Supplementary data accompanying:

Urocortin3 mediates somatostatin-dependent negative feedback control of insulin secretion

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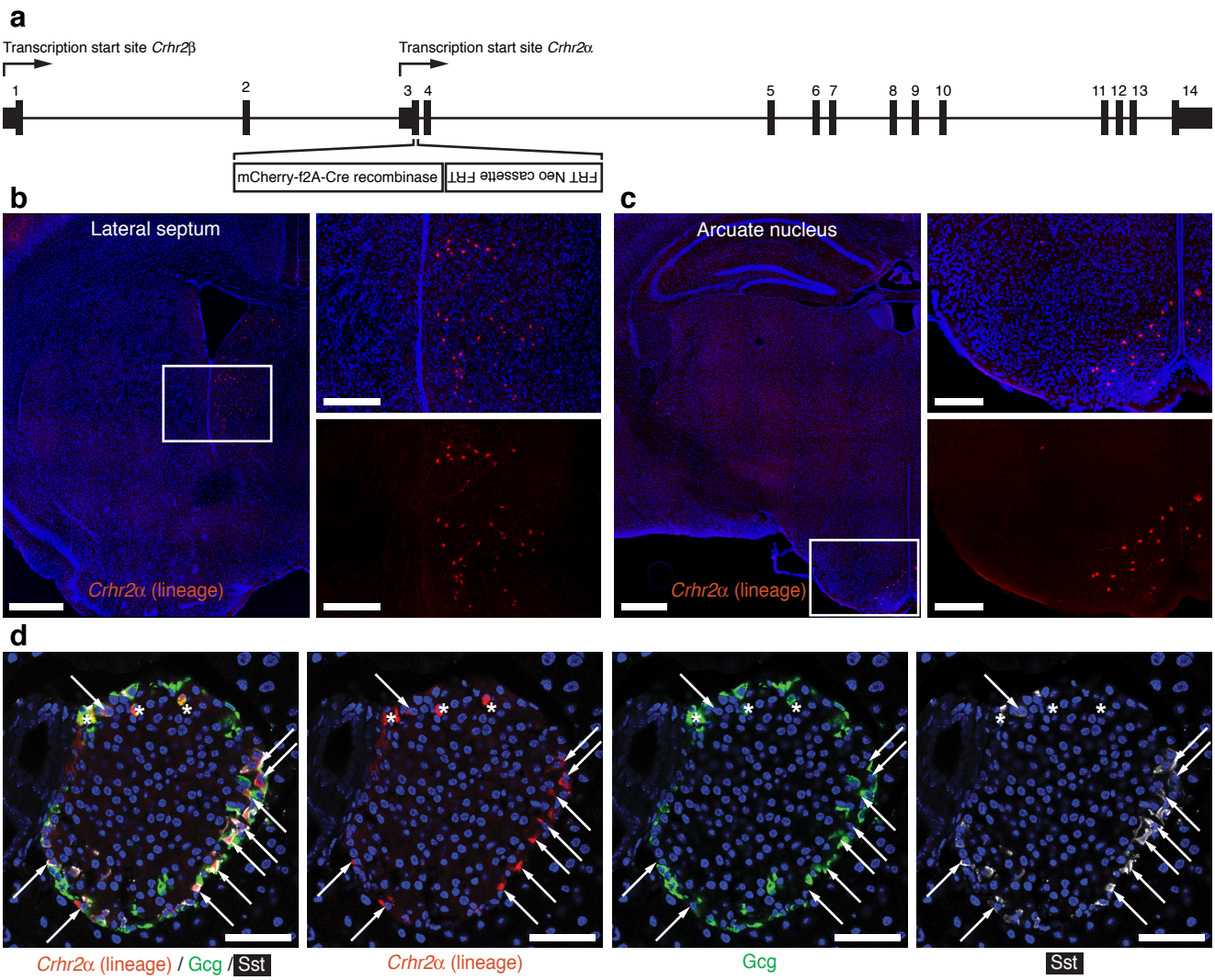
Supplementary Figure 1

