The Obesity Susceptibility Gene *Carboxypeptidase E* Links FoxO1 Signaling in Hypothalamic Pro–opiomelanocortin Neurons with Regulation of Food Intake

Leona Plum, Hua V. Lin, Roxanne Dutia, Jun Tanaka, Kumiko S. Aizawa, Michihiro Matsumoto, Andrea J. Kim, Niamh X. Cawley, Ji–Hye Paik, Y. Peng Loh, Ronald A. DePinho, Sharon L. Wardlaw and Domenico Accili

SI Guide

Supplementary Results

Seven Supplementary Figures

One Supplementary Table

Supplementary Results

α–MSH inhibits hypothalamic *Pcsk1*

We found reduced hypothalamic *Pcsk1* mRNA and protein levels in refed *Pomc-Foxo1*$^{-/-}$ mice (**Supplementary Fig. 6a–e**). Unlike *Cpe*, the decrease of *Pcsk1* was not confined to the ARC, but was also seen in the PVN, consistent with a paracrine effect of increased α–Msh (**Supplementary Fig. 6b,c**). This decrease was unexpected, given that leptin sensitivity was increased (Fig. 2g), and that leptin increases *Pcsk1* in hypothalamic neurons. We therefore investigated whether the reduction of hypothalamic *Pcsk1* results from feedback inhibition by α–Msh. We first analyzed the effect of α–Msh on *Pcsk1* promoter activity in Neuro2A cells. Incubation with α–Msh suppressed *Pcsk1* reporter gene activity by ~60% (**Supplementary Fig. 6f**). Likewise, α–Msh reduced endogenous *Pcsk1* expression by ~50% in primary MBH cells (**Supplementary Fig. 6g**). To test if repression also occurs *in vivo*, we injected NDP–α–Msh ICV and analyzed *Pcsk1* expression. Consistent with the *in vitro* findings, NDP–α–Msh reduced *Pcsk1* in ARC and PVN (**Supplementary Fig. 6h,i**), but failed to affect *Cpe* (data not shown). These data demonstrate feedback inhibition of hypothalamic *Pcsk1* by α–Msh and suggest that reduced Pc1 in refed *Pomc-Foxo1*$^{-/-}$ mice is the result of increased α–Msh.

Supplementary Figure Legends

Supplementary Figure 1 POMC neuron-specific FoxO1 ablation. **(a)** Representative hypothalamic GFP immunohistochemistry in *PomcCre-Gt(ROSA)26Sor*^{tm2Sho} mice. 3V: third ventricle. **(b)** *Foxo1* and *Cre* genotyping. Arrows indicate loxP-flanked (*flox*, lower arrow) and recombined (Δ, upper arrow) *Foxo1* alleles. Pi: pituitary; Hy: mediobasal hypothalamus; Bs: brainstem; Cx: cortex; Cb: cerebellum; Li: liver; Pa: pancreas; Wa: white adipocyte; Ba: brown adipocyte; Sm: skeletal muscle; Co: control. **(c)** Relative pituitary *Foxo1* expression in adult mice ($n = 6$). **(d)** MBH *Pomc* and *Agrp* promoter ChIP. **(e)** Relative *Pomc* expression in pituitary of mice in (c). **(f)** Serum corticosterone levels in adult mice in the basal state and after 1-h restraint stress (females only) ($n = 6$). Data are presented as means ± SEM. * = $P < 0.05$; *** = $P \leq 0.001$ by t-test.

Supplementary Figure 2 Body length and body mass index. **(a)** Body mass index (BMI) of NCD–fed, 18-week-old female ($n = 40–71$) and **(b)** male ($n = 35–67$) mice. **(c, d)** Naso-anal body length of mice in (a, b). Data are presented as means ± SEM. ** = $P \leq 0.01$; *** = $P \leq 0.001$ by unpaired t-test.

Supplementary Figure 3 Energy expenditure and food intake. **(a)** Energy expenditure, plotted as 1-h running averages, **(b)** average energy expenditure during the light/day and dark/night phase, **(c)** total locomotion in the cage periphery, **(d)** respiratory quotient (RQ), and **(e)** percentage of NCD ingested during the light (light grey) and dark (dark grey) phase in adult male mice ($n = 8$).

Supplementary Figure 4 POMC neuron counts and neuropeptide mRNA levels. **(a)** POMC neuron numbers in 15-week-old male mice (n = 9). **(b)** Ad libitum (n = 5) and **(c)** refed (n = 14–17) MBH mRNA expression in 18–week–old male mice. Data represent mean ± SEM and are normalized by *Actb* levels. * = P <0.05 by t-test.

