Supplementary data

**Generation of GPRC6A knockout mice**

For the targeted disruption of *Gprc6a*, a genomic fragment of the mouse *Gprc6a* including exon VI was isolated from a P1 bacteriophage artificial chromosome (PAC) 129 library (RZPD, German Ressourcezentrum) (thus isogenic to the embryonic stem (ES) cells used) by screening with a human *GPRC6A* exon VI cDNA probe. A 9.8 kb *SpeI*/*SacI* DNA fragment including exons V and VI, intron V of *Gprc6a*, and 5’ and 3’ homology arms of 4.6 and 4.0 kb, respectively, was subcloned into pBluescript to generate the template for the targeting vector. Using the technique of ET cloning (homologous recombination (HR) in *E. coli* cells mediated by *recE* and *recT* proteins) (Muyrers et al. 1999), the *Loxp* and *Frt* sites were engineered. To generate the targeting vector, an upstream *Loxp* site was first introduced (Figure 1). By PCR we generated a *Loxp* flanked kanamycin resistance (*Kana*) gene flanked on each side by 50-60 bp homology region at the target point in *Gprc6a* and an engineered *SacI* site. The PCR product was then engineered into the template by HR in recombinant positive *E. coli* cells (strain JC8657) (Zhang et al. 1998), followed by removal of the floxed *Kana* gene by transformation into CRE-expressing *E. coli* (Buchholz et al. 1996), thus leaving one *Loxp* site at the desired location desired location (300 bp upstream of exon VI). Correspondingly, a second ET recombination allowed insertion of a second *Kana* 300 bp downstream of exon VI (Fig. 2), and flanked by the unique restriction sites, *SgrAI* and *SphI*. Excision of the *Kana* gene and subsequent ligation of an available construct (Casanova et al. 2002) consisting of 5’-*Loxp*- *SgrAI*-Frt-Neo-Frt-*Loxp*-AscI-3’ resulted in the final gene targeting vector. Correct orientation and functionality of the *Loxp* and *Frt* sites was verified by DNA sequencing and by transformation of the targeting construct into *E. coli* expressing CRE or FLP recombinases (Buchholz et al. 1996).

*GPRC6A*⁻/⁻ mice were generated by homologous gene targeting in E14.1 ES cells (129P2/OlaHsd). The targeting vector was linearized and electroporated into ES cells grown in medium containing G418 (300 µg ml⁻¹).
Body weight measurements

For analysis of body weight, 8 GPRC6A wild type and knockout mice of each gender fed an a regular chow diet were weighed every week from week 6 to week 16, and every two weeks from week 18 to week 38. At no time point was there any statistical difference in body weight or percent weight gain (Figure 3) between wild type and knockout animals (student’s t test).

References

Buchholz F, Angrand PO & Stewart AF 1996 A simple assay to determine the functionality of Cre or FLP recombination targets in genomic manipulation constructs. *Nucleic Acids Research* 24 3118-3119.

Casanova E, Fehsenfeld S, Greiner E, Stewart AF & Schutz G 2002 Conditional mutagenesis of CamKIV. *Genesis* 32 161-164.


Legends for figures

**Figure 1.** Introduction of the first *Loxp* site into the targeting vector. (A) Top, PCR to amplify a floxed *Kana* gene with engineered 5’ and 3’ homology regions. *Loxp* sites are indicated by black triangles. Primers were designed to contain 50-60 nucleotide GPRC6A sequence (green and blue) at the desired positions in the genomic DNA (300 bp upstream of exon VI), and an artificial *SacI* site to allow for diagnosis of the construct. Middle, homologous recombination (HR) in recombinant positive *E. coli* cells between the PCR product and the template construct containing the targeted part of *Gprc6a* (1), yielding a product as shown (2). This product was selected for, using both ampicillin and kanamycin. Bottom, transformation into rec⁺ CRE-expressing *E. coli* cells allowed for deletion of *Kana* but left one *Loxp* site at the intended position (3). (B) Verification of the introduction of the floxed kanamycin in (2) by a restriction digest and envisaged by gel electrophoresis. Due to the insertion of a *SacI* site, the upper band at 8.3 kb was split into two bands of 4.3 and 4.0 kb.

**Figure 2.** Introduction of the second *Loxp* site and *Frt* flanked (flrtered) neomycin (neo) resistance cassette in the *Gprc6a* targeting vector. Top, PCR to amplify a *Kana* gene with engineered 5’ and 3’ homology regions. *Loxp* sites are indicated by black triangles. Primers were designed to contain 50-60 nucleotides of GPRC6A sequence (orange and purple) at the desired positions in the genomic DNA (300 bp downstream of the polyadenylation site in exon VI), and artificially engineered *SgrAI*, *SphI* and *AscI* restriction enzyme recognition sites. Homologous recombination in recombinant positive *E. coli* cells between the PCR product and the template construct containing *Gprc6a* genomic DNA and one *Loxp* site, yielding a product with an inserted *Kana* gene and restriction sites as shown. This product was selected for, using both ampicillin and kanamycin. The targeting vector was finally assembled by a *SgrAI/AscI* mediated excision and subsequent ligation with a fragment containing a flrtered *Neo* cassette and the second *Loxp* site.

**Figure 3.** Comparison of relative change in body weights between female and male GPRC6A+/+ and −/− mice, fed on a regular chow diet, as a function of age. Results are means ± SEM of 8 mice from each genotype. Data have been normalized to percent of weight at age 6 weeks. The body weight gain of either gender of GPRC6A−/− mice was not significantly different from +/+ littermates (student’s *t* test, P > 0.05).
Supp. data Figure 2
Supp. data Figure 3

- Male
  - +/-
  - +/-

- Female
  - +/-
  - +/-