
Supplementary information

**Constitutively active glucagon receptor
drives high blood glucose in birds**

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authors and unedited

Supplementary Information

Supplementary Information Figures

Supplementary Information Fig. 1 Phylogenetic tree of all synthesized GCGR genes and constitutively active ancestral GCGR genes. Related to Fig. 1.

a, Green: mammals; red: birds; orange: reptiles; grey: amphibians; blue: fish. The boxed species indicate that the corresponding synthetic GCGRs were not expressed well. The red line distinguishes placental mammals from non-placental mammals.

b, Phylogenetic tree of GCG receptor family. Black bold font represents the ancestral gene, *Phallusia mamillata*-GCGR/GLP1R-like (pmGCGR/GLP1R-like) and *Ciona intestinalis*-GCGR/GLP1R-like (ciGCGR/GLP1R-like).

c, pmGCGR/GLP1R-like and ciGCGR/GLP1R-like have constitutive activity. A higher value of slope indicates a higher level of Gs constitutive activity. Data are mean $\pm$ SEM from at least three independent experiments, performed in technical triplicate.

Supplementary Information Fig. 2 Sequence alignment of mammalian and avian GCGRs. Related to Fig. 1.

Sequence alignment of mammalian and avian GCGRs. Red highlighted amino acids are selected residues with high conservation in birds while different in placental mammals and blue characters represent the positions after which the C-terminus was truncated. The upper red broken line separates placental mammals from non-placental mammals and the lower red broken line separates mammals from birds.

Supplementary Information Fig. 3 AlphaFold structural predictions of GCGRs in different species or those with mutations. Related to Fig. 1.

a-c, Structural prediction of mdGCGR (**a**) and mdGCGR including domain swapping with ggGCGR (**b**) (mdGCGR-Stalk-ECL1-ICL2 (gg)) and hsGCGR (mdGCGR-Stalk-ECL1-ICL2 (hs)) (**c**). An upward curvature at the N-terminus of mdGCGR-Stalk-ECL1-ICL2 (gg), bringing it into closer proximity to the intracellular architecture resembling ggGCGR.

d-f, Structural prediction of ggGCGR (**d**), and ggGCGR-V284G, V249L (**e**), ggGCGR-V284G, V251T mutations of ggGCGR (**f**).

g-i, Structural prediction of hsGCGR (**g**), and hsGCGR-G254V, L255V (**h**), hsGCGR-G254V, T257V mutations of hsGCGR (**i**).

j, Differences when merging scGCGR and ggGCGR.

k, Differences when merging scGCGR and hsGCGR.

l. Differences when merging scGCGR, hsGCGR, and ggGCGR. **m**, Differences when merging hsGCGR and hsGCGR-435-496(gg) (hsGCGR including domain with C-terminus of ggGCGR). The TM6 (transmembrane 6) of hsGCGR turned outward after C-terminal domain swapping with ggGCGR. **n**, Differences when merging hsGCGR, hsGCGR-417-496(gg), hsGCGR-435-496(gg), hsGCGR-444-496(gg) (the three C-terminal truncations are shown in **Extended Data Fig. 4d**). Structural predictions of GCGRs of the ECD domain (dark blue), seven TM domains (TM1: blue-green TM2: dark green TM3: light green, TM4: light yellow, TM5: dark yellow, TM6: light orange, TM7; dark orange and tangerine: C-terminal domain).

Supplementary Information Fig. 4 Functional tests of GCGR and determination of downstream signaling pathways. Related to Fig. 1 and Fig. 2.

- a**, Endogenous GCGR expression level in liver of *Gallus gallus*.
 - b**, *Gallus gallus* (gg) GCGR was exogenously overexpressed in HEK293 with similar expression levels to that of endogenous GCGR in the liver of *Gallus gallus*.
 - c**, ggGCGR at similar endogenous GCGR gene expression levels in the liver of *Gallus gallus* showed similar activity to hsGCGR stimulated by 100pM hsGCG in HEK293 cells.
 - d**, Endogenous expression of GCG and GCGR mRNAs are hardly detected in AML12 and HEK293 cells.
 - e**, Inhibition effect of H89 and adomeglivant on increased *Pck1* and *G6pc1* transcription in AML12 cells transfected with constitutively active GCGR (scGCGR). **f-h**, Adomeglivant only had inhibition effect on Gs signaling induced by GCGR constitutive activity (**f,g**), not on the other GCG receptor family members in AML12 cells (**h**). H89: PKA (Protein Kinase A) inhibitor; Ad: adomeglivant, allosteric inhibitor of constitutively active GCGR; YM-254890: novel Gq/11 inhibitor; Des: desHis¹Pro⁴-glucagon amide, a glucagon analog; pc: *Phascolarctos cinereus*; pk: *Paramormyrops kingsleyae*. Related to **Fig. 1**.
 - i**, Adomeglivant (Ad) injection exhibited lower blood glucose levels and higher body weight than the control group in chicks. Related to **Fig. 2**.
- Data are mean $\pm$ SEM from at least three independent experiments, performed in technical triplicate and analyzed by one-way ANOVA and Dunnett's post hoc test (**e,f,h**), two-tailed unpaired Student's *t* test (**g**), two-way ANOVA followed by Šídák's post hoc test (**i**).