Supplementary Figure 5 MBH neuropeptide mRNA and peptide expression. **(a)** *Pomc* levels in 3– to 4–week–old mice (n = 4–6) and **(b)** in 4– to 5–week-old (n = 3–4) refed mice. **(c)** *Agrp* and *Pomc* in *ad libitum*-fed (n = 5) and **(d)** refed (n = 14–17) 18–week–old male mice. **(e, f)** *Agrp/Pomc* ratios in the animals shown in (c, d). Data represent mean ± SEM. mRNA levels (but not ratios) are normalized by average neuron number in each genotype. * = P <0.05 by unpaired t-test.

Supplementary Figure 6 α–MSH inhibits hypothalamic PC1. **(a)** Hypothalamic *Pcsk1* in *ad libitum*-fed (n = 5) and refed (n = 14–15) male mice. Data are normalized by *Actb* and plotted as % of *ad libitum*-fed levels in WT. **(b)** *Pcsk1* levels in ARC (n = 10–11) and **(c)** PVN (n = 5–6) of refed male mice. **(d)** Representative hypothalamic Pc1 and β-actin western blot and **(e)** quantitation of Pc1 protein levels in refed male mice (n = 13–14). **(f)** α–Msh regulates *Pcsk1*-luciferase activity in Neuro2A cells co-transfected with plasmid *pEGFP-Mc4r* to express melanocortin-4 receptors. **(g)** α–Msh regulates *Pcsk1* in primary MBH cultures (n=6–21). **(h)** *Pcsk1* expression in ARC and **(i)** PVN of adult mice following ICV injection of NDP–α–Msh or saline control (n = 9–10). Data are presented as means ± SEM. # = $P \leq 0.01$ in *ad libitum* vs. refed (same genotype) by ANOVA. * = P

<0.05; ** = $P \leq 0.01$ WT vs. *Pomc-Foxo1*$^{-/-}$ untreated vs. treated (same condition) by ANOVA.

Supplementary Figure 7 Pcsk2 expression and ghrelin levels. **(a)** MBH *Pcsk2* in refed male mice (n = 14–15). Data are normalized by *Actb* levels. **(b)** Western blot of the 64–66kDa Pc2 isoform in MBH extracts of refed male mice. Actin was used as loading control. **(c)** Serum ghrelin levels in adult male mice. Samples were obtained one hour before lights off (n = 7). Data are presented as means ± SEM.