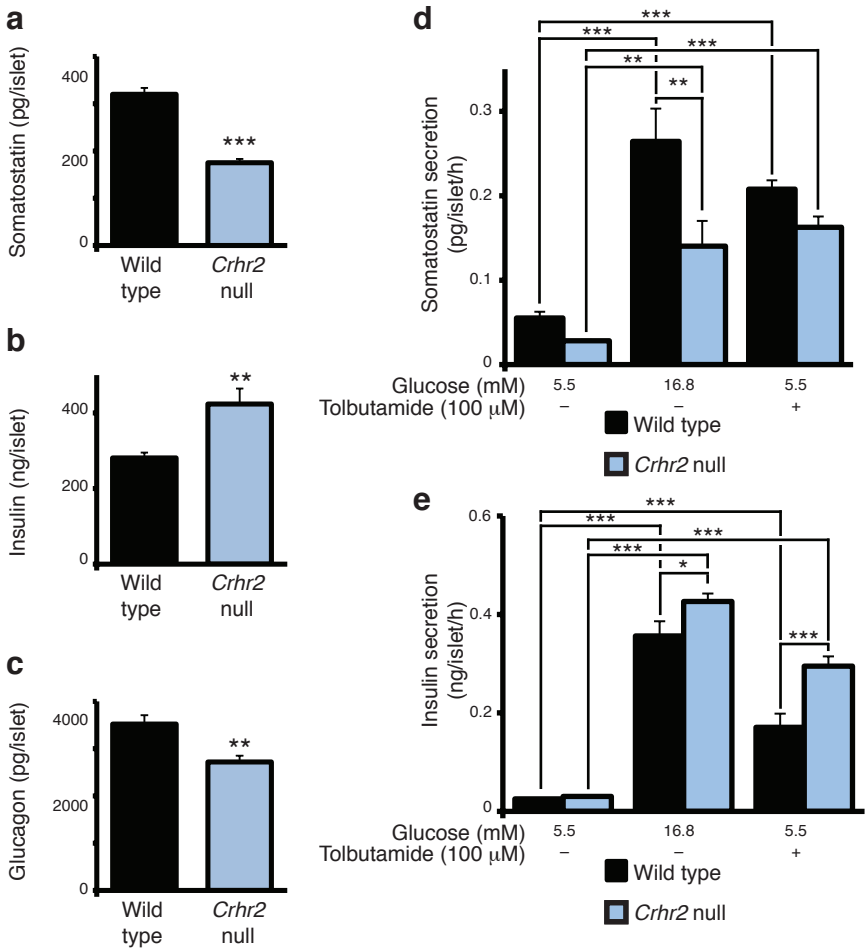
Supplementary Figure 1 Ucn3 expression in islets compared to a range of tissues. Ucn3 expression is markedly more abundant in islets compared to any other tissue, with expression in most other tissues undetectable. Islet data obtained from wild type islets (**Fig. 2**); all other data obtained from the Encode project[55] (Geo: GSE30567). All data are normalized to $1x10^7$ reads.

Supplementary Figure 2



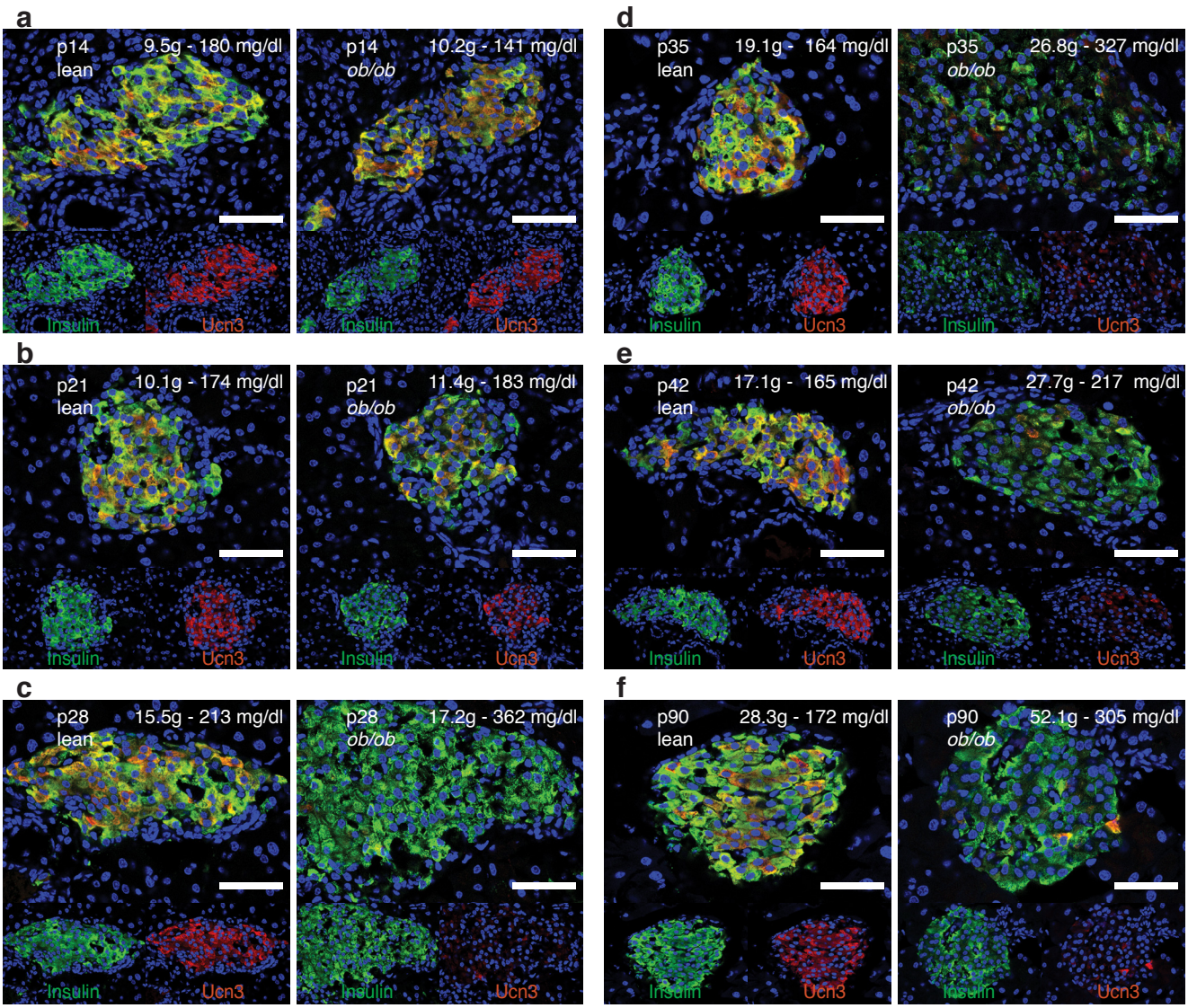
Supplementary Figure 2 Generation of a BAC−transgenic Crhr2α reporter. (**a**) A 140 kb BAC clone was manipulated by homologous recombination to replace the start codon of the alpha isoform of the *Crhr2* gene in exon 3, which is skipped in the beta isoform of Crhr2, with a reporter cassette containing mCherry fused to Cre recombinase by a constitutively proteolytic f2A processing site. A neomycin selection marker, flanked by FRT sites was used for positive selection and was later removed by crossing our founder to an actin FLP1 mouse. While mCherry was silent, this mouse crossed to an appropriate floxed reporter mouse (here tdTomato or LacZ) enables the visualization of Crhr2α lineage expression at established expression sites in the brain such as the lateral septum (**b**) and the arcuate nucleus (**c**). (**d**) The same Crhr2α reporter line demonstrates lineage expression of Crhr2α on delta (arrows) and alpha (asterisks), but not beta cells. Scale bars indicate 500 μm in overviews and 200 μm in details (**b**,**c**) and 50 μm (**d**).

