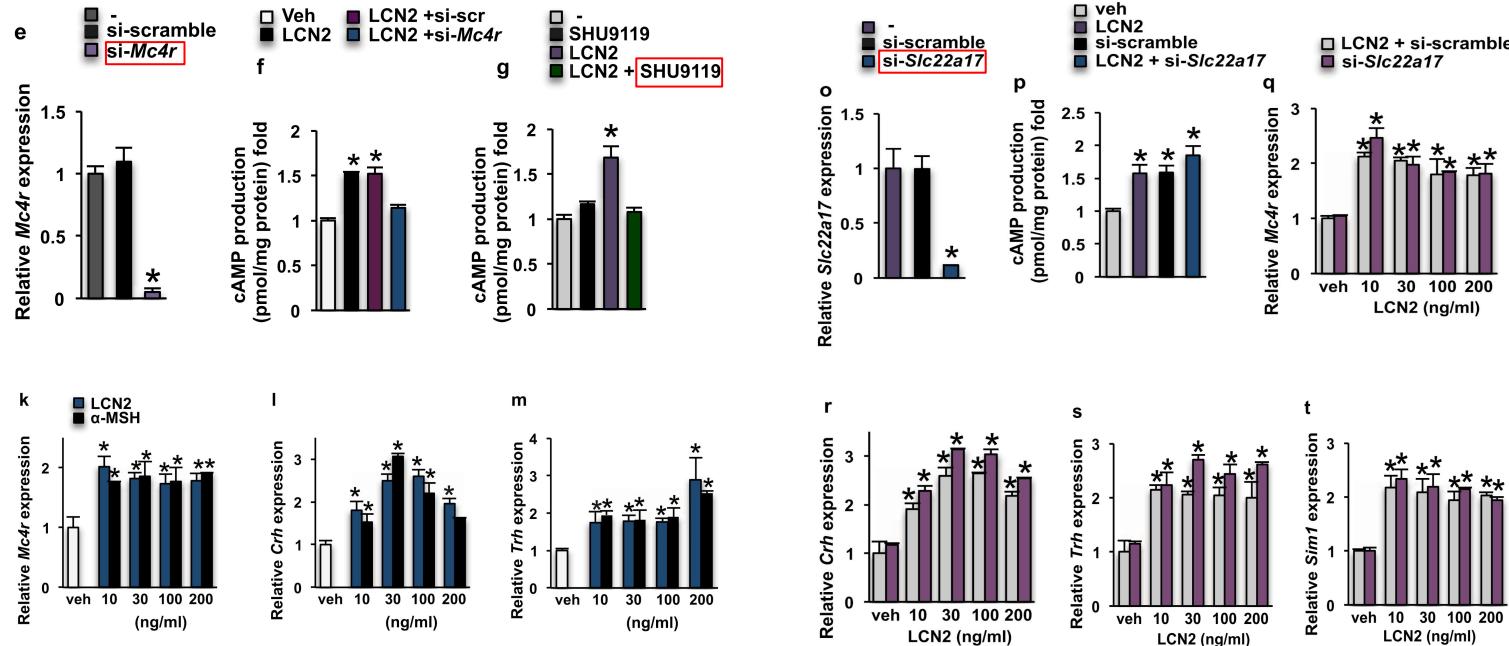
Key Points:

- Among different neuropeptides which regulate appetite in hypothalamus, only MC4R-signalling associated neuropeptides are affected upon *Lcn2* depletion or i.p. Injection of LCN2 for 16 weeks.
- LCN2 specifically binds PVH neurons, which is further strengthened from the injection of non-labelled (un-tagged) LCN2 in excess. The loss of fluorescence signal indicates that all MC4R are bound by un-tagged LCN2 instead.

∴ Localization of LCN2 and physiological effects of LCN2 are dependent on MC4R. 38

MC4R is ‘required’ for LCN2-mediated effects

Extended Figure 7



Key Points:

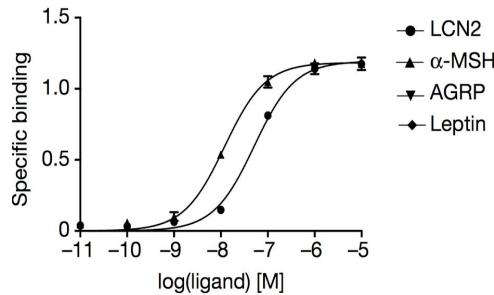
- siRNA-mediated knockdown or pharmacological inhibition of ‘ONLY’ MC4R leads to the failure of increasing cAMP production seen from LCN2 administration alone.

∴ Although LCN2 may bind several receptors, its effects seen thus far are specifically due to its binding to MC4R.