Supplementary Information Fig. 5 Broilers were selected according to the level of blood glucose and screening of chickens with point mutations in the GCGR promoter. Related to Fig. 3.

a, Schematic of chicken selection. F1 was bred by selection for high or low blood glucose concentrations of F0 (outbred); potential sites on the promoter that may affect GCGR expression were comprehensively analyzed by sequencing and blood glucose data of F0 and F1.

b, Significant differences in body weight and blood glucose between the low glucose (LG) and the high glucose (HG) individuals of the F1 generation.

c,d, Body weight, blood glucose (**c**) and hepatic expression of *GCGR* gene (**d**) in HG and LG.

e, mRNA expression of hepatic glycogenolysis genes (*G6pc2* and *Pck1*) in males (M) and females (F).

f, Lipolysis genes (*Atgl*, *Hsl* and *Lpl*) expression levels in 90-day-old F1 generation in males (M) and females (F).

g,h, Blood glucose and body weight measurements of the F2 generation at 60 and 90 days showed that LG had significantly lower blood glucose and higher body weight compared to HG in males (M) and females (F).

i, Promoter activity of mutation constructs in chicken hepatocellular carcinoma epithelia (LMH).

j,k, Heterozygotes of 60-day-old (**b**) and 90-day-old (**c**) LG descendants (LG-C/G) had higher body weight and lower blood glucose than G/G of HG descendants (HG-G/G) but there was no significant difference between the LG-C/G and LG-G/G.

l,m, Blood glucose and body weight of 90-day-old C/G had higher body weight and lower blood glucose than G/G littermates. Littermates C/G and G/G were LG descendants.

n, Hepatic *GCGR* gene expression of C/C homozygotic chicks of F2 generation that died during hatching. Data are presented as the mean $\pm$ SEM and analyzed two-tailed unpaired Student's *t* test.

o,p, Image of 60-day-old (**g**) and 90-day-old (**h**) male C/C of F2 generation in comparison with the G/G strain.

q,r, Blood glucose and body weight of 60-day-old male C/C compared with their littermates.

s, Body weight gain rate of male C/C and the G/G individuals at 40, 60 and 90 days. Data are mean $\pm$ SEM from at least three independent experiments, performed in technical triplicate, and analysed by two-tailed unpaired Student's *t* test (**b-h**, **l-n**), one-way ANOVA and Tukey's post hoc test (**i-k**). Cartoons were created with BioRender.com.

Supplementary Information Fig. 6,7,8 The raw image for all data obtained by western blots.

For western blots source data, see Supplementary Figure 6-8.

Supplementary Video 1 Intrahepatic injection of *Eublepharis macularius*.

Schematic of AAV intrahepatic injection (*in situ*) of *Eublepharis macularius*.

Supplementary Tables

Supplementary Table 1: Blood glucose level in different species using the published for the species based on searches of the literature.

Supplementary Table 2: The CDS sequences of 84 GCGRs from various vertebrate species (thirty mammals, twenty-six birds, ten reptiles, seven amphibians, nine fishes. two urochordata).

Supplementary Table 3: Information of animals. Supplementary Table 4: The list of Abbreviations.

Supplementary Table 5: RNA-seq preproject information for liver of different speicies. Data information for Extended Data Figure 3c.

Supplementary Table 6: Sample information on RNA-seq for liver of mice and Single-nucleus RNA-sequencing for liver of budgerigar.

Supplementary Table 7: The list of upregulated genes in AAV-shGCGR group from Single-nucleus RNA-sequencing for liver of budgerigar.

Supplementary Table 8: The list of downregulated genes in AAV-shGCGR group from Single-nucleus RNA-sequencing for liver of budgerigar.

Supplementary Table 9: The list of differentially expressed genes in the AAV-scGCGR group from RNA-sequencing of wild type mice. Data for Figure 4f and Extended Data Figure 9e-f.

Supplementary Table 10: The list of differentially expressed genes in the AAV-scGCGR group from RNA-sequencing of HFD mice after 5 days AAV injection. Data for Extended Data Figure 10x-z.

Supplementary Table 11: The list of differentially expressed genes in the AAV-scGCGR group from RNA-sequencing of HFD mice after 18 days AAV injection. Data for Extended Data Figure 10x-z.

Supplementary Table 12: Basal metabolism rate (BMR) in different species using the published for the species based on searches of the literature.

Supplementary Table 13: The list of peptide sequences.

Supplementary Table 14: The list of primer sequences and shRNA sequences.

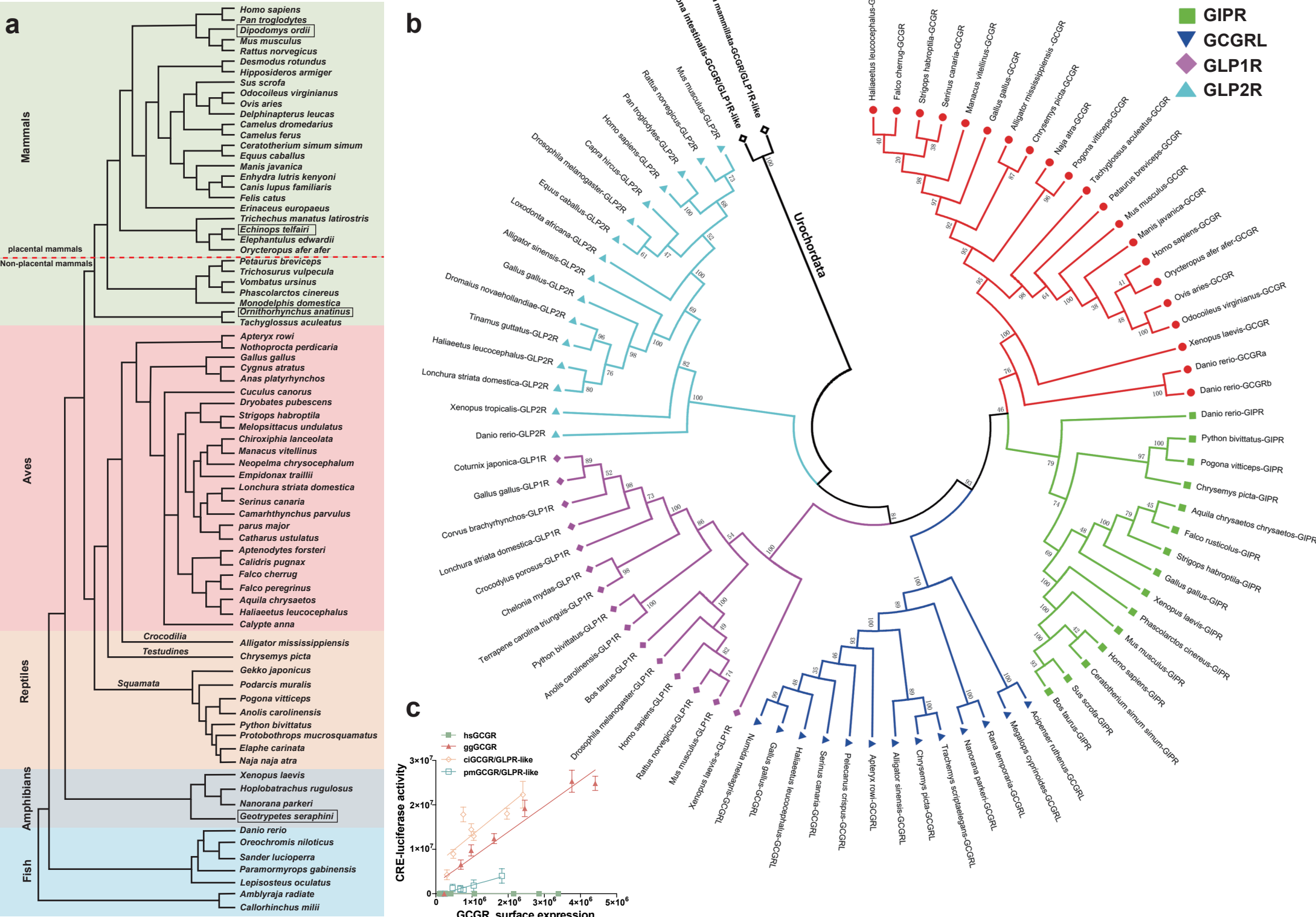
Supplementary Table 15: The list of Statistics.