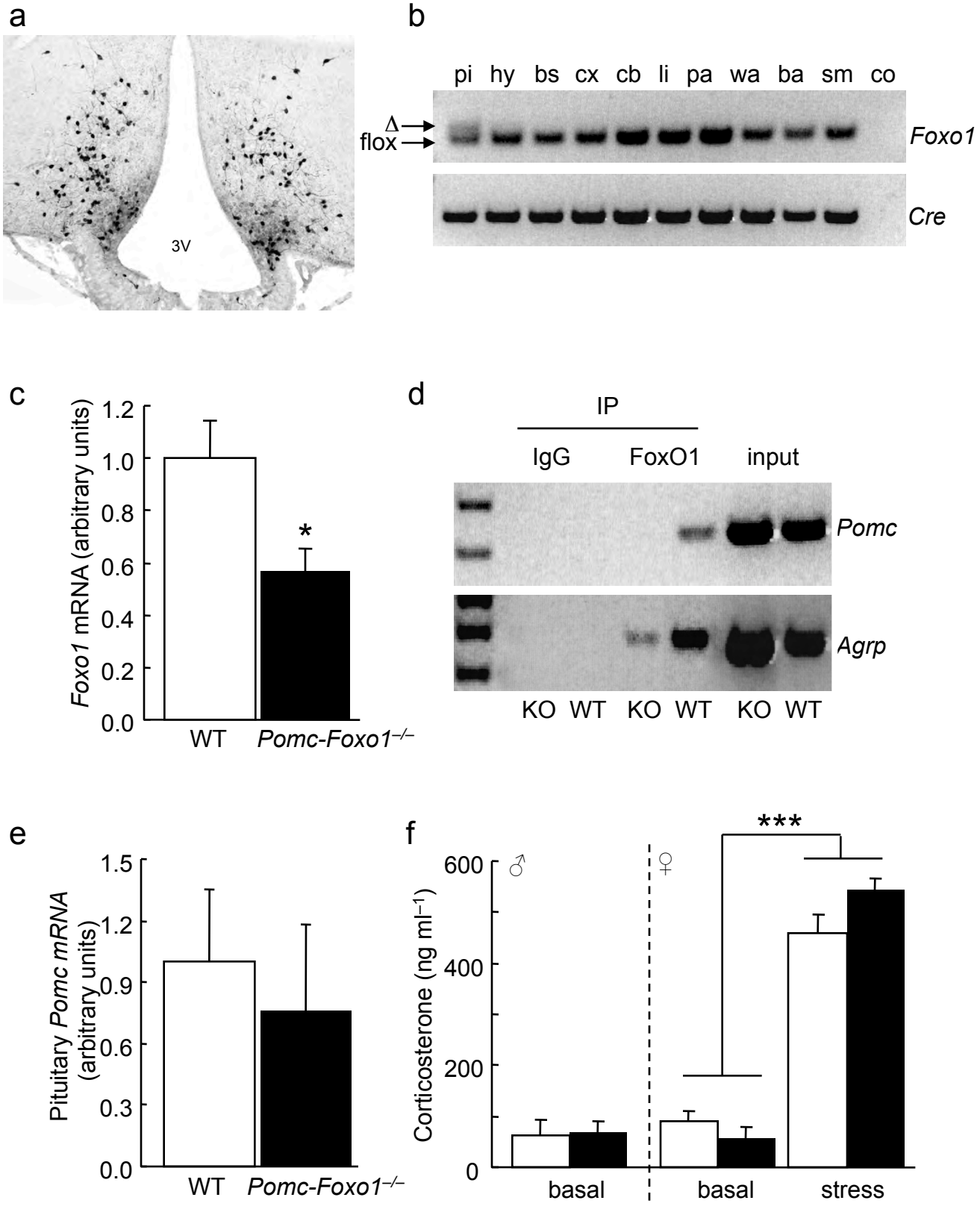
Supplementary References

1. Fox, D.L. & Good, D.J. Nescient helix-loop-helix 2 interacts with signal transducer and activator of transcription 3 to regulate transcription of prohormone convertase 1/3. *Mol Endocrinol* **22**, 1438-1448 (2008).
2. Nakashima, K., Narazaki, M. & Taga, T. Overlapping and distinct signals through leptin receptor (OB-R) and a closely related cytokine signal transducer, gp130. *FEBS Lett* **401**, 49-52 (1997).
3. Sanchez, V.C.*, et al.* Regulation of hypothalamic prohormone convertases 1 and 2 and effects on processing of prothyrotropin-releasing hormone. *J Clin Invest* **114**, 357-369 (2004).
4. Kitamura, T.*, et al.* Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. *Nat Med* **12**, 534-540 (2006).
5. Kim, E.M., Grace, M.K., O'Hare, E., Billington, C.J. & Levine, A.S. Injection of alpha-MSH, but not beta-endorphin, into the PVN decreases POMC gene expression in the ARC. *Neuroreport* **13**, 497-500 (2002).
6. Lee, M.*, et al.* Effects of selective modulation of the central melanocortin-3-receptor on food intake and hypothalamic POMC expression. *Peptides* **29**, 440-447 (2008).