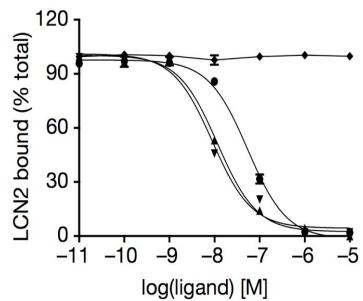
LCN2 shows specificity for MC4R in ligand binding

Figure 5

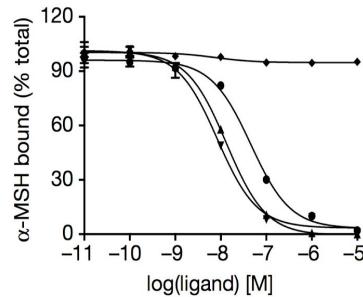
c



d



e



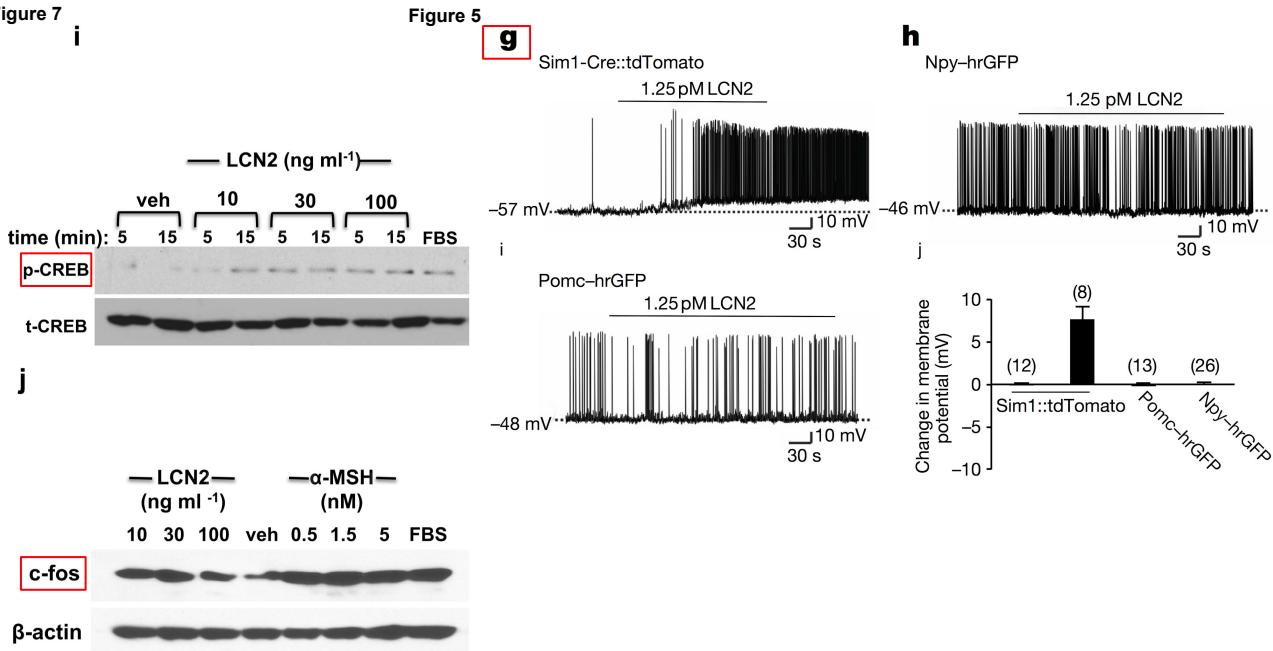
Key Points:

- Receptor-ligand binding assay illustrates that LCN2 binds MC4R at a similar specificity (affinity) compared to α-MSH, a known ligand of MC4R involved in anorexigenic signalling.
- Competitive binding assay further indicates that LCN2 or α-MSH bound MC4R can be effectively displaced by one another.

∴ Effects of LCN2 (i.e. suppression of appetite, changes in body weight, and glucose tolerance) may be the consequence of LCN2-mediated activation of MC4R signalling

How are LCN2-mediated effects carried out?

Extended Figure 7



Key Points:

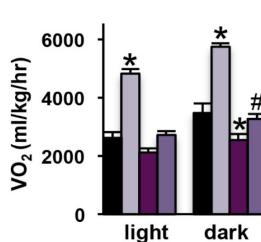
- LCN2-mediated increase in cAMP production and signalling leads to the phosphorylation of CREB (cAMP response element binding protein),

∴ LCN2-mediated effects manifested are tightly associated with neuronal activity.