Supplementary Table 16: Information of instruments, soft wares, and antibodies.

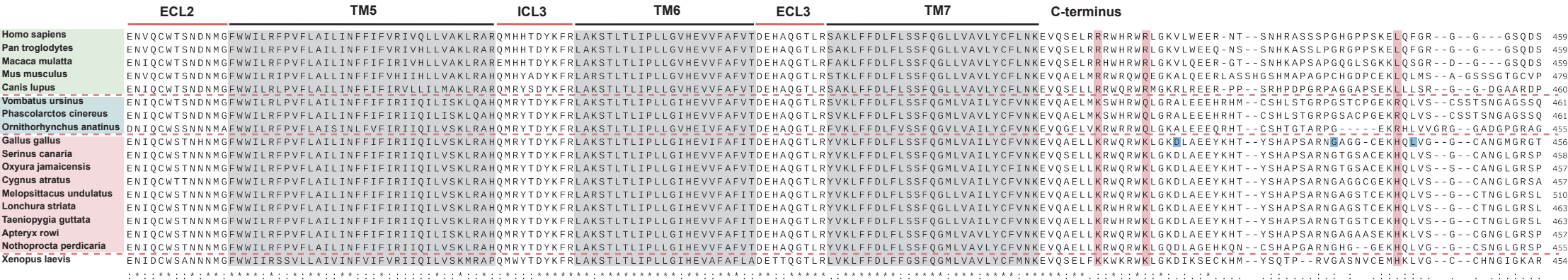
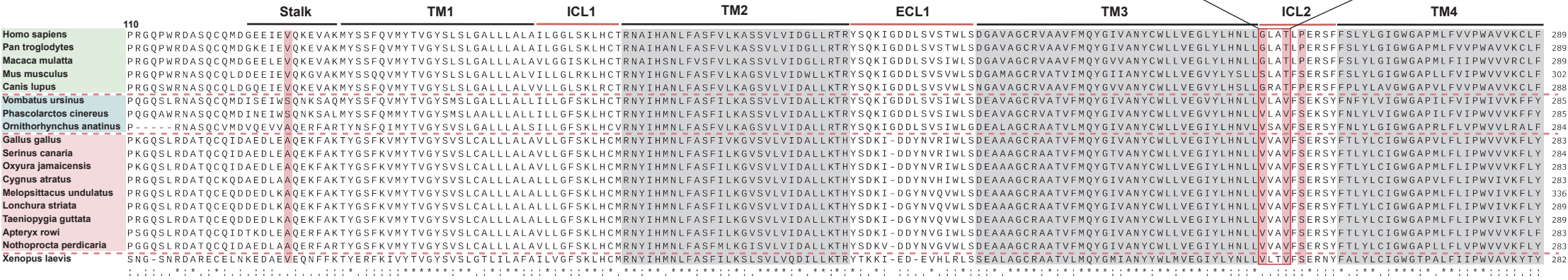
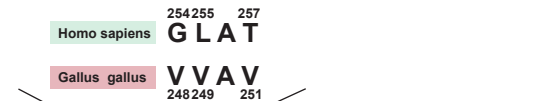
Supplementary Video

Supplementary Video 1: Schematic of intrahepatic injection (*in situ*) of *Eublepharis macularius*.