Supplementary Figure 3



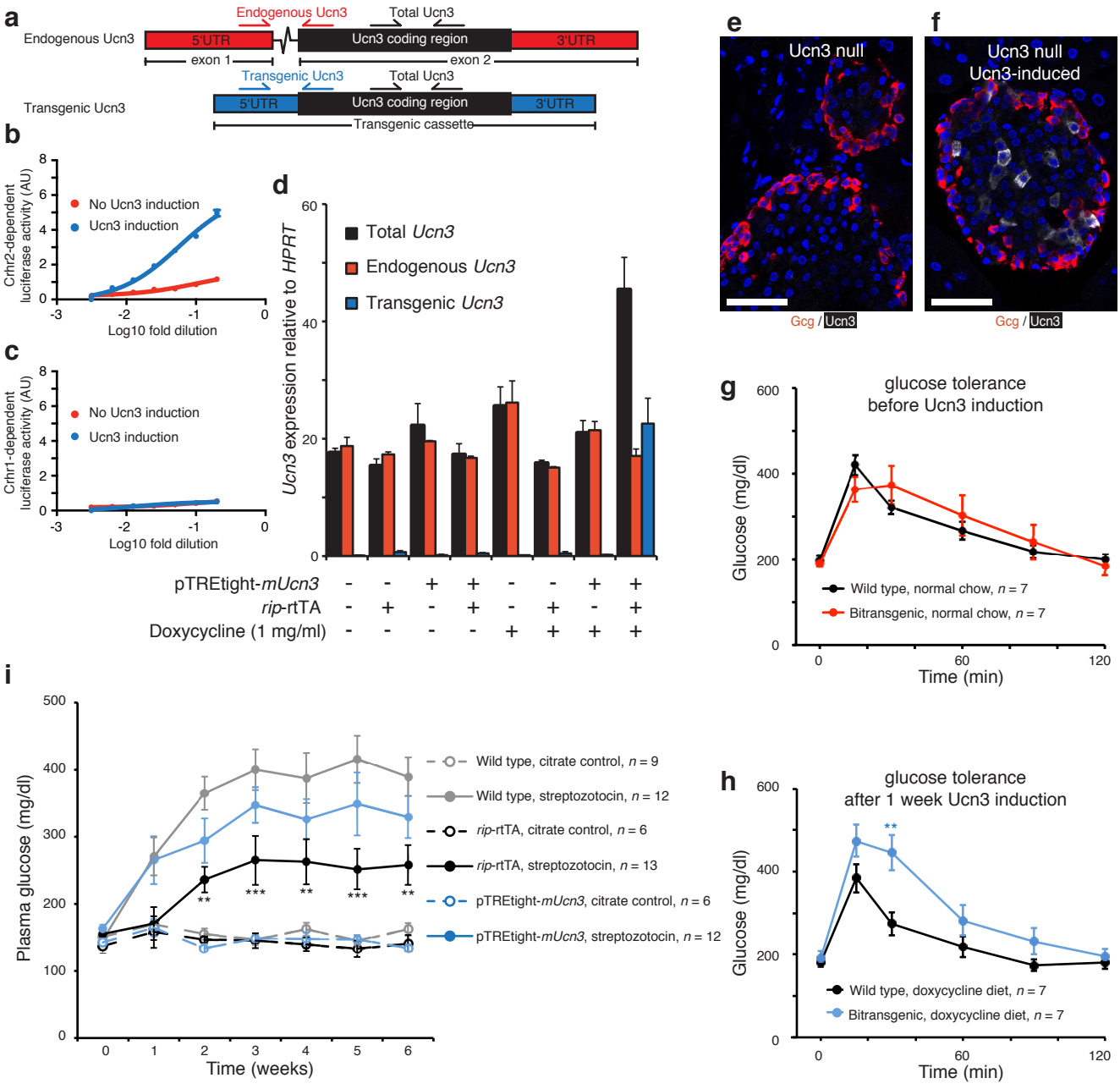
Supplementary Figure 3 *Crhr2* null islets are delta cell−deficient and demonstrate impaired somatostatin secretion. *Crhr2* null islets demonstrate reduced somatostatin content (**a**), increased insulin content (**b**) and reduced glucagon content (**c**) (n = 12). (**d**) *Crhr2* null islets are deficient in somatostatin secretion and display modest impairment of somatostatin release in response to tolbutamide (n = 4, 50 or 100 islets/well). (**e**) Conversely, the lack of Crhr2 is associated with increased tolbutamide−stimulated insulin secretion (n = 4). Significance determined by Student's t−test (**a**,**b**,**c**) or two−way ANOVA for treatment and genotype, followed by Holm−Sidak's multiple comparison test (**d**,**e**, asterisks indicate significance with the 5.5 mM glucose group for the same genotype, unless indicated otherwise). All values are mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary figure 4



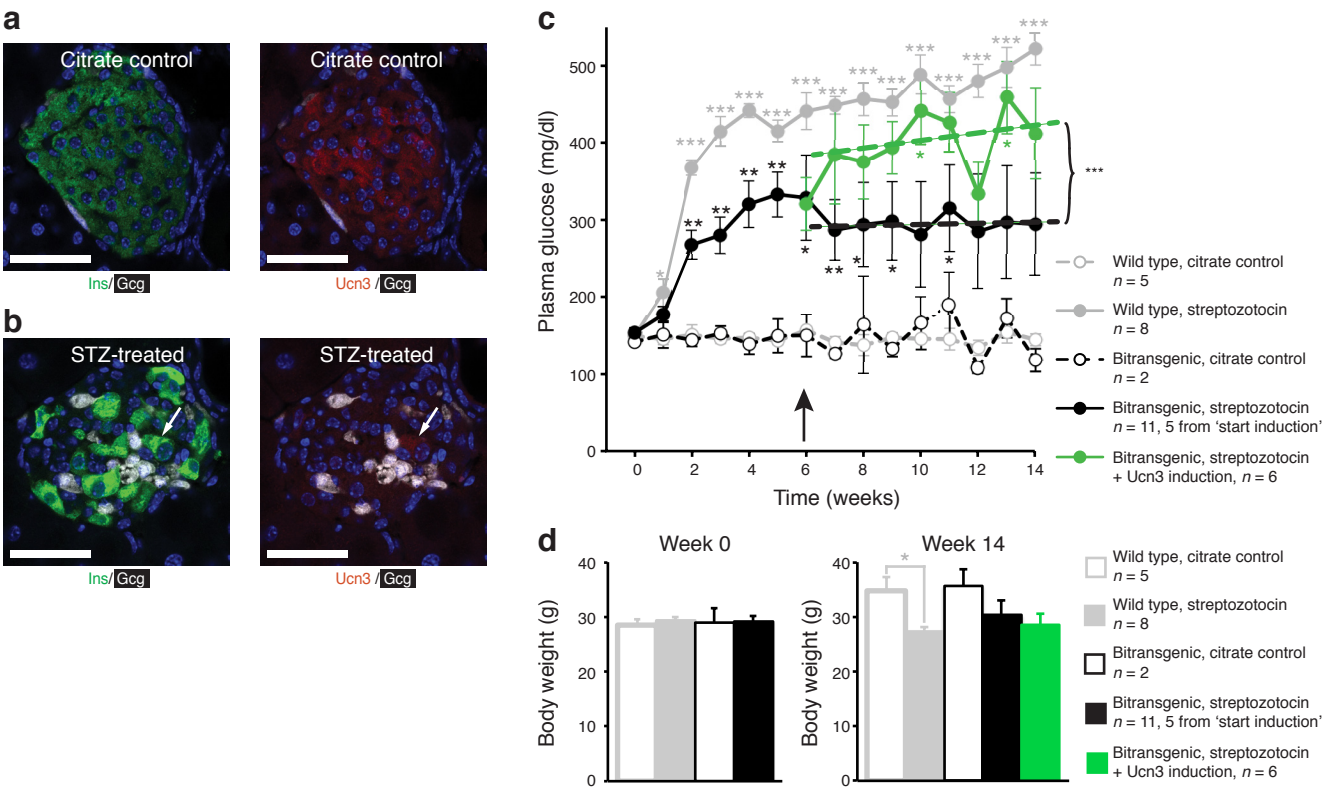
Supplementary Figure 4 The disappearance of Ucn3 expression coincides with the onset of obesity and diabetes in *ob/ob* mice. Ucn3 expression in beta cells is comparable between lean and *ob/ob* animals at p14 (**a**) and p21 (**b**), but disappears from beta cells of *ob/ob* mice around p28 (**c**), coincident with the onset of hyperglycemia. Ucn3 expression remains markedly reduced in *ob/ob* mice at p35 (**d**), p42 (**e**) and p90 (**f**), although residual Ucn3 immunoreactivity is occasionally detected. Scale bars indicate 50 μm.

