Maternal deletion of the NESP55 differentially methylated region (DMR) (delNESP55/A5delE3-4Δ, delNE2AS) from the GNAS locus in humans causes autosomal dominant pseudohypoparathyroidism type Ib (AD-PHP-Ib), a disorder of proximal tubular parathyroid hormone (PTH) resistance associated with loss of maternal GNAS methylation imprints. Mice carrying a similar, maternally inherited deletion of the Nes55 DMR (ΔNesp55) replicate these Gnas epigenetic abnormalities and show evidence for PTH resistance, yet these mice demonstrate 100% mortality during the early postnatal period. We investigated whether the loss of extra-large αs (XLαs) imprinting and the resultant biallelic expression of XLαs are responsible for the early postnatal lethality in ΔNesp55 mice. First, we found that ΔNesp55 mice are hypoglycemic and have reduced stomach-to-body weight ratio. We then generated mice having the same epigenetic abnormalities as the ΔNesp55 mice but with normalized XLαs expression due to the paternal disruption of the exon giving rise to this Gnas product. These mice (ΔNesp55/mNASm−m+/p−) showed nearly 100% survival up to postnatal day 10, and a substantial number of them lived to adulthood. The hypoglycemia and reduced stomach-to-body weight ratio observed in 2-d-old ΔNesp55 mice were rescued in the ΔNesp55/mNASm−m+/p− mice. Surviving double-mutant animals had significantly reduced Gnas mRNA levels and showed hypoccaemia, hyperphosphataemia, and elevated PTH levels, thus providing a viable model of human AD-PHP-Ib. Our findings show that the hypoglycemia and early postnatal lethality caused by the maternal deletion of the Nes55 DMR result from biallelic XLαs expression. The double-mutant mice will help elucidate the pathophysiological mechanisms underlying AD-PHP-Ib.

stimulatory G protein | renal proximal tubule | cyclic AMP

Most autosomal genes are expressed equally from both parental alleles, but in a subset of mammalian genes, the transcription from one allele is epigenetically repressed based on its parent of origin; this process is called genomic imprinting (1, 2). The proper dosage of imprinting genes is critical for survival, and aberrant expression of normally imprinted alleles is responsible for several human disorders, including, but not limited to, Beckwith–Wiedemann syndrome [Mendelian Inheritance in Man (MIM) 130650], Prader–Willi syndrome (MIM 176270), Angelman syndrome (MIM 105830), Silver–Russell syndrome (MIM 180860), transient neonatal diabetes (MIM 601410), and autosomal dominant pseudohyoparathyroidism type Ib (AD-PHP-Ib; MIM 603233).

The genes encoding human and mouse Gs (GNAS and Gnas) are complex, imprinted loci located within chromosomal regions of conserved synteny (distal chromosome 2 in mice, 20q13.32 in humans) and have similar overall organizations (3–5). GNAS/Gnas generates multiple gene products through the use of different alternative promoters and first exons that splice onto common exons (2–13 in humans and 2–12 in mice) (Fig. S1). The most downstream alternative first exon is Gox exon 1, which generates transcripts encoding the ubiquitously expressed Gox (6). The Gox promoter resides within a nonmethylated CpG island, but despite the absence of differential methylation at its promoter, Gox shows predominantly maternal expression in some tissues, including pituitary, thyroid, renal proximal tubules, and gonads (7–10); Gox expression is biallelic in most other tissues (11–13). The furthest upstream alternative promoter generates transcripts that encode the neuroendocrine-specific protein of 55 kDa (NESP55; mouse Nes55), a chromogranin-like protein, the coding sequence of which is located within a specific upstream exon; Gox exons 2–13 reside within the 3′ untranslated region of NESP55 transcripts (14). This mRNA shows exclusive maternal expression, because its promoter is methylated on the paternal allele (12, 15, 16). A third alternative promoter generates transcripts encoding the extralarge Gox isoform (XLαs) (17). XLαs has a long amino-terminal extension encoded by its specific first exon, whereas the remainder of the protein is identical to Gox. XLαs is imprinted oppositely to NESP55; i.e., its promoter is methylated on the maternal allele and transcriptionally active only on the paternal allele (12, 15, 16). Within the same differentially methylated region (DMR) and just upstream of the XLαs promoter is a promoter driving expression of a paternally expressed antisense transcript, which is noncoding and traverses the NESP55 exon from the opposite direction (Aβ; mouse Nespas) (18, 19). Like XLαs, this Aβ transcript is expressed from the paternal allele. A fifth alternative promoter and first exon (Aβ; mouse exon 1A) also splices onto exons 2–13 (mouse 2–12) (20). The Aβ transcript is paternally expressed and presumed to be untranslated (21), although a recent study has shown that it can lead to an amino-terminally truncated Gox variant that may have biological activity (22). Loss of methylation at the maternal Aβ exon and promoter leading to biallelic A/B expression is found in patients with PHP-Ib, who show renal parathyroid hormone (PTH) resistance presumably due to Gox deficiency in the renal proximal tubule...
This finding suggests that exon A/B controls tissue-specific \( \alpha \text{G} \)s imprinting via the presence of one or more regulatory \( \text{cis} \)-acting elements that are both tissue-specific and methylation-sensitive. Genetic microdeletions identified in AD-PHP-Ib suggest that \( \text{cis} \)-acting elements within the nearby \( \text{STX16} \) locus and the upstream \( \text{NESP55} \) DMR or transcription from the \( \text{NESP55} \) promoter may be critical for the establishment and/or maintenance of exon A/B maternal-specific methylation (25–29).