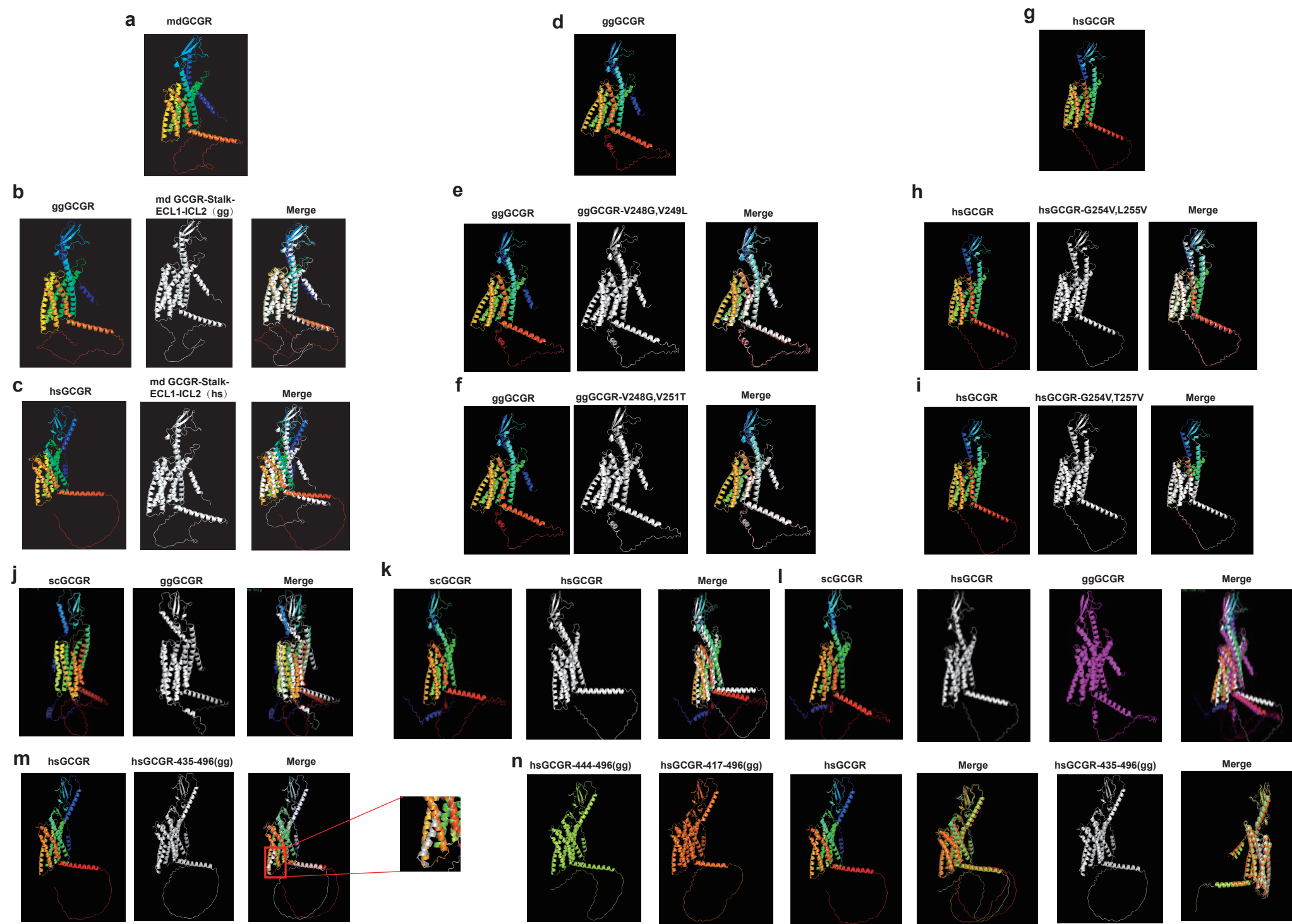
Supplementary Information Fig.1



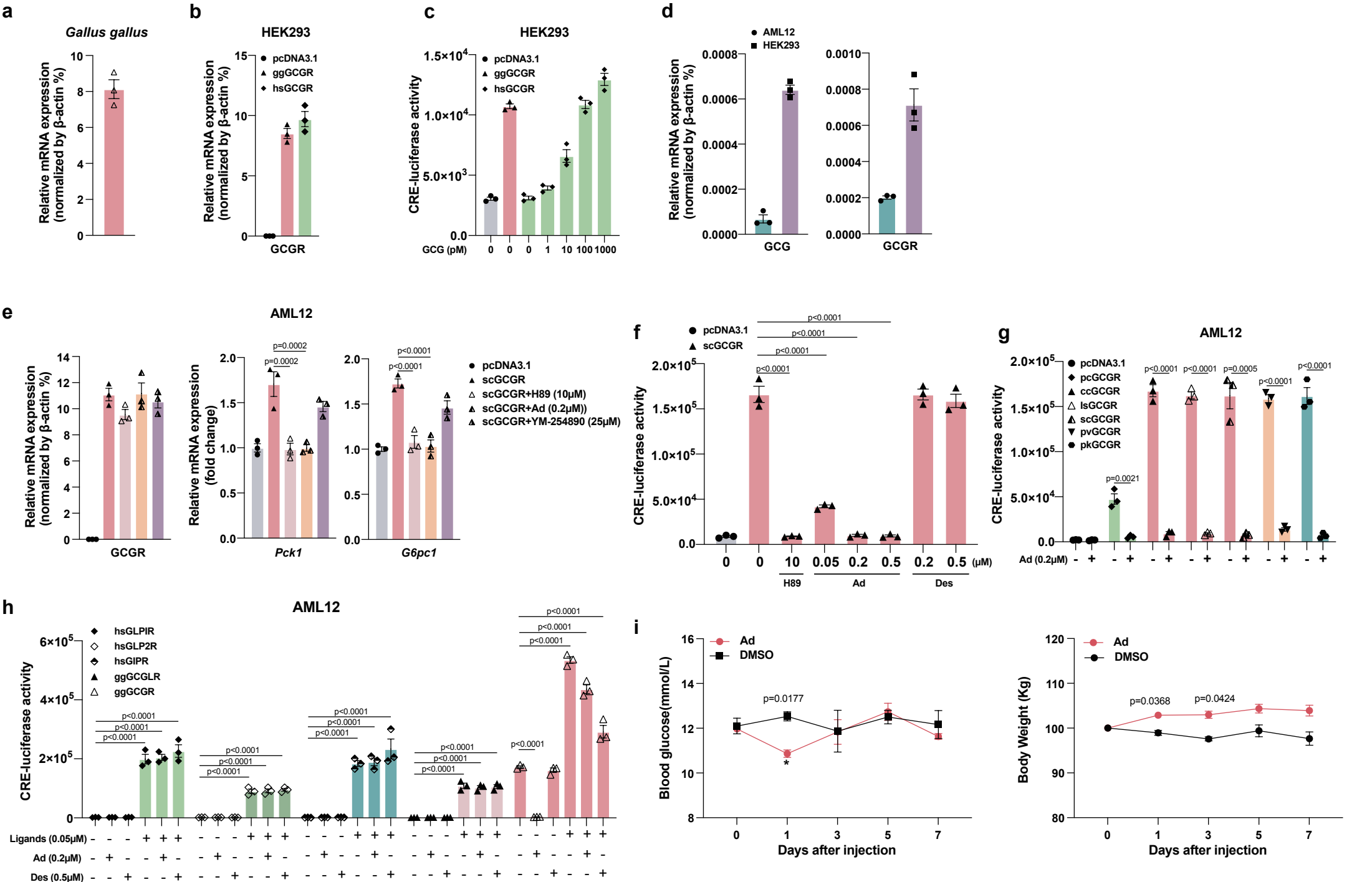
Supplementary Information Fig.2