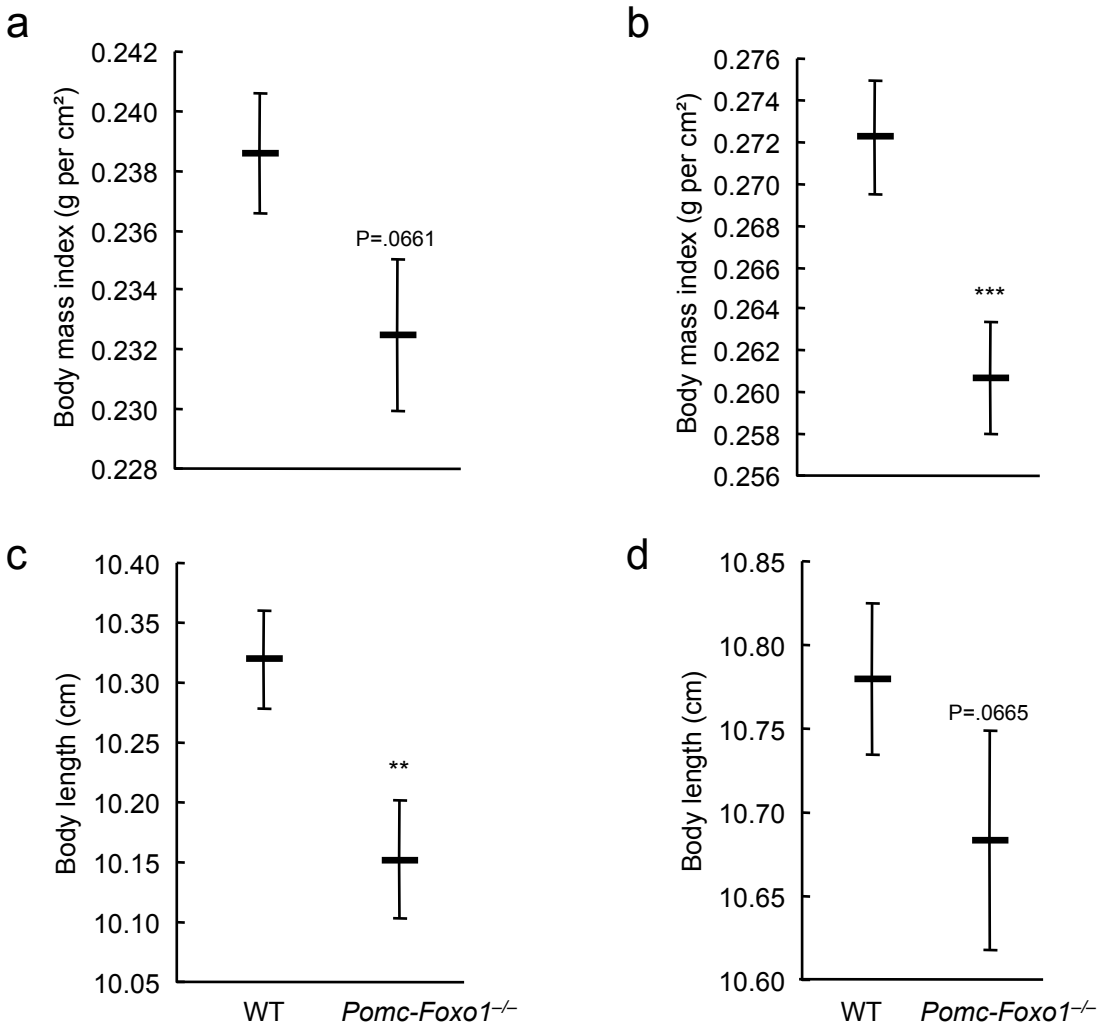
Supplementary Table 1 Neuropeptide levels in MBH of refed mice

Peptide	WT	*Pomc-Foxo1*$^{-/-}$
ACTH	166 ± 19	149 ± 14
POMC	220 ± 20	195 ± 16
β–EP	555 ± 28	543 ± 32
αMSH	306 ± 20	309 ± 20
AgRP	343 ± 25	299 ± 18
POMC/αMSH	0.75 ± 0.06	0.66 ± 0.06

