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ORIGINAL ARTICLE

Adult Tph2 knockout mice without brain serotonin have moderately elevated spine trabecular bone but moderately low cortical bone thickness

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Disruption of serotonin synthesis in neurons and the periphery by knockout (KO) of mouse genes for tryptophan hydroxylases (peripheral *Tph1* and neuronal *Tph2*) has been claimed to decrease (*Tph2* KO) and increase (*Tph1* KO) bone mass. In this report, adult male and female *Tph2* KO mice were observed to have elevated spine trabecular bone. Female *Tph2* KO mice have reduced midshaft femur cortical bone thickness. Bone mass was normal in male and female *Tph1* KO mice examined as part of a *Tph1*/*Tph2* double knockout (DKO) mouse cohort.

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Introduction

Tryptophan hydroxylase (TPH) catalyzes the rate-limiting step in serotonin synthesis from tryptophan. There are two TPH enzymes, with TPH1 and TPH2 expressed in the periphery and neurons, respectively. Serotonin does not cross the blood–brain barrier, and thus serotonin actions are independently regulated in the brain and periphery. Brain serotonin influences mood, and selective serotonin reuptake inhibitors are used to treat depression. Platelets avidly accumulate serotonin, maintaining low free circulating serotonin levels. 5-hydroxyindoleacetic acid (5-HIAA) is the major metabolite of serotonin metabolism, and urinary 5-HIAA excretion is an index of the release of peripheral and neuronal serotonin. Historical developments involving serotonin discovery and actions have been reviewed.[1]

Knockout (KO) of *Tph1* in mice during 2003[2,3] led to the discovery that *Tph1* and *Tph2* are separate genes,[4] and several groups have studied *Tph2* KO mice since 2008. Intestinal enterochromaffin cells are the primary depot of peripheral serotonin. Although gastrointestinal motility is minimally affected in *Tph1* KO mice studied under standard laboratory conditions,[5] detailed examination of colonic motility shows that serotonin secreted by enterochromaffin cells robustly stimulates mucosal colonic migrating motor complexes and fecal pellet propulsion while only minimally affecting stretch-induced peristalsis.[6] Intestinal enteric neurons express TPH2, and *Tph2* KO mice have enhanced gastric emptying, decreased intestinal motility and reduced numbers of enteric neurons.[7] The multiple phenotypes observed in *Tph1* and *Tph2* KO mice have been elegantly reviewed.[8–10]

Decreased trabecular bone mass in spine and femur has been reported in growing *Tph2* KO mice.[11] This laboratory[12] previously reported that disruption of *Tph1* in mice resulted in high trabecular bone mass at 4 to 12 weeks of age by removing the normal suppression of bone formation promoted by gut-derived circulating serotonin. A second group[13] observed high trabecular bone mass in *Tph1* KO mice at 6 weeks of age, but not at 16 weeks of age, resulting from reduced bone resorption through an osteoclast autonomous mechanism. High bone mass in *Tph1* KO mice was not confirmed by two other laboratories, including Lexicon.[14,15] Interestingly, double knockout (DKO) of *Tph1* and *Tph2* was reported to result in low bone mass, suggesting that neuronal *Tph2* might have a more critical role than peripheral *Tph1* in skeletal metabolism.[11]

Using dual energy X-ray absorptiometry (DEXA) and micro-computed tomography (microCT) high-throughput screening, Lexicon examined 3762 viable gene KO mouse lines and found no obvious skeletal phenotypes in *Tph1* and *Tph2* KO mice.[16] Given continued interest in serotonin and bone,[17] we examined bone mass in additional cohorts of adult *Tph1* KO, *Tph2* KO and *Tph1*/*Tph2* DKO mice.

Results

Male (18–21 weeks) and female (83 weeks) *Tph2* KO mice fed a high-fat diet were examined in the first study, which involved bone measurements by both DEXA and microCT. The second study used quantitative magnetic resonance (QMR) body composition and microCT analyses of bones from male (33 weeks) and female (69 weeks) *Tph1* KO, *Tph2* KO and *Tph1*/*Tph2* DKO mice. Brain serotonin content was greatly reduced in