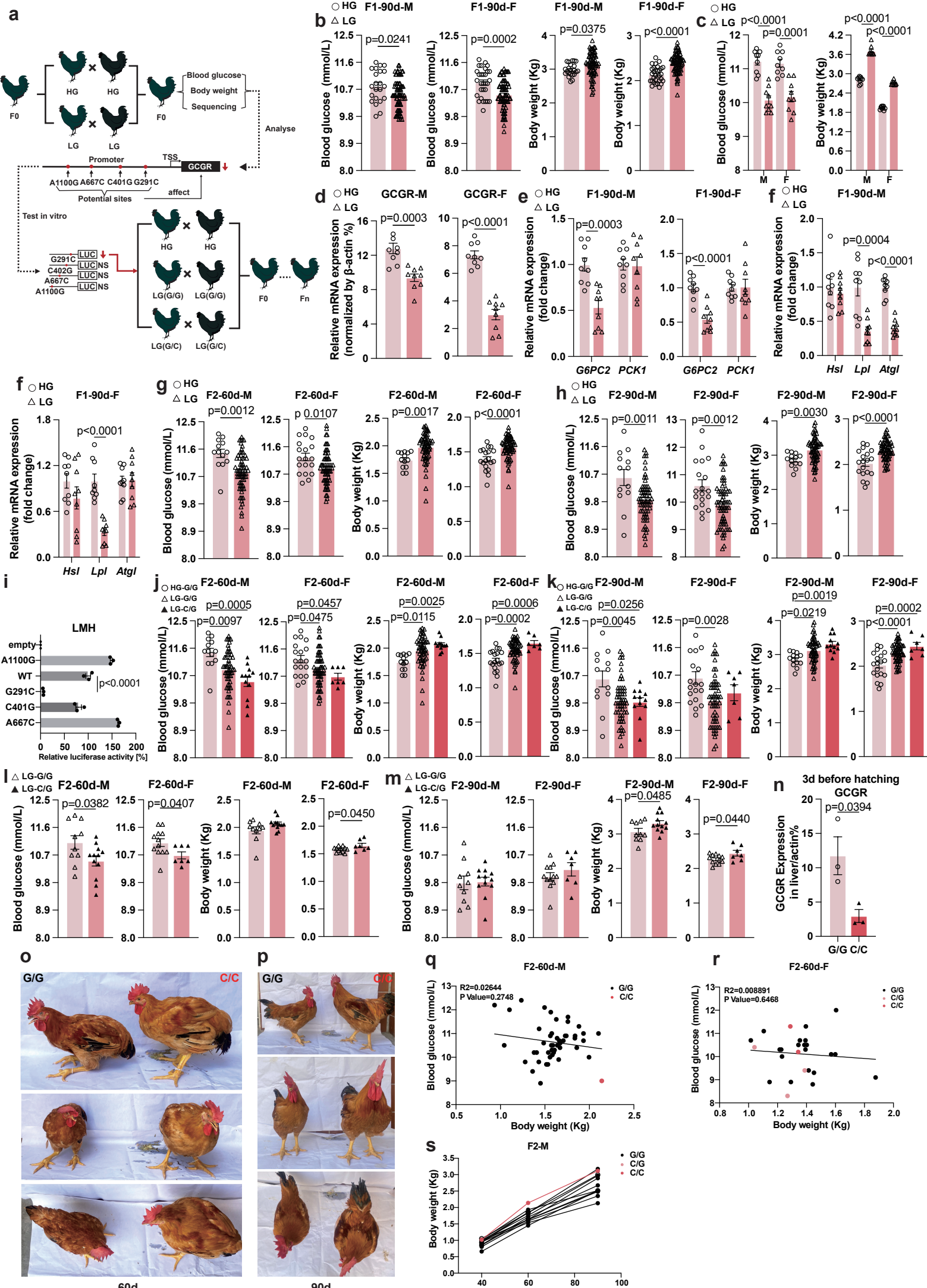
Supplementary Information Fig.3



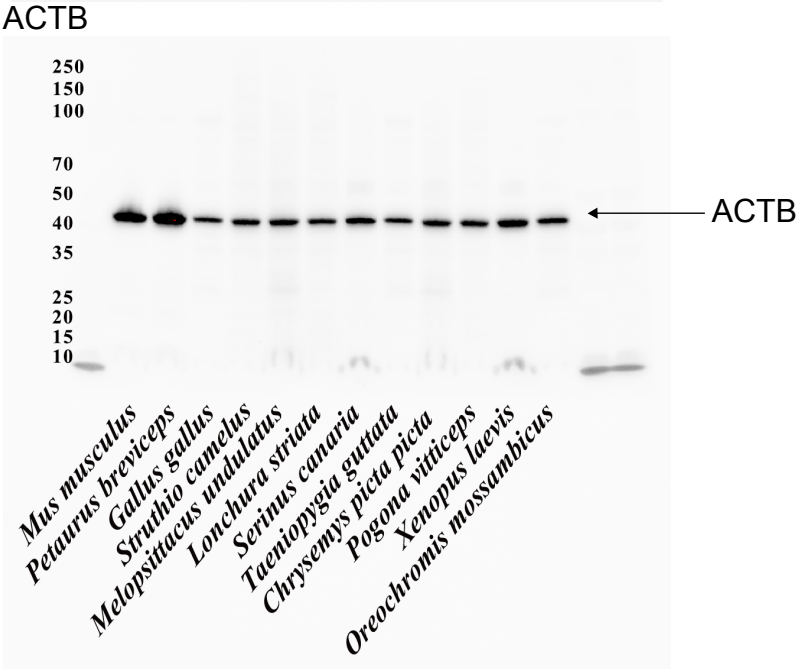
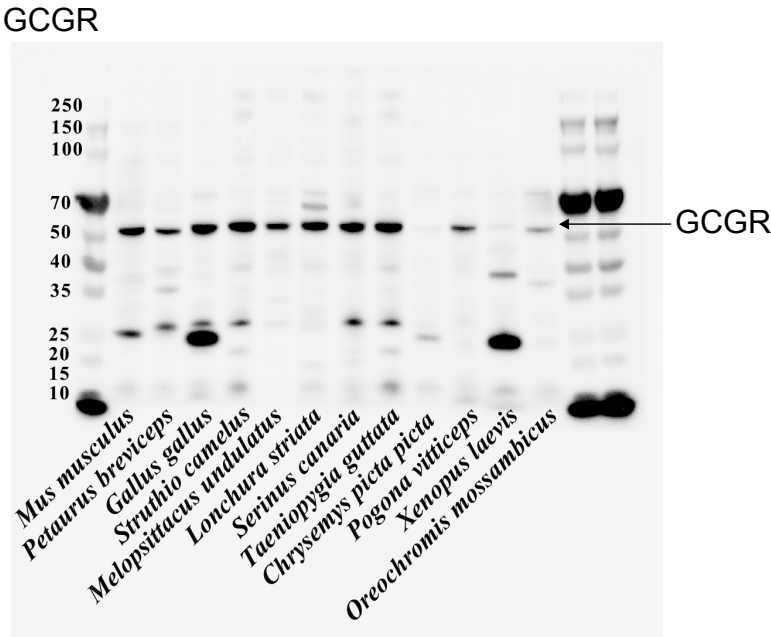
Supplementary Information Fig.4



Supplementary Information Fig.5

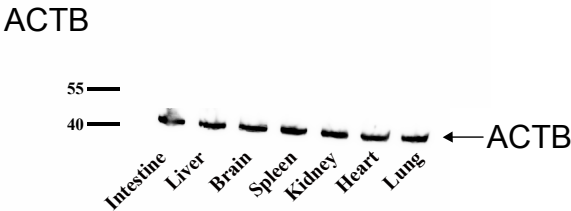
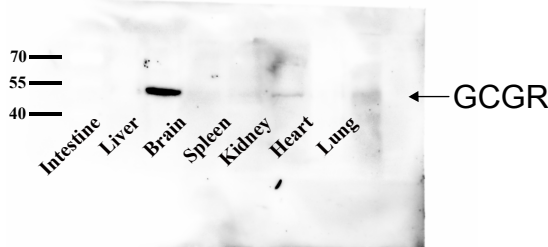