MC4R is necessary for the regulation of appetite by LCN2 (1)

Extended Figure 10

a



■ WT veh □ WT LCN2 ■ $\text{Mc4r}^{-/-}$ veh □ $\text{Mc4r}^{-/-}$ LCN2

b

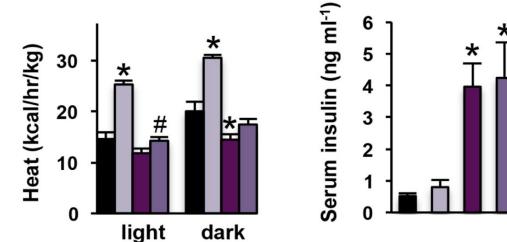
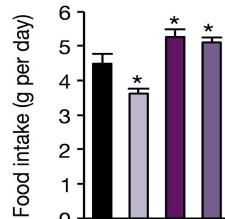


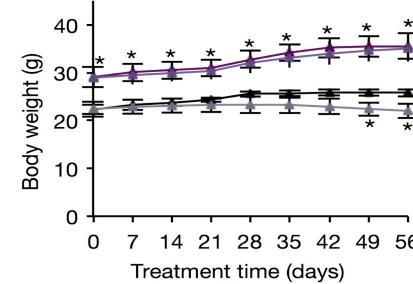
Figure 6

■ WT vehicle □ WT LCN2 ■ $\text{Mc4r}^{-/-}$ vehicle □ $\text{Mc4r}^{-/-}$ LCN2

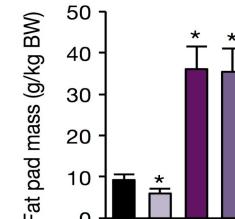
a



b

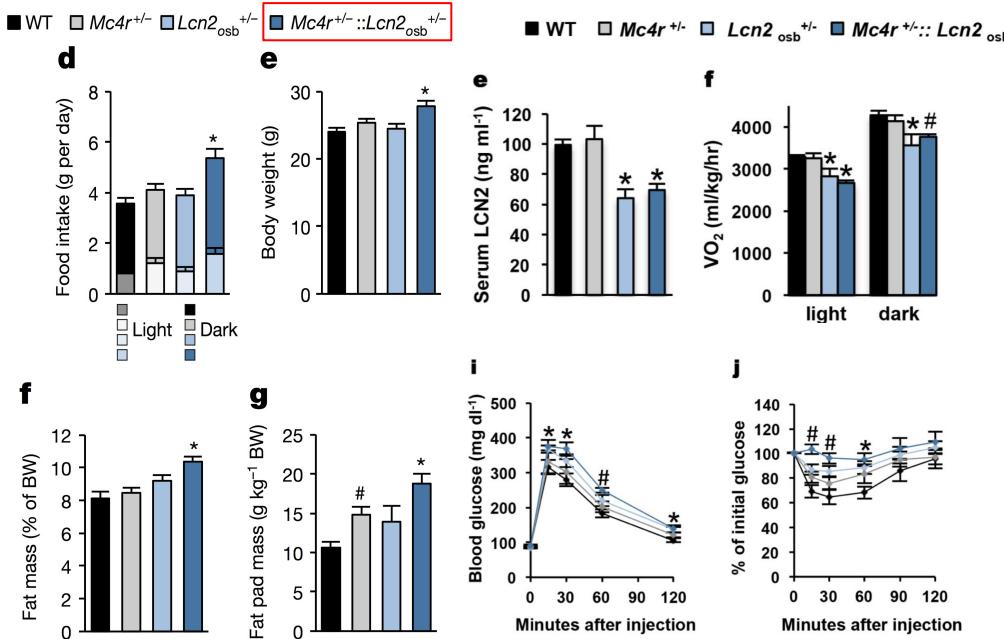


c



MC4R is necessary for the regulation of appetite by LCN2 (2)

Extended Figure 10



Key Points:

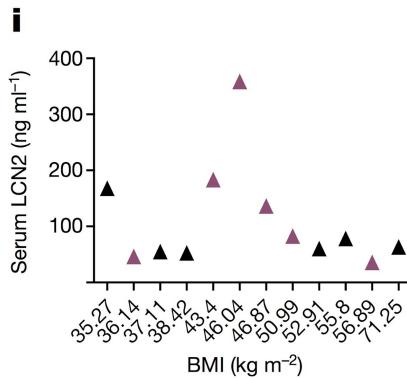
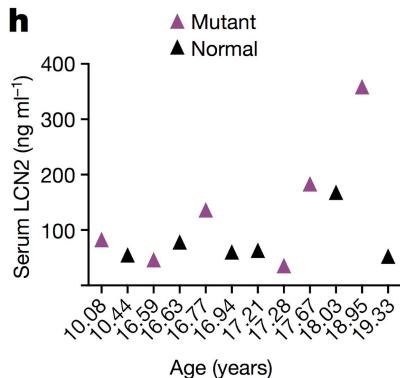
- LCN2-mediated suppression of appetite, leading to modulation of body weight gain does not occur in $Mc4r$ KO mice.
- Mouse with heterozygotic mutation on $Mc4r$ or $Lcn2$ (osteoblast-specific) were mated to produce an offspring with heterozygotic mutations on both genes.
- Similar loss of LCN2's effects are manifested in offspring mouse bearing heterozygotic mutation on both $Mc4r$ and $Lcn2$ (osteoblast-specific), where glucose tolerance is impaired.