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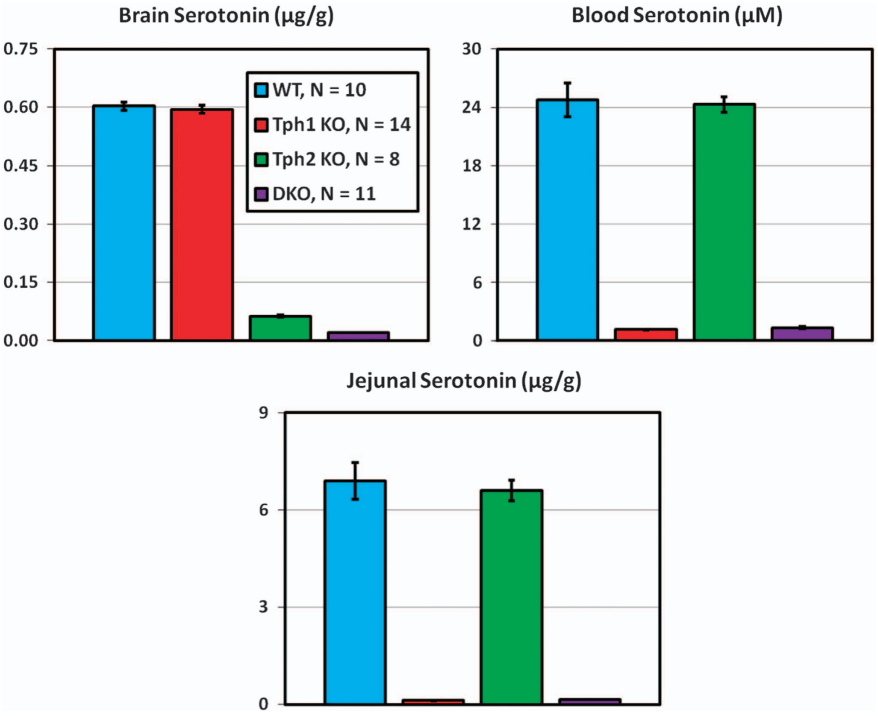

Figure 1 Brain, whole blood and jejunal serotonin contents in *Tph1* KO, *Tph2* KO and *Tph1/Tph2* DKO mice. Male mice were examined at 33 weeks of age.

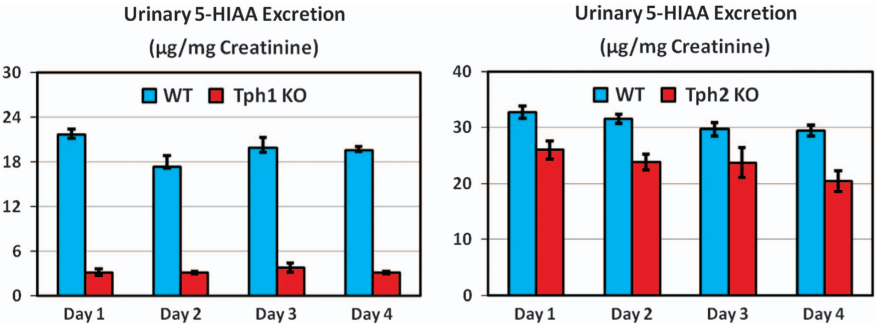

Figure 2 Urinary 5-HIAA excretion for *Tph1* and *Tph2 KO* mice. Male *Tph1* KO mice studied at 14 months of age (left) and female *Tph2* KO mice at 6–7 months of age (right). *Tph1* KO mouse data were published previously.[24] Data are means ± s.e.m. for six mice per group. For *Tph1* KO mice, $P<0.001$ for Days 1, 2, 3 and 4. For *Tph2* KO mice, $P<0.01$ for days 1, 2 and 4.

Tph2 KO and *Tph1/Tph2* DKO mice. Whole blood and jejunal serotonin contents were greatly reduced in *Tph1* KO and *Tph1/Tph2* DKO mice (**Figure 1**). Twenty-four-hour urinary excretion (**Figure 2**) of 5-HIAA (measured during 4 consecutive days) was reduced 24% in *Tph2* KO mice. For *Tph1* KO mice (exon 3 disrupted), urinary 5-HIAA excretion was reduced 83%. Urine volumes and creatinine excretions were identical in WT and KO mice in both studies (data not shown). This greater reduction in urinary 5-HIAA excretion in *Tph1* compared with *Tph2* KO mice reflects the greater contribution of TPH1 to body serotonin turnover. Body weight (obtained during high-throughput screening) was reduced during growth in *Tph2* KO mice (**Figure 3**).

Tph1 and *Tph2* KO mice had reduced body fat (**Tables 1 and 2**), and studies examining this lean phenotype in *Tph2* KO mice will be reported separately. Femur length was decreased by 3–4% in male *Tph2* KO and *Tph1/Tph2* DKO mice

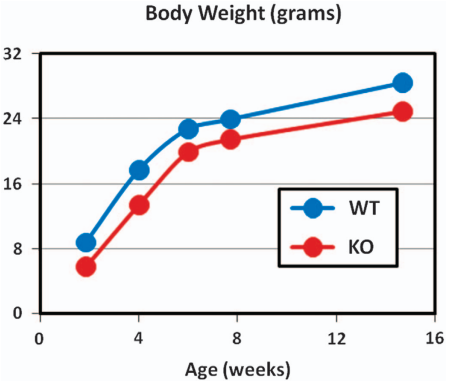

Figure 3 Body weight during growth in *Tph2* KO mice. Data obtained during high-throughput screening.[16] Equal numbers of male and female mice were examined for each genotype at each age, with Ns = 4 for WT and Ns = 8 for KO mice. $P<0.001$ at 2 weeks and $P=0.02$ at 4 weeks of age.