Supplementary Information Fig.6
Extended Data Fig.3a



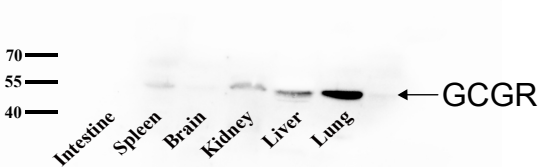
Extended Data Fig. 3k

Mus musculus
GCGR

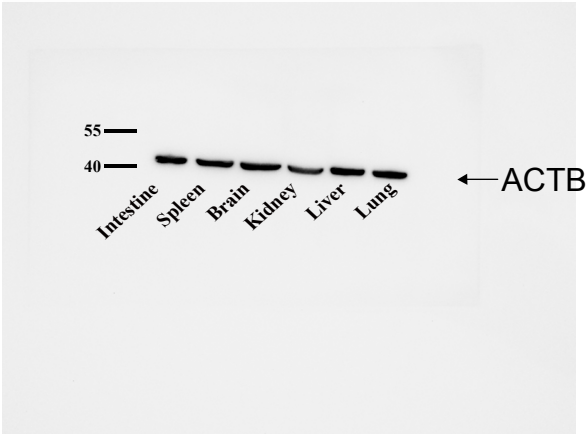


Extended Data Fig. 3l

Petaurus breviceps
GCGR

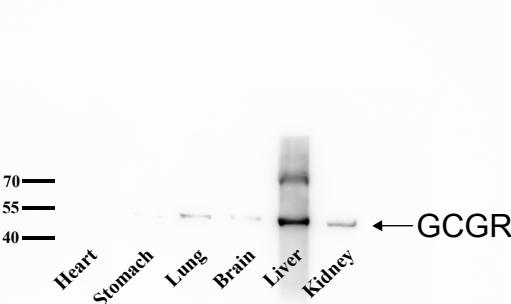


ACTB

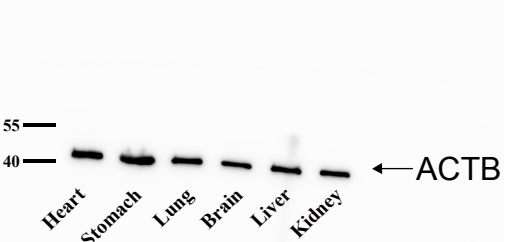


Extended Data Fig. 3m

Gallus gallus
GCGR

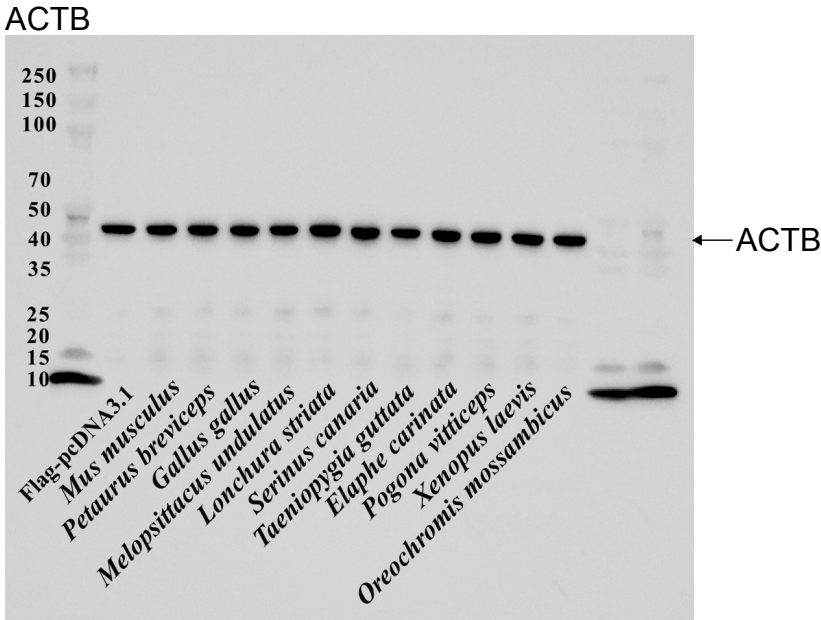
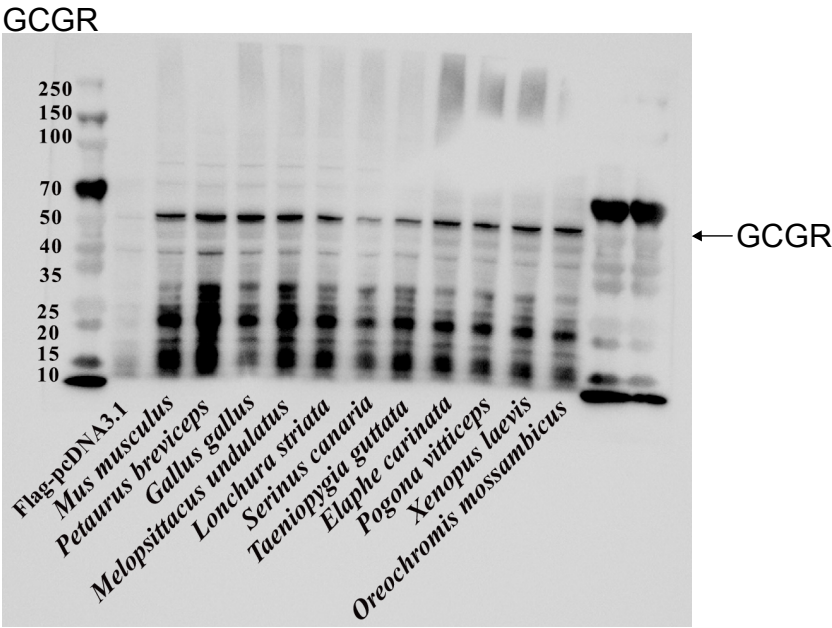
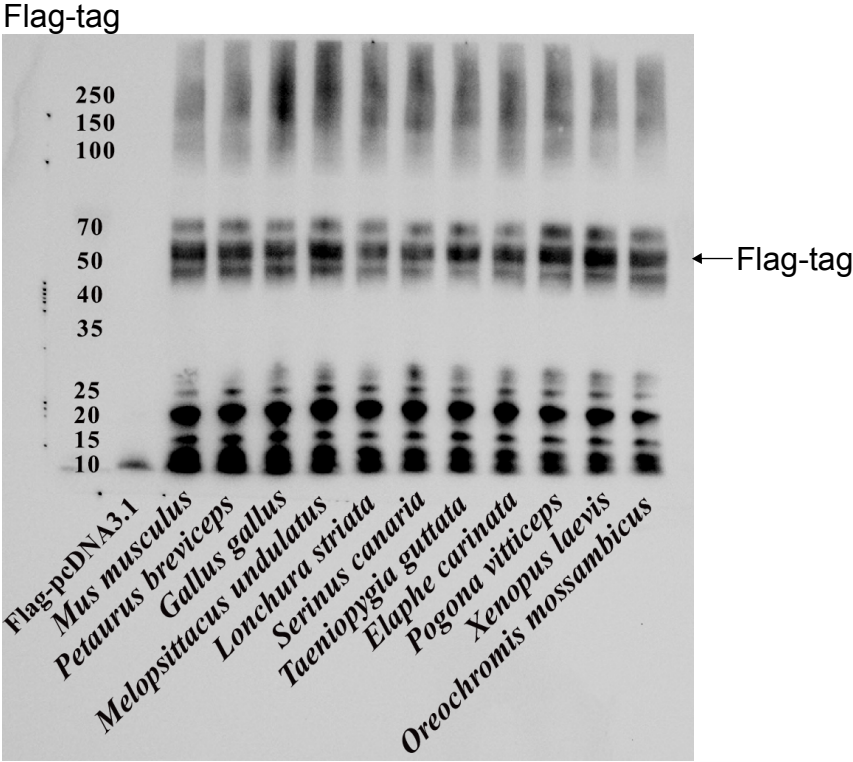