We recently developed a mouse model of AD-PHP-Ib by deleting the maternal \( \text{Nesp55} \) DMR (30) in a manner similar to the deletions described in some patients with this disease (26). This mouse strain, \( \Delta \text{Nesp55}^{\text{m}} \), phenocopies AD-PHP-Ib with respect to the \( \text{GNAS} \) imprinting defects—i.e., loss of all of the maternal \( \text{Gnas} \) methylation imprints combined with increased (biallelic) 1A transcription—and with respect to the abnormal regulation of mineral ion homeostasis—i.e., hypocalcemia, hyperphosphatemia, and secondary hyperparathyroidism (30). However, unlike the findings in patients with deletions involving \( \text{NESP55} \) and antisense exons 3 and 4 (AD-PHP-Ib-de\( \text{NESP55}\)m), there is 100% early postnatal lethality in \( \Delta \text{Nesp55}^{\text{m}} \) mice, whereas mice in which the paternal \( \text{Nesp55} \) DMR is deleted (\( \Delta \text{Nesp55}^{\text{p}} \) mice) show no epigenetic and biochemical abnormalities and have an apparently normal phenotype and life span. The lethality in \( \Delta \text{Nesp55}^{\text{m}} \) mice, which was assumed to reflect worsening hypocalcemia during the first 5 d of life, prevented additional investigation of this mouse model of AD-PHP-Ib regarding the mechanisms underlying PTH resistance.

In this study, we further investigated the cause of the early postnatal death in \( \Delta \text{Nesp55}^{\text{m}} \) mice to reach a better understanding of the consequences of abnormal \( \text{Gnas} \) methylation and, thereby, to generate hypotheses as to how to extend the life span of this AD-PHP-Ib mouse model. Both 1A and \( \text{XLas} \) transcripts are expressed biallelically in these mice. Biological roles of the \( \text{XLas} \) protein remain almost completely unknown, but data from mouse models have shown that it is essential for postnatal adaptation to feeding and for glucose and energy metabolism. Moreover, some in vivo data indicate that \( \text{XLas} \) can oppose the actions of \( \text{Gnas} \) (31–33). We therefore reasoned that overexpression of \( \text{XLas} \) contributes to the phenotype in the \( \Delta \text{Nesp55}^{\text{m}} \) mice by further antagonizing the already diminished \( \text{Gnas} \) actions. For example, hypocalcemia associated with lowered \( \text{Gnas} \) levels in the kidney, and the resultant PTH resistance, could be exacerbated by increased \( \text{XLas} \) levels. To address whether the loss of \( \text{XLas} \) imprinting is involved in the phenotypes observed in the \( \Delta \text{Nesp55}^{\text{m}} \) mice, we generated mice in which the \( \text{Gnas} \) methylation profile of the \( \Delta \text{Nesp55}^{\text{m}} \) mouse is preserved but the expression of \( \text{XLas} \) is confined to a single parental allele. Our investigations revealed that the loss of \( \text{XLas} \) imprinting contributes significantly to the early postnatal lethality phenotype observed in \( \Delta \text{Nesp55}^{\text{m}} \) mice.