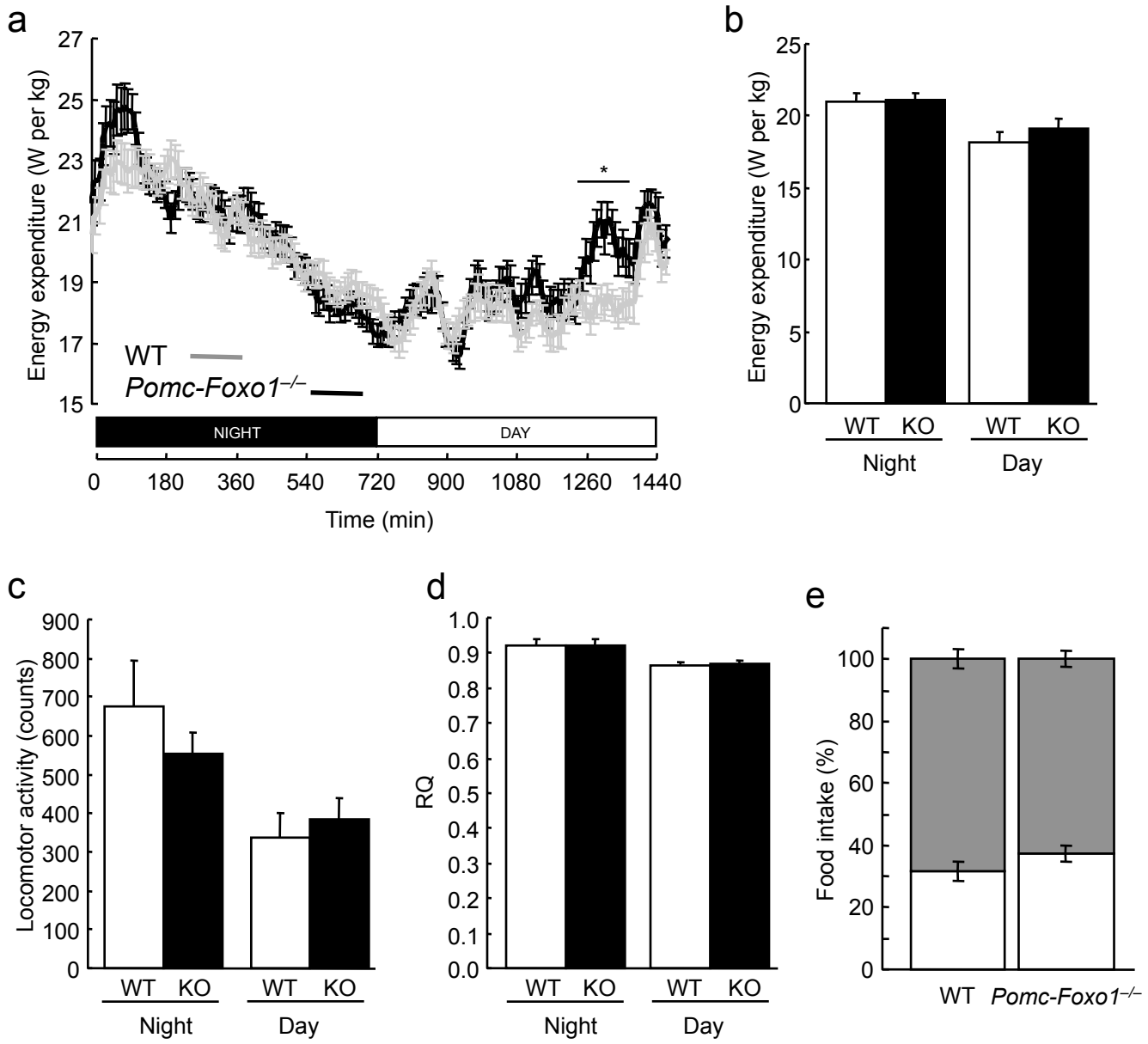
Data are presented as mean fmol/mg protein ± SEM (n = 13–18), P = NS.