Supplementary Figure 5



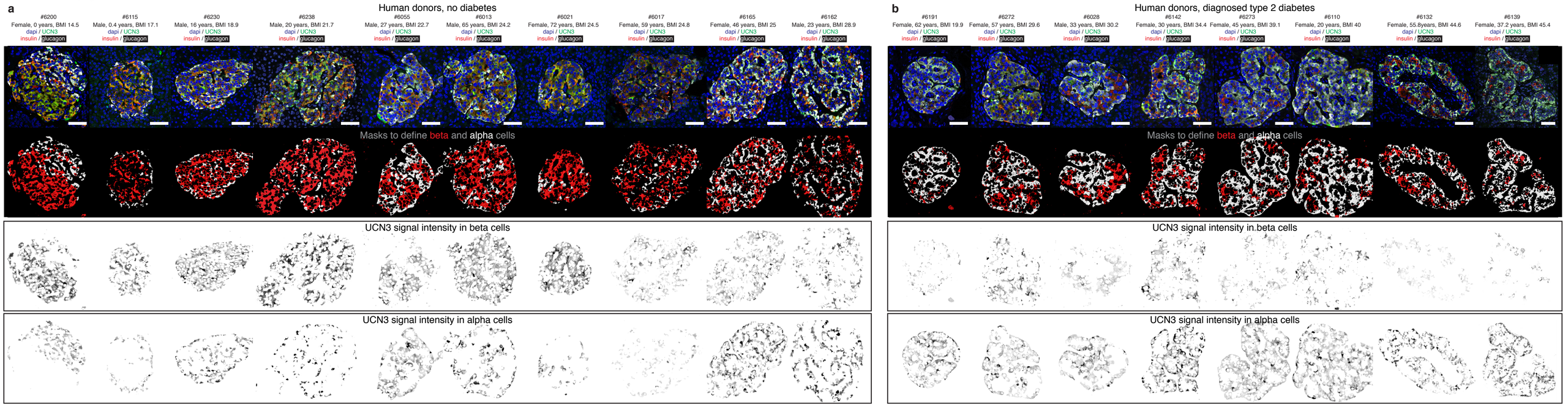
Supplementary Figure 5 Development of a beta cell−specific doxycycline inducible Ucn3 transgenic model. (**a**) Schematic of the strategy used to develop and validate a beta cell−specific doxycycline inducible Ucn3 transgenic model. (**b**,**c**) Conditioned media from doxycycline−stimulated rat Ins1 insulinoma cells transfected with the pTREtight−*mUcn3* doxycycline−inducible transgenic vector and a helper vector encoding reverse tetracycline transactivator protein (rtTA) selectively stimulated Crhr2 as measured by cAMP response element−dependent luciferase activity in HEK293T cells[56]. Conditioned media from non−induced Ins1 cells has residual activity on Crhr2, likely attributable to endogenous rat Ucn3 that is secreted by Ins1 cells. (**d**) A pTREtight−*mUcn3* transgenic mouse supported robust expression of transgenic *Ucn3 in vivo*, when crossed to mice that express the reverse tetracycline transactivator protein under control of the rat insulin promoter (*rip*−rtTA) and received doxycycline. (**e**,**f**) Backcrossing of pTREtight−*mUcn3* x *rip*−rtTA bitransgenic animals on a *Ucn3* null background reveals that the doxycycline−mediated induction of Ucn3 is mosaic and restricted to a relatively small subset of beta cells. Bitransgenic animals, when naive to doxycycline, show normal intra−peritoneal glucose tolerance (**g**), but after one week of doxycycline exposure develop glucose intolerance (**h**) (n = 7). The *rip*−rtTA, but not pTREtight−*mUcn3* single transgenic animals are protected from STZ−induced diabetes (**i**) (n for each treatment indicated in panel, asterisks reflect significant differences with wild type STZ−treated animals). AU = arbitrary units. Significance determined by two−way ANOVA for treatment and its interaction with time, followed by Holm−Sidak's multiple comparison test (**g**,**h**). Scale bars indicate 50 μm. All values are mean ± s.e.m. across technical replicates (**b**–**d**) or biological replicates (**g**–**i**), *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary figure 6