**Results**

The \( \Delta \text{Nesp55}^{\text{m}} \) mice die within 5 d of postnatal life in 129/S2 and C57BL/6j backgrounds (30). As an attempt to improve survival of these mice, we crossed them into the outbred CD1 strain, but the early postnatal lethality did not change significantly, with no pups surviving until postnatal day 10 (P10). We have previously shown that \( \Delta \text{Nesp55}^{\text{m}} \) mice are hypocalcemic at P2 and attributed the lethality to the possible worsening of the low calcium levels by day 5 (30). To determine whether other factors could underlie or contribute to the early postnatal lethality of the \( \Delta \text{Nesp55}^{\text{m}} \) mice, we measured blood glucose levels at P2. Compared with wild-type littermates, \( \Delta \text{Nesp55}^{\text{m}} \) pups were hypoglycemic [81.8 ± 5.0 mg/dL (\( n = 21 \)] vs. 54.0 ± 4.0 mg/dL (\( n = 28 \)); \( P < 0.05 \) and had considerably lower, albeit readily measurable, insulin levels (Fig. S2). Corticosterone levels were markedly higher than in wild-type littermates (Fig. S2), thus ruling out adrenal insufficiency as the cause of hypoglycemia. Most \( \Delta \text{Nesp55}^{\text{m}} \) mice had visible milk in their stomach, but compared with wild-type littermates, there was a ~50% reduction in their stomach-to-body weight ratio [0.0572 ± 0.0034 (\( n = 10 \) vs. 0.0278 ± 0.0026 (\( n = 20 \)); \( P < 0.05 \), an indirect measure that has previously been used to assess food intake (31). Thus, these results indicated that \( \Delta \text{Nesp55}^{\text{m}} \) pups were feeding insufficiently.
To determine whether the loss of XLαs imprinting and the consequently doubled XLαs expression level could underlie any of these early postnatal phenotypes in the ΔNesp55<sup>m</sup> mice, we mated female ΔNesp55<sup>m</sup> mice with male Gnasα<sup>−/−</sup> or Gnasα<sup>−/−</sup> mice, which resulted in four genotypes, including double mutants (ΔNesp55<sup>m</sup>Gnasα<sup>−/−</sup>), in which paternal XLαs expression was abolished and maternal XLαs expression was derepressed (Fig. 1A). Consistent with this reestablished monoallelic expression, the level of XLαs expression was abolished and maternal XLαs expression was derepressed (Fig. 1A). As expected from ΔNesp55<sup>m</sup>2-d-old mice (Fig. 1A), the Gnasα methylation status in ΔNesp55<sup>m</sup>Gnasα<sup>−/−</sup> pups was identical to that of ΔNesp55<sup>m</sup>, showing a loss of all of the maternal imprints and an apparent gain of methylation at Nesp55 (Fig. S3).

Subcutaneous edema noted in early postnatal ΔNesp55<sup>m</sup> pups (30) also existed in ΔNesp55<sup>m</sup>Gnasα<sup>−/−</sup> littermates (Fig. 1C). However, the latter animals were visibly bigger (Fig. 1C) and weighed significantly more at P2 (Fig. 1D) than both ΔNesp55<sup>m</sup> and Gnasα<sup>−/−</sup> littermates. At this age, approximately equal numbers of pups of each genotype were alive (Table 1). Two-day-old double-mutant mice had normal blood glucose levels, unlike their ΔNesp55<sup>m</sup> or Gnasα<sup>−/−</sup> littermates, which were both hypoglycemic (Fig. 2A). During a 3-h fasting period, wild-type and double-mutant mice displayed a similar blood glucose profile; ΔNesp55<sup>m</sup> or Gnasα<sup>−/−</sup> mice showed much lower blood glucose levels at baseline than wild-type and double-mutant mice; all strains, with the exception of ΔNesp55<sup>m</sup> mice, showed a drop in the levels within the first hour, which was followed by a rise to the initial values by the second hour (Fig. 2B). Only ΔNesp55<sup>m</sup> mice seemed to maintain constant glucose levels during fasting (Fig. 2B). In Gnasα<sup>−/−</sup> pups, however, the blood glucose level diminished in the first hour, but no recovery to the initial value was observed, with the hypoglycemia after 3 h being more severe than that observed before fasting (Fig. 2B). The ΔNesp55<sup>m</sup>Gnasα<sup>−/−</sup> and wild-type littermates had similar stomach weights, unlike ΔNesp55<sup>m</sup> and Gnasα<sup>−/−</sup> littermates, which both showed a similar degree of reduction in stomach weight (Fig. 2C). Consistent with inadequate feeding, ΔNesp55<sup>m</sup> and Gnasα<sup>−/−</sup> pups had significantly lower liver glycogen content than double-mutant and wild-type littermates (Fig. 2D).