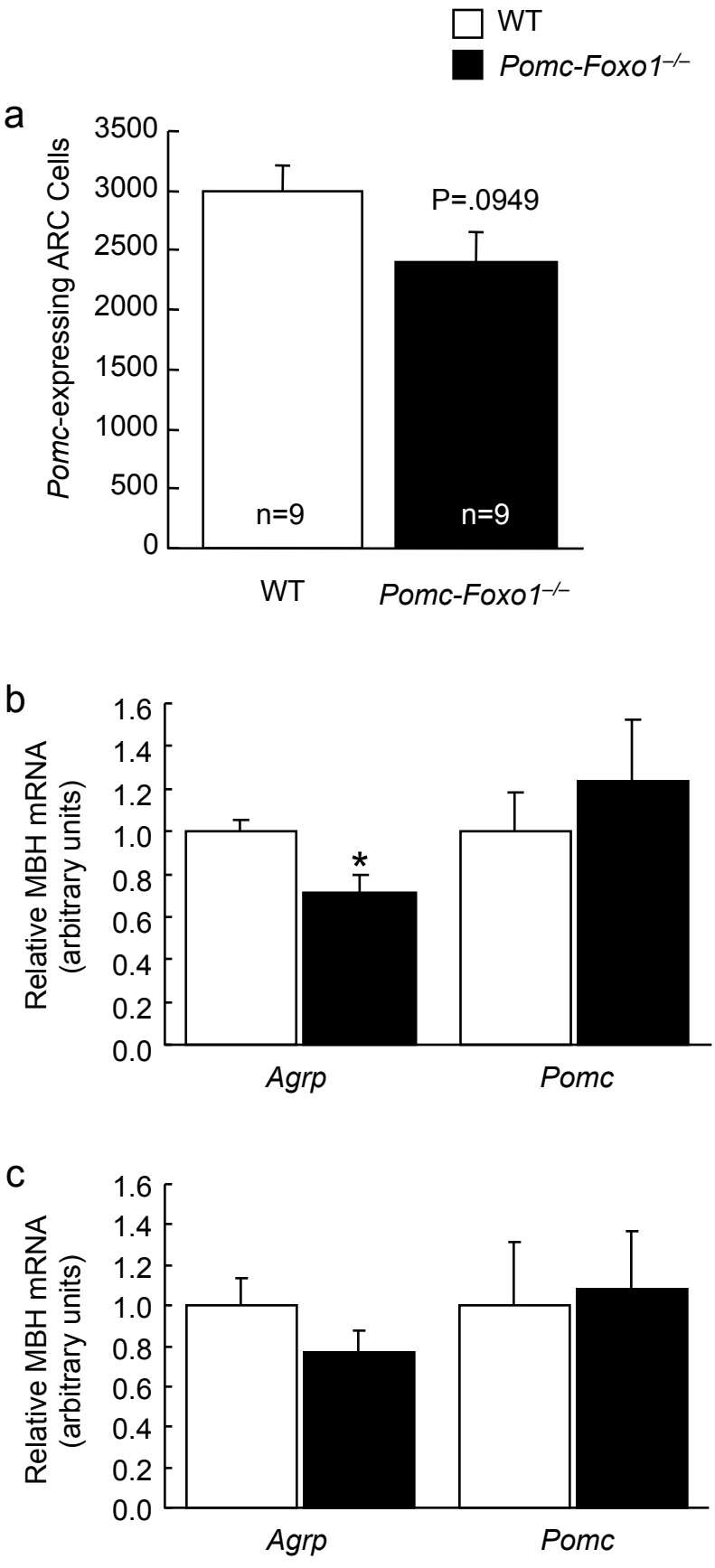
Plum, Supplementary Figure 1



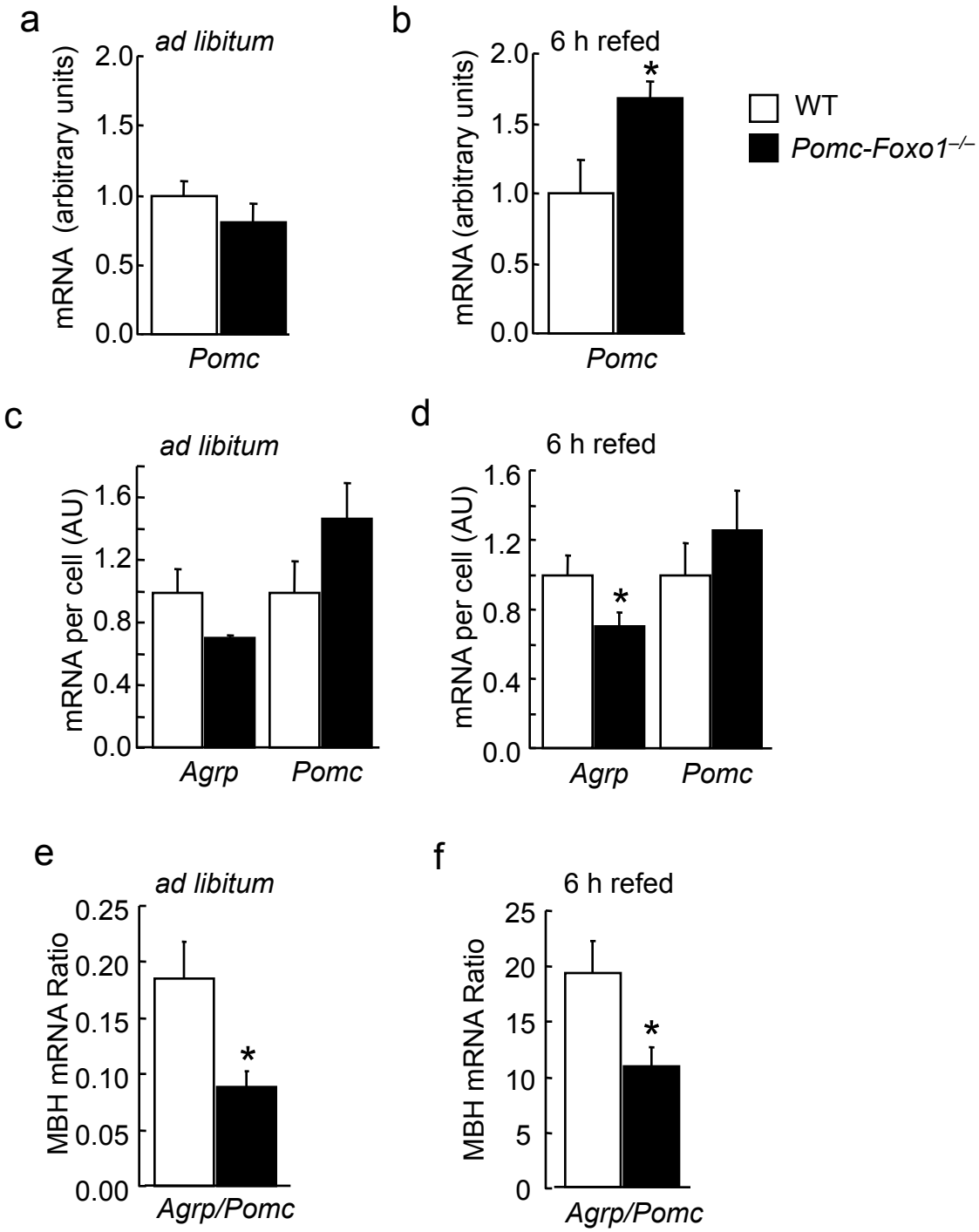