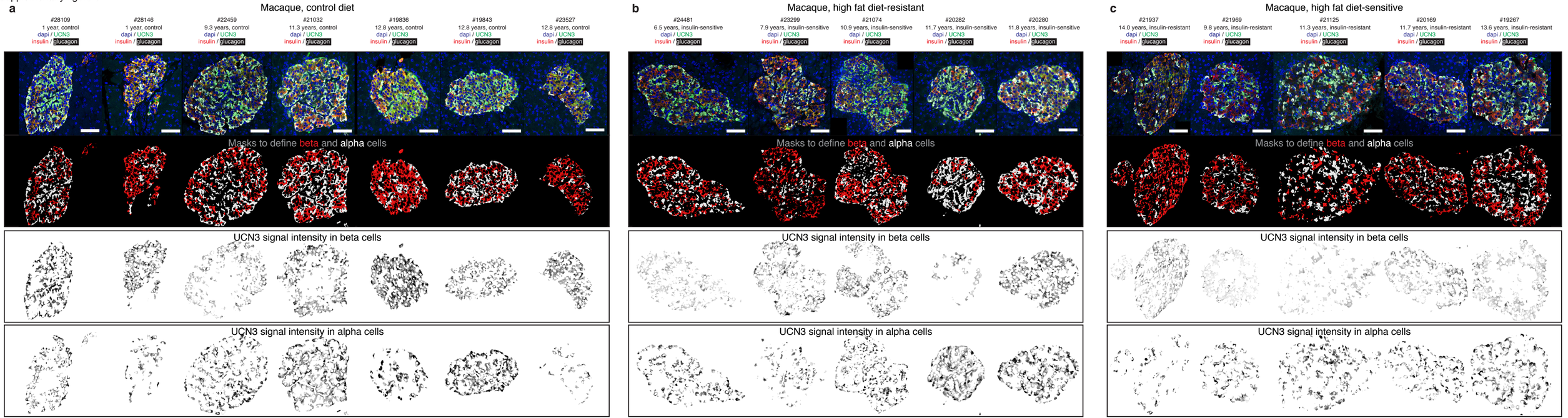
Supplementary Figure 6 Ucn3 aggravates hyperglycemia in STZ−induced diabetes. (**a**,**b**) Diabetes induced by the multiple low−dose STZ model led to the loss of Ucn3 expression in residual beta cells (arrow indicates a single Ucn3+ beta cell). (**c**) Doxycycline−inducible expression of Ucn3 specifically in beta cells after the onset of moderate but stable STZ−induced hyperglycemia aggravated diabetes and markedly increased plasma glucose levels compared to bitransgenic animals without doxycycline to levels indistinguishable from STZ−treated wild−type animals (*n* for each treatment indicated in panel). Body weights for each cohort prior to STZ administration (**d**) and at the conclusion of the experiment (**e**). All asterisks reflect significant differences with wild type citrate controls, except green asterisks that reflect significance between bitransgenic, STZ−treated groups with or without Ucn3 induction. Scale bars indicate 50 μm. Significance determined by linear regression between non−induced and induced bitransgenics following induction (**c**) and by Student's t−test to compare individual time points. Significance of body weights (**d**,**e**) was determined by one−way ANOVA followed by Student's t−test with Welch's correction as necessary. All values are mean ± s.e.m., **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Supplementary figure 7



Supplementary Figure 7 UCN3 expression is lost in beta cells of human donors with diagnosed type 2 diabetes. (**a**) UCN3 is expressed at approximately equal levels in both alpha and beta cells in a cohort of ten non−diabetic pancreas donors. (**b**) Beta cells from a cohort of eight type 2 diabetic donors across a range of BMIs displayed varying degrees of depletion of Ucn3 from beta cells, while UCN3 expression in alpha cells remained. We applied masks based on insulin (red) and glucagon (white) to isolate UCN3 staining in beta and alpha cells. Donor nPOD ID numbers (#) are provided for each donor, along with pertinent donor information. Scale bars indicate 50 μm.