At P10, no ΔNesp55<sup>m</sup> mice were found alive. In contrast, double-mutant littermates were observed at a frequency close to that predicted from Mendelian inheritance, although the number of surviving ΔNesp55<sup>m</sup>Gnasα<sup>−/−</sup> was slightly lower than wild-type littermates (Table 1). These results indicated a marked improvement in the survival of double-mutant mice compared with ΔNesp55<sup>m</sup> mice. The 10-d-old ΔNesp55<sup>m</sup>Gnasα<sup>−/−</sup> mice weighed significantly less than wild-type littermates (6.62 ± 0.64 vs. 8.60 ± 0.25 g; n = 10; P < 0.05) and were significantly hypocalcemic (Fig. 3A). The mean plasma phosphorus and PTH levels in 10-d-old ΔNesp55<sup>m</sup>Gnasα<sup>−/−</sup> mice tended to be higher than wild-type littermates, although statistical significance could not be reached (Fig. 3B and C). Interestingly, 10-d-old Gnasα<sup>−/−</sup> littermates also showed mild but significant hypocalcemia, combined with hyperphosphatemia (Fig. 3A and B).

Discussion

We have previously generated mice in which the Gnas Nesp55 DMR was ablated (30). Maternal deletion of this DMR led to a loss of all maternal Gnas imprint marks and biochemical abnormalities consistent with PTH resistance, similar to that observed in AD-PHP-β<sub>3</sub>Δ<sub>INAnim</sub> patients, who carry the equivalent deletion in the same locus. Loss of A/B imprinting is thought to be due to lack of calcium in the bloodstream, thereby reducing Gs expression levels and leading to PTH resistance. Because ΔNesp55<sup>m</sup> mice, unlike patients with AD-PHP-β<sub>3</sub>Δ<sub>INAnim</sub>, showed 100% early postnatal lethality, we further investigated these mice to search for the cause of this unexpected phenotype. The ΔNesp55<sup>m</sup> mice show loss of XLαs imprinting (30), and we therefore asked whether the early postnatal phenotype of the ΔNesp55<sup>m</sup> mice resulted from the loss of XLαs imprinting. Our findings revealed that ΔNesp55<sup>m</sup> mice are hypoglycemic and that this phenotype, as well as the early postnatal demise of these animals, can be prevented by normalizing XLαs expression. These observations indicate that restricting the expression of XLαs (or any of the other Gnas products that use exon XL, e.g., XLαs-N1, XXLαs, and ALEX; refs. 34–37) to a single parental allele is critical for survival and maintaining normal blood glucose levels during the early postnatal period.

Table 1. Frequency of each genotype among offspring from ΔNesp55<sup>m</sup> and Gnasα<sup>−/−</sup> intercrosses

<table>
<thead>
<tr>
<th>Age</th>
<th>Wild type, n (%)</th>
<th>ΔNesp55&lt;sup&gt;m&lt;/sup&gt;, n (%)</th>
<th>ΔNesp55&lt;sup&gt;m&lt;/sup&gt;Gnasα&lt;sup&gt;−/−&lt;/sup&gt;, n (%)</th>
<th>Gnasα&lt;sup&gt;−/−&lt;/sup&gt;, n (%)</th>
<th>Total born</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>56 (23.9)</td>
<td>50 (21.4)</td>
<td>50 (21.4)</td>
<td>54 (23.1)</td>
<td>234</td>
</tr>
<tr>
<td>P10</td>
<td>41 (25.3)</td>
<td>0 (0)</td>
<td>36 (22.2)*</td>
<td>31 (19.1)**</td>
<td>162</td>
</tr>
<tr>
<td>Adult</td>
<td>33 (26.6)</td>
<td>0 (0)</td>
<td>9 (7.3)</td>
<td>16 (12.9)</td>
<td>124</td>
</tr>
</tbody>
</table>

The values represent the number of surviving pups and percentage (in parentheses) relative to the total number of pups that were born. Data were obtained from 18, 12, and 10 litters for P2, P10, and adult mice, respectively. Number of dead pups for individual genotypes was estimated according to the number of wild-type pups, assuming 100% survival for the latter. *P < 0.05; **P < 0.001 compared with wild type by 2 test analysis using live and dead animals.
involutions of these sites and, thereby, leads to a feeding defect through a related mechanism. Alternatively, the feeding difficulty in ΔNesp55m mice can result from a generalized neurological or motor defect that prevents the pups from getting access to the mother. In fact, some of the ΔNesp55m pups appear hyperactive (30), which may reflect a neurological defect.