Plum, Supplementary Figure 2



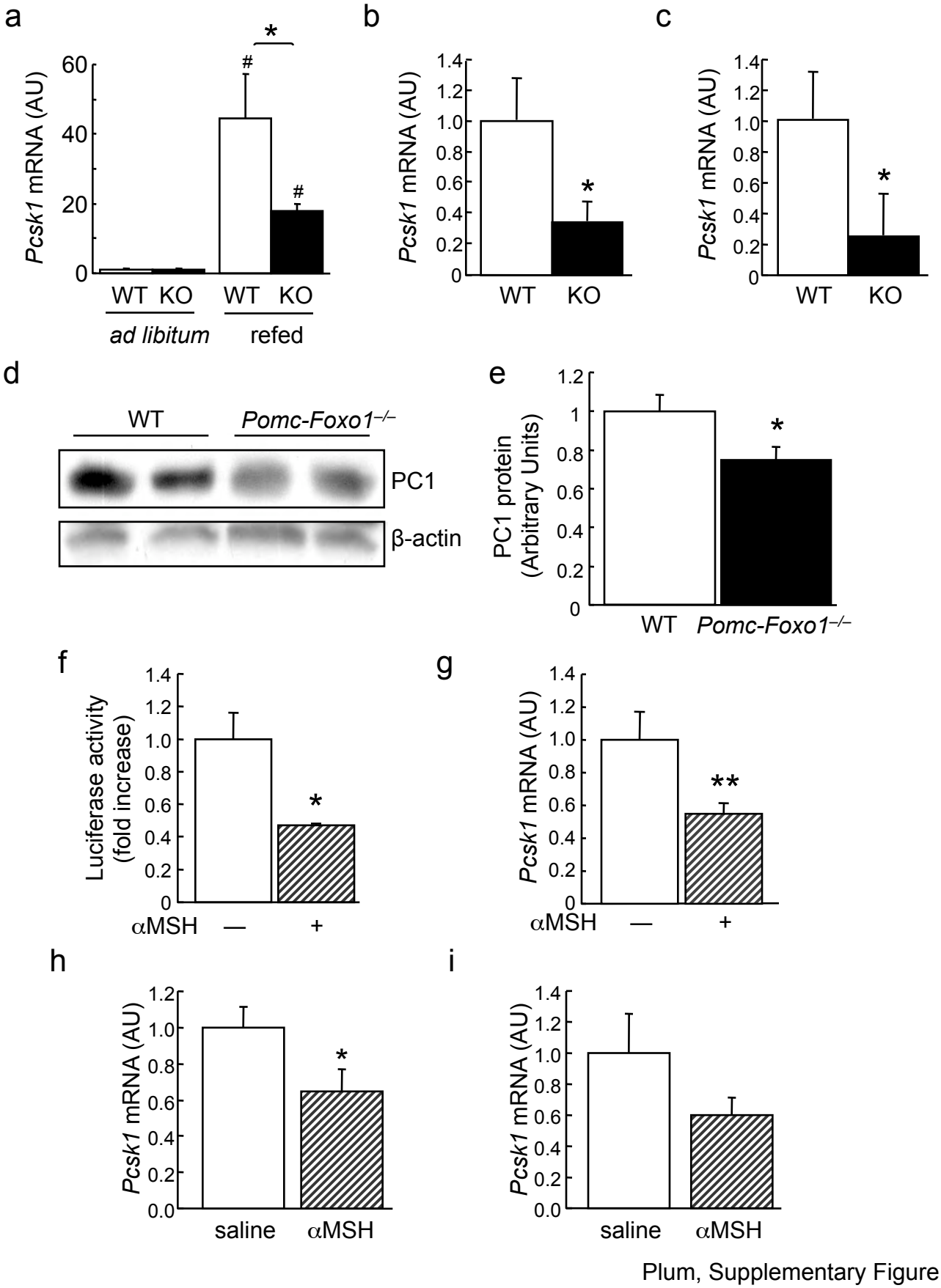
Plum, Supplementary Figure 3



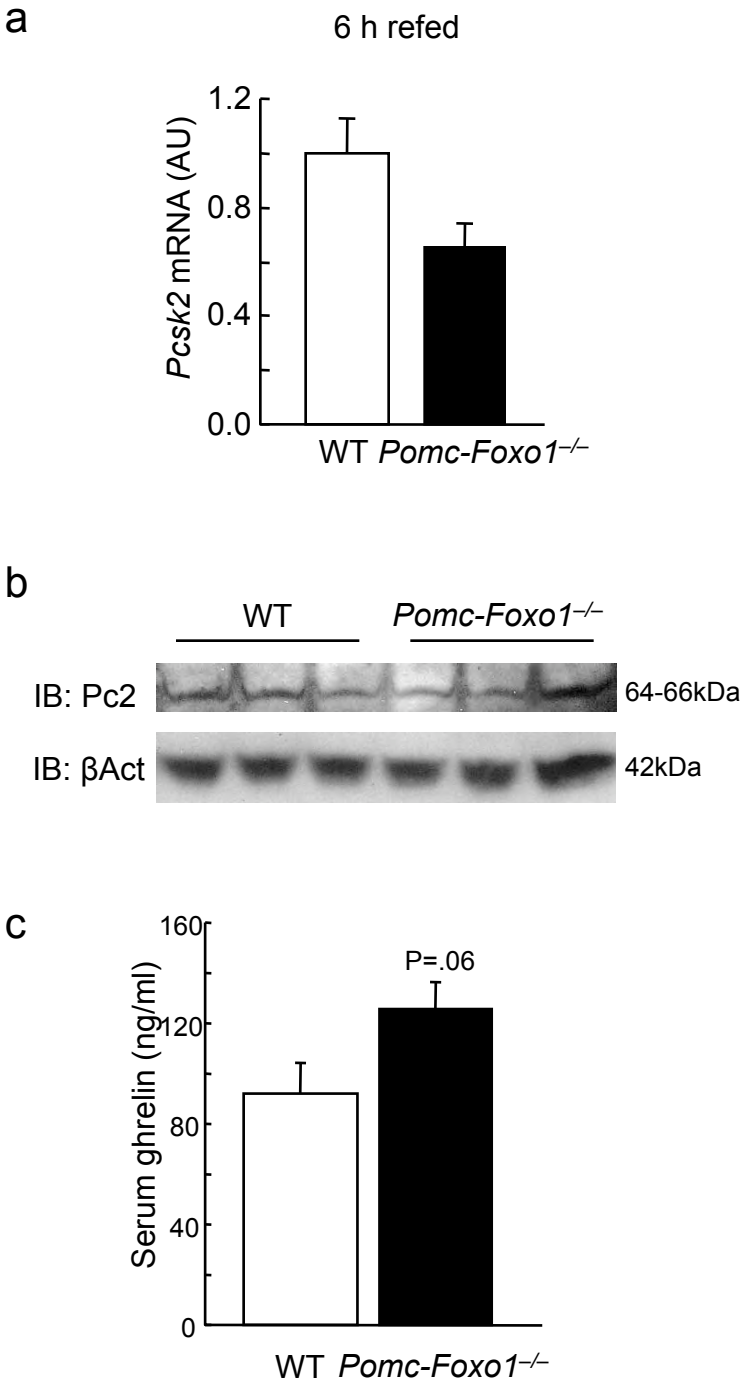
Plum. Supplementary Figure 4



Plum, Supplementary Figure 5



Plum, Supplementary Figure 6



Plum, Supplementary Figure 7