Supplementary figure 8



Supplementary Figure 8 UCN3 expression is lost in beta cells of pre−diabetic macaques on high−fat diet. (**a**) Islets from control macaques demonstrate intense UCN3 immunoreactivity in both beta and alpha cells. (**b**) Macaques on a high−fat diet classified as 'diet−resistant' demonstrated a varying degree of loss in UCN3 in beta cells, while alpha cell UCN3 expression persisted. (**c**) Macaques classified as 'diet−sensitive', for their development of insulin resistance, increased body weight and dyslipidemia (**Supplementary Table 3**), demonstrated marked and selective depletion of Ucn3 in their beta cells. Macaque ID numbers (#) are provided for each animal. Scale bars indicate 50 μm.

Supplementary Table 1: Top 20 most significantly altered genes in Ucn3 null islets compared to wild type islets

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RefSeq ID	Symbol	normalized mean expression in wild type islets	normalized mean expression in Ucn3 null islets	fold depletion in Ucn3 null islets	adjusted p-value
NM_031250	Ucn3	10356.37	119.96*	86.33	9.01E-187
NR_028405	Marcksl1-ps4	178.03	24.37	7.31	1.20E-20
NM_001033879	Tubal3	1.05	47.16	0.02	1.62E-10
NM_001252620	Spock3	278.53	111.28	2.50	4.64E-08
NM_008245	Hhex	679.60	360.82	1.88	2.72E-07
NM_007914	Ehf	1203.57	675.04	1.78	1.94E-06
NM_001081306	Ptprz1	415.05	208.31	1.99	2.47E-06
NM_001013785	Akr1c19	1160.04	2308.71	0.50	7.13E-06
NM_011255	Rbp4	2924.10	1739.26	1.68	7.64E-06
NM_134072	Akr1c14	2001.13	1283.06	1.56	1.59E-05
NM_009215	Sst	56983.61	23838.63	2.39	4.73E-05
NM_007911	Efnb3	434.82	243.28	1.79	1.61E-04
NM_172800	Sdk2	2078.29	3025.00	0.69	4.45E-04
NM_001252293	Mest	339.55	186.07	1.82	7.16E-04
NM_146050	Oit1	228.67	113.62	2.01	1.13E-03
NM_001252292	Mest	339.91	189.30	1.80	1.16E-03
NM_009511	Vipr2	26.33	86.11	0.31	2.60E-03
NM_001163691	Cacna1h	311.03	174.87	1.78	2.60E-03
NM_001163584	Prom1	2968.21	4882.28	0.61	3.88E-03
NM_007976	F5	187.72	96.51	1.95	8.70E-03

* residual Ucn3 message is detected because the promoter and first (non-coding) exon of the Ucn3 gene were left intact in the Ucn3 null mouse and drive expression of a partial transcript.

Supplementary Table 2: Statistical comparison of continuous glucose monitoring data

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Differences in variance (t-test for differences in mean\Brown Forsythe test for differences in variance)

Plasma glucose (mg/dl)

	mean	**st. dev.**	**sample size**	ob/ob 1	+/ob 1	ob/ob 2	+/ob 2	
ob/ob 1	737.13	125.10	852		**1.0597E-119**	1.2984E-16	**5.0888E-91**	differences
+/ob 1	146.61	33.47	848	**7.1470E-04**		**4.8361E-97**	2.5224E-13	in
ob/ob 2	720.80	88.21	852	0.06048	**6.1670E-07**		**6.5657E-59**	variance
+/ob 2	152.34	45.32	851	**6.7803E-03**	0.48231	**5.5256E-06**		

differences in mean plasma glucose

Period length (minutes)

	mean	**st. dev.**	**sample size**	ob/ob 1	+/ob 1	ob/ob 2	+/ob 2	
ob/ob 1	37.26	22.14	113		**0.01913**	0.20786	**0.00554**	differences
+/ob 1	29.34	14.73	143			**0.00006**	0.50664	in
ob/ob 2	43.16	23.27	98				**0.00001**	mean period length
+/ob 2	30.58	14.91	138					

Supplementary Table 3: macaque cohort metabolic information[§]