The poor feeding and the depletion of liver glycogen likely contribute to the hypoglycemia observed in ΔNesp55m mice and their Gnasxl+/p− littermates. Interestingly, however, our analysis of Gnasxlm+/p− pups at P2 revealed a fasting glucose profile that is consistent with a defect in glucose counterregulation. Such a defect has been suggested for Gnasxl+/p− mice based on inappropriately low glucagon and inappropriately normal epinephrine, norepinephrine, and corticosterone levels (31). Some Gnasxl+/p− mice, despite having apparently defective glucose counterregulation, are able to survive in the outbred CD1 mouse strain (31), whereas no ΔNesp55m mice are rescued by crossing into different strains (30). Thus, mechanisms other than hypoglycemia likely contribute to the early postnatal lethality in the ΔNesp55m mice. Nonetheless, our findings show that the survival of these mice is markedly improved by limiting XLαs expression to a single allele. This finding accords with observations made in mice with disruption of Nesp55 transcription (Nesp55Δp−), in which there is variable loss of methylation of the XLαs and A/B DMRs (29). Mice with loss of methylation of both DMRs, and therefore with overexpression of XLαs, die within a few days of birth. In contrast, survival to weaning is observed in some mice with loss of methylation restricted to the A/B DMRs, which retain normal XLαs expression from only the paternal allele. Thus, the mechanism leading to the early postnatal lethality in ΔNesp55m mice remains to be investigated. Availability of mice in which XLαs is disrupted in a tissue-specific manner would be valuable in those investigations, but generation of mice with conditional XLαs ablation could not be accomplished yet (38).

Loss of XLαs imprinting is observed in most patients with PHP-Ib who show broad defects in GNAS methylation (23, 24, 39–45). Hypoglycemia is not a typical feature of this disease, but neonatal hypoglycemia has been documented in a PHP-Ib patient who had patUPD20q and, consequently, broad GNAS methylation defects (24). It is therefore possible that transient neonatal hypoglycemia occurs more frequently in these patients but is not perceived clinically as a presentation of PHP-Ib. Clinical characterizations of PHP-Ib patients during the early postnatal period will be important to verify whether loss of XLαs imprinting causes hypoglycemia in humans as well.

Unlike hypoglycemia and reduced stomach-to-body weight ratio, the s.c. edema observed around the necks of early postnatal ΔNesp55m mice was not rescued by the normalization of XLαs expression. This finding is not surprising, because neonatal edema was also observed in several other mouse models in which the maternal Gos allele was disrupted (10, 32, 46). Some of these models have normal food intake, including our double-mutant ΔNesp55m/Gnasxlm+/p− pups, and edema was also noted in utero (47); therefore, insufficient food intake is unlikely to contribute to the edema. Furthermore, 2-d-old ΔNesp55m pups, which were edematous, did not reveal any overt abnormalities in heart or liver. The mechanism underlying the edema remains to be determined, but it likely involves Gos deficiency in a tissue where maternal Gos expression is normally silenced.

The hypocalcemia and hyperphosphatemia with elevated PTH in ΔNesp55m/Gnasxlm+/p− mice is consistent with renal PTH resistance. This finding is also supported by the blunted PTH-induced elevation of urinary cAMP, which is consistent with the reduction in Gos mRNA in the renal proximal tubule. However, despite careful isolation and analysis of proximal...
set of proximal tubular cells. Our conditions, and/or this regulatory event may occur only in a sub-
can mimic G

microscopy. Data are expressed as mean
to the levels in wild-type (wt) mice. Tubules were isolated by laser capture
expression levels were normalized to
re
as is also true in patients with PHP-Ib (48). This result likely
demonstrated a normal calcemic response to PTH administra-
tion, indicating that the actions of PTH on bone are not impaired,
demonstrated a normal calcemic response to PTH administra-


tubes through the use of laser capture microscopy, we found that the reduction in Gds levels was only 50%. Thus, the silencing of paternal Gαs expression may not be complete under normal conditions, and/or this regulatory event may occur only in a subset of proximal tubular cells. Our ΔNesp55m/Gnasx−/− mice demonstrated a normal calcemic response to PTH administra-
tion, indicating that the actions of PTH on bone are not impaired, as is also true in patients with PHP-Ib (48). This result likely reflects the absence of paternal Gαs silencing in this tissue (13).