∴ MC4R mediates anorexigenic function of LCN2 *in vivo*, where suppression of appetite by LCN2 is carried out through MC4R.

LCN2-mediated regulation of MC4R signalling in humans

I

MC4R results	Sex	Age	BMI (Kg/m ²)	LCN2 (ng/ml)
Normal	f	18.0	35.3	168.207
Normal	m	16.9	52.9	60.616
Normal	m	19.3	38.4	52.957
Normal	f	17.2	71.3	63.658
Normal	f	16.6	55.8	78.465
Normal	f	10.4	37.1	55.056
p.Phe202Leu	m	19.0	46.0	358.713
p.Cys271Arg	m	17.3	56.9	35.927
p.Asp146His	f	17.7	43.4	183.602
p.Leu139CysfsTer22	f	16.6	36.1	46.553
p.Ile251TrpfsTer34	f	16.8	46.9	136.504
p.Ile251TrpfsTer34	f	10.1	51.0	82.991



Question:

- How does LCN2 regulate MC4R signalling in humans?

Key Points:

- Plasma level of LCN2 from patients or normal individuals show 2~4 fold increased LCN2 levels in adult or pediatric patient with MC4R mutations.
- 3 out of 5 young-adult patients and 'single' pediatric patient with MC4R mutations specifically, show increased plasma LCN2 levels.

∴ LCN2-MC4R mediated effects are conserved *in vivo* and are seen in humans.

∴ While plasma LCN2 levels are increased significantly, patients with mutations in MC4R compared to control individuals (normal MC4R sequences), perfect correlation is NOT observed.

Questions left unanswered

- No experimental evidence that explains why bone would suppress food intake
 - Suggested that LCN2 may have a homeostatic role similar to that of leptin
 - Decrease in bone mass → decrease in LCN2 → increase in food intake to restore nutrients and maintain skeletal growth
- Mechanisms that regulate appetite, and the abnormal glucose metabolism and obesity are **not yet fully analyzed**
- The role of the skeleton in energy uptake and metabolism may provide new insights into the pathogenesis of these metabolic diseases

What was previously reported...

- Two papers mentioned in the discussion
 - 1. Law, I. K. et al. Lipocalin-2 deficiency attenuates insulin resistance associated with aging and obesity. *Diabetes* 59, 872–882 (2010).
 - 2. Guo, H., et al. Lipocalin-2 deficiency impairs thermogenesis and potentiates diet-induced insulin resistance in mice. *Diabetes* 59, 1376–1385 (2010).
- **Different results are still not clear**
- Methodology → differences in generating Lcn2-deficient mice (?) measurements (?)
- But... effects of addition of exogenous LCN2 support conclusions drawn from loss of function experiments

Shortcomings and pitfalls

- The study
 - Maybe additional experiments less behavioral in nature to corroborate
 - Maybe trying different diets (seeing what happens on higher fat diet?)
 - No mention of regulators upstream of LCN2 (barely touched upon TF FOXO1)
 - Insufficient to conclude that bone phenotype unaffected - more bone analysis required
 - Only looked at osteoblasts, not osteoclast involvement
 - Did not look at bone loading and unloading effects of LCN2
 - Only looked at vertebrae, not long bones
 - Should section tibia - do micro CT, 3 point bending test, trabecular thickness, etc.
- The paper
 - A large amount of supplemental figures (this could be because of the journal)
 - Making it hard to follow
 - Data could be better organized
 - Some data of the same topic was split between a main figure and extended figures
 - Arbitrarily decided which ones were main figures and which were extended

Novel contributions to the field

- Novel role of LCN2 in bone (previously only in adipocytes)
 - A tenfold increase of expression in bone when compared to fat
- Novel role of bone in glucose metabolism and appetite
- Novel bone-derived endocrine protein
- Novel ligand of MC4R

Further research to be done

- RNA processing pathway mechanism
 - Alternative splicing?
 - Post-translational modifications?
- Human studies
 - Check for mutations in *Lcn2* gene or its regulating factors
 - Is there a cohort of people with Type II Diabetes / obesity that have SEVERELY decreased LCN2 serum levels?
- Protein pathway mechanism
 - Chicken or the egg: Is it the loss of function of LCN2 causing the increase in appetite or is it the decrease in LCN2 (something upstream of LCN2) that is causing the increase in appetite
 - Is the LCN2 loss of function from *Lcn2* gene mutation or upstream regulator mutation?
 - ??? (FOXO1?) → LCN2 → MC4R → cAMP → ???

Questions?