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	control (average ± s.e.)	diet-resistant (average ± s.e.)	diet-sensitive (average ± s.e.)
fasting glucose (mg/dl)	57.4 (± 1.81)	70.6 (± 3.08) *	64 (± 5.98)
fasting insulin (μg/ml)	20.67 (± 6.61)	27.42 (± 5.75)	357.9 (± 209.98)
AUC i.v. GTT glucose	10363 (± 431)	10409 (± 513)	13043 (± 614) **
AUC i.v. GTT insulin	6399 (± 1694)	8216 (± 984)	51778 (± 11394) ***
fat mass (kg)	2.41 (± 0.47)	4.38 (± 0.75)	7.06 (± 0.67) ***
lean mass (kg)	8.52 (± 0.34)	9.70 (± 0.48)	12.18 (± 0.49) ***
total body weight (kg)	11.18 (± 0.59)	14.36 (± 1.15)	19.54 (± 0.96) ***
percent body fat	21.0 (± 3.29)	29.7 (± 3.10)	35.9 (± 1.97) **
percent trunk fat	25.1 (± 4.91)	36.8 (± 3.81) *	44.0 (± 1.95) **
plasma triglycerides (mg/dl)	49.8 (± 4.21)	82.0 (± 18.59)	261.8 (± 35.25) ***
total plasma cholesterol (mg/dl)	125.0 (± 20.64)	190.0 (± 8.60) *	218.2 (± 19.46) **
plasma VLDL (mg/dl)	10.0 (± 0.84)	16.4 (± 3.72)	52.4 (± 7.05) ***
plasma LDL (mg/dl)	47.5 (± 9.15)	54.0 (± 4.68)	75.2 (± 9.89) *
plasma HDL (mg/dl)	66.5 (± 15.20)	119.2 (± 9.72) *	90.6 (± 15.29)

[§] data from Nygaard et al., International Journal of Obesity 2014.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control.

Supplementary Table 4: mouse body weight information

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Corresponding figure	group	weight (mean ± s.e.m.)
Figure 3g	saline control	29.4 (± 1.10)
	Ucn3	30.0 (± 0.98)
	Ast2b	30.1 (± 1.35)
Figure 3i	saline control	27.9 (± 1.15)
	Ucn3	29.9 (± 1.24)
	Sst ant.	29.1 (± 0.80)
	Ucn3 + Sst ant.	28.2 (± 1.31)
Supplementary figure 5g	wild type, normal chow	27.1 (± 0.79)
	bitransgenic, normal chow	27.7 (± 1.15)
Supplementary figure 5h	wild type, doxycycline diet	27.7 (± 0.45)
	bitransgenic, doxycycline diet	26.6 (± 0.77)
Supplementary figure 5i	wild type, citrate control	29.3 (± 0.90)
	wild type, STZ	29.4 (± 0.80)
	rip-rtTA, citrate control	31.6 (± 1.39)
	rip-rtTA, STZ	30.3 (± 0.87)
	pTREtight-mUcn3, citrate control	28.6 (± 0.82)
	pTREtight-mUcn3, STZ	29.9 (± 1.13)

Supplementary Table 5: qPCR primer information

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RefSeq ID	Gene	primer	sequence 5' ➔ 3'	amplicon size (bp)
NM_008100	Gcg	qrodGcg.fwu1	TCACAGGGCACATTCACCAG	121
		qrodGcg.rvu1	CATCATGACGTTTGGCAATGTT	
NM_001185(	Ins2	qrodIns2.fwu1	GCTCTCTACCTGGTGTGTGGG	128
		qrodIns2.rvu1	CAAGGTCTGAAGGTCACCTGC	
NM_031250	Ucn3	qmUcn 3.fwu1	GCTGTGCCCCTCGACCT	71
		qmUcn 3.rvu1	TGGGCATCAGCATCGCT	
NM_021332	Glp1r	qmGLP-1R.fwu1	CCCTGGGCCAGTAGTGTG	88
		qmGLP-1R.rvu1	GCAGGCTGGAGTTGTCCTTA	
NM_009215	Sst	qrodSst.fwu1	GACCCCAGACTCCGTCAGTTT	112
		qrodSst.rvu1	TCTCTGTCTGGTTGGGCTCG	
NM_001159(	Rbp4	qmRbp4.fwu1	AGACACGGAGGCTGGTGA	96
		qmRbp4.rvu1	AGGGCCTGCTTTGACAGTAA	
NM_008245	Hhex	qmHhex.fwu1	CTACACGCACGCCCTACTC	76
		qmHhex.rvu1	CAGAGGTCGCTGGAGGAA	
NM_009953	Crhr2	qmCRFR2.fwu1	AAGTGCACGAGGGCAATG	69
		qmCRFR2.rvu1	TGGTGACCACAAAATAGTTGAAG	
NM_013556	Hprt	qmHPRT.fwu	TCCTCCTCAGACCGCTTTT	90
		qmHPRT.rvu	CCTGGTTCATCATCGCTAATC	