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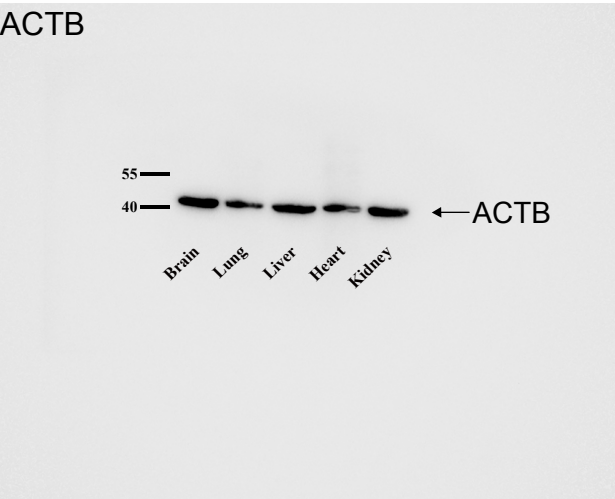
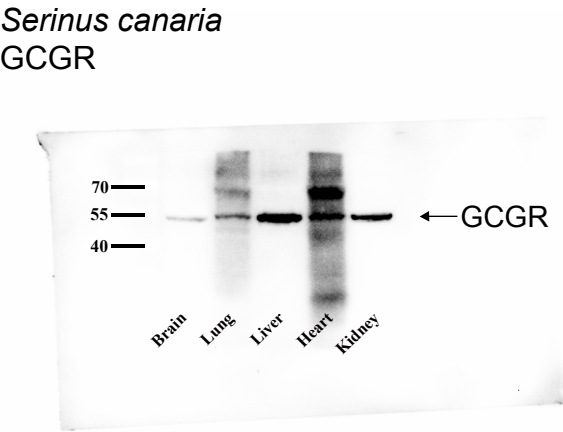


Supplementary Information Fig.7

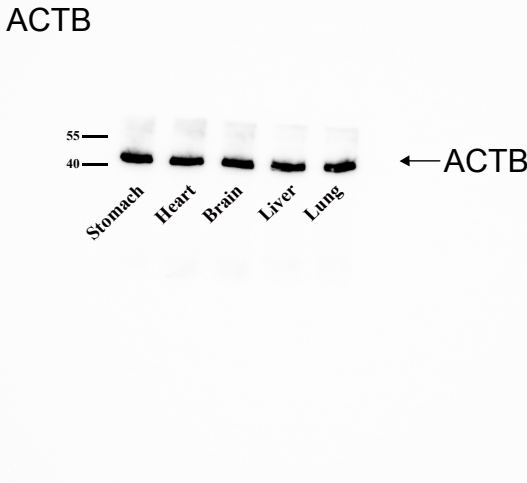
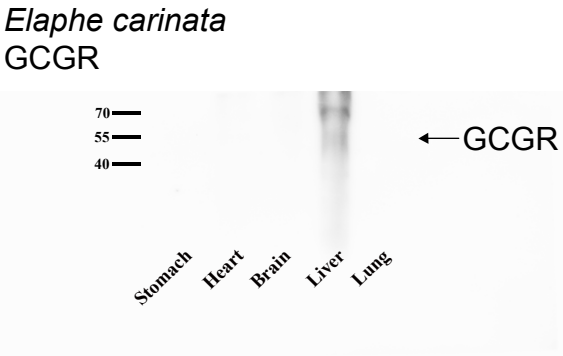
Extended Data Fig. 3t



Extended Data Fig. 3q



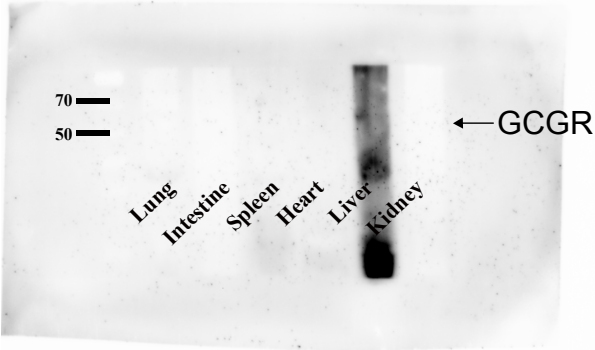
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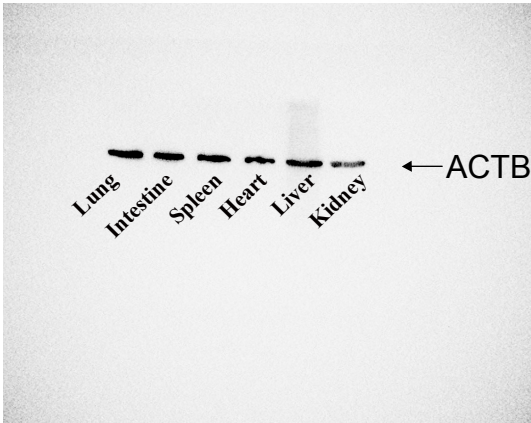
Supplementary Information Fig.8

Extended Data Fig. 3s

Xenopus laevis
GCGR

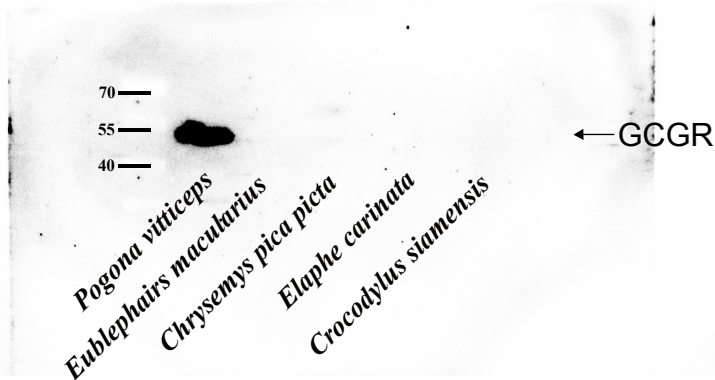


ACTB



Extended Data Fig. 4i

GCGR



ACTB