Like ΔNesp55m/Gnasx−/− mice, 10-d-old Gnasx−/− mice are also hypocalcemic and hyperphosphatemic, suggesting per-
haps that XLαs contributes to the renal actions of PTH. This explanation would be consistent with previous reports that XLαs can mimic Gαs actions (49, 50), and the absence of hypocalcemia and hyperphosphatemia in adult Gnasx−/− mice may indicate that the contribution of XLαs protein to mediating the renal effects of PTH may decline with age (50). Conversely, the cal-
cemic response to PTH is blunted in adult Gnasx−/− mice (Fig. 4D), suggesting that XLαs might still play a role in medi-
at ing PTH actions in bone. Consistent with this interpretation, XLαs protein expression has been detected in adult mouse osteocytes (51), which are importantly involved in the PTH-


dependent regulation of bone remodeling and calcium homeo-

stasis (52–54).

By generating the ΔNesp55m/Gnasx−/− mice, we were able to establish a viable mouse model of AD-PHP-Ib. Although their survival rate was found to be diminished, a substantial number of these double-mutant mice (currently >30) survived to adulthood and had seemingly normal life spans. The mechanisms underlying the pathogenesis of hypocalcemia and hyperphosphatemia result-
sing from PTH resistance could now be investigated further in the surviving ΔNesp55m/Gnasx−/− mice. Furthermore, pre-
weaning lethality of these double-mutant mice may indicate that paternal Gαs silencing occurs in more tissues than previously recognized or that overexpression of the other paternally expressed Gnas products—e.g., 1A and Nespas—has a negative effect on survival. These questions remain to be addressed.

Materials and Methods

Mouse Models. Gnasα−/− and ΔNesp55m mice were described (30, 31). The ΔNesp55m mice were crossed into the CD1 strain for more than six genera-
tions before mating female ΔNesp55m mice with male Gnasα−/− or Gnasα−/− mice, which were also maintained in the CD1 background. ΔNesp55m mice of the different genotypes were analyzed between 2 and 4 mo of age. These studies were carried out under Institutional Animal Care and Use Committee guidelines and approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Glucose, Insulin, and Corticosterone Analysis. Glucose was measured from truncal blood by using a glucose strip reader (Precision XceedPro Blood Glucose and β-Ketone Monitoring System; Abbott Laboratories). Truncal blood was furthermore collected into heparinized tubes to generate plasma for additional measurements. Insulin was measured by using the Ultra Sensitive Rat Insulin ELISA Kit (CrystalChem). Corticosterone was measured by using a radioimmunoassay (RIA; MP Biomedical).

Quantification of Liver Glycogen. Glycogen content was measured according to Roehrig and Allred (55) after dissolving 20–100 mg of liver tissue in 2 M NaOH. After neutralization (by 2 M HCl), an aliquot of dissolved liver was digested with amyloglucosidase (Sigma) at 55 °C for 15 min. Glucose content was measured by using the Glucose GO Kit (Sigma). Glucose was undetect-
able in nondigested liver. Values were normalized to the amount of liver protein, determined by the BCA reagent (Pierce). To combine data from different experiments, each value (micrograms of glucose per millgram of protein) was divided by the mean wild-type value obtained in the same experiment.

Gene Expression Analyses. Isolation of the renal proximal tubules and ex-
traction of total RNA were as described (50). Total RNA from 2-d-old mouse whole kidney was extracted by using the Qiagen RNeasy Mini kit. Quanti-
tative gene expression analysis for XLαs and Gαs was performed by TaqMan real-time RT-PCR with β-actin as a reference gene. Calculations were per-
formed by using the accurate cycle threshold method (56). Primers, probes, and conditions are available on request.

Calcium, Phosphorus, and PTH Measurements. Blood ionized calcium, plasma
phosphorus, and plasma PTH in P10 and adult mice were measured as de-
scribed (50). In assays to determine PTH responsiveness in vivo, blood and
urine were collected from mice before and after s.c. injection of 50 mU/kg human PTH(1–34). CAMP was quantified by using a RIA (50), and urinary CAMP values were normalized to urinary creatinine measured by using a STANBIO kit.

Statistical Analyses. Differences between means were evaluated for statistical significance by using the Student t test or, for multiple comparisons among groups of three or more, one-way ANOVA followed by Tukey’s post hoc test. The effect of genotype in fasting blood glucose levels was tested by two-way
ANOVA. χ2 test was used to compare the survival of genotypes at P10. P <
0.05 was considered to be significant. Statistical tests were performed by using GraphPad Prism Software.

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