Table 1 DEXA data for male and female *Tph2* KO mice

Parameter	*Male mice WT = 12, KO = 13*	*Statistics*	*Female mice WT = 15, KO = 13*	*Statistics*
Body BMD ($mg\,cm^{-2}$)	WT: 57.1 ± 0.9	Δ = Zero	WT: 54.9 ± 0.9	Δ = −1%
	KO: 57.1 ± 0.7	$P = 0.94$	KO: 54.6 ± 0.9	$P = 0.82$
Spine BMD ($mg\,cm^{-2}$)	WT: 60.6 ± 1.7	Δ = 11%	WT: 46.0 ± 3.2	Δ = 22%
	KO: 67.4 ± 1.7	$P = 0.01$	KO: 56.0 ± 3.6	$P = 0.05$
Femur BMD ($mg\,cm^{-2}$)	WT: 88.3 ± 1.6	Δ = 2%	WT: 85.9 ± 2.1	Δ = NC
	KO: 89.5 ± 1.5	$P = 0.55$	KO: 85.8 ± 1.8	$P = 0.97$
Body weight (grams)	WT: 32.8 ± 1.3	Δ = −2%	WT: 35.2 ± 1.6	Δ = −8%
	KO: 31.0 ± 0.9	$P = 0.24$	KO: 32.3 ± 2.2	$P = 0.29$
Lean body mass (grams)	WT: 24.0 ± 0.7	Δ = 10%	WT: 19.6 ± 0.5	Δ = 3%
	KO: 26.2 ± 0.6	$P = 0.02$	KO: 20.3 ± 0.7	$P = 0.46$
Body fat (percent)	WT: 28.0 ± 1.4	Δ = −43%	WT: 39.4 ± 1.7	Δ = −23%
	KO: 16.1 ± 1.1	$P < 0.001$	KO: 30.2 ± 3.1	$P = 0.01$

Abbreviations: BMD, bone mineral density; DEXA, dual energy X-ray absorptiometry; KO, knockout; NC, no change; WT, wild type. Male and female mice were examined at 18 and 83 weeks of age, respectively.

Table 2 QMR body composition data for *Tph1* KO, *Tph2* KO and *Tph1*/*Tph2* DKO Mice

Parameter	*Body weight (grams)*	*Lean body mass (grams)*	*Body fat (%)*
Male WT ($N = 10$)	42.6 ± 2.5	31.1 ± 1.4	26.1 ± 2.1
Male *Tph1* KO ($N = 14$)	39.9 ± 0.9	31.5 ± 0.8	20.1 ± 1.9
Male *Tph2* KO ($N = 8$)	35.5 ± 0.8	29.9 ± 1.1	14.8 ± 2.9
Male *Tph1*/*Tph2* DKO ($N = 11$)	32.2 ± 0.8	29.9 ± 0.7	9.2 ± 0.5
Statistics	$P = 0.13$ for *Tph1* $P < 0.001$ for *Tph2* interaction $P = 0.86$	$P = 0.89$ for *Tph1* $P = 0.07$ for *Tph2* interaction $P = 0.59$	$P = 0.005$ for *Tph1* $P < 0.001$ for *Tph2* interaction $P = 0.92$
Female WT (N = 15)	38.2 ± 2.0	24.0 ± 0.6	35.6 ± 2.3
Female *Tph1* KO (N = 9)	40.8 ± 2.9	26.1 ± 0.9	34.7 ± 2.8
Female *Tph2* KO (N = 14)	39.0 ± 2.9	25.0 ± 0.8	32.9 ± 3.1
Female *Tph1*/*Tph2* DKO ($N = 6$)	31.6 ± 1.9	24.9 ± 1.2	20.6 ± 3.4
Statistics	$P = 0.13$ for *Tph1* $P < 0.001$ for *Tph2* interaction $P = 0.86$	$P = 0.26$ for *Tph1* $P = 0.91$ for *Tph2* interaction $P = 0.24$	$P = 0.04$ for *Tph1* $P = 0.01$ for *Tph2* interaction $P = 0.04$

Abbreviations: DKO, double knockout; KO, knockout; QMR, quantitative magnetic resonance; WT, wild type. Male and female mice were examined at 33 and 65 weeks of age, respectively.

Table 3 MicroCT bone data for *Tph1* KO, *Tph2* KO and *Tph1*/*Tph2* DKO mice

Parameter	*LV5 Tb number (1*/N)	*Femur length (mm)*	*Femur total area (mm^2)*
Male WT ($N = 10$)	4.50 ± 0.28	16.5 ± 0.1	2.22 ± 0.09
Male *Tph1* KO ($N = 14$)	4.46 ± 0.19	16.7 ± 0.1	2.27 ± 0.07
Male *Tph2* KO ($N = 8$)	5.62 ± 0.43	16.0 ± 0.1	2.23 ± 0.07
Male *Tph1*/*Tph2* DKO ($N = 11$)	4.59 ± 0.28	16.0 ± 0.2	2.04 ± 0.05
Statistics	$P = 0.07$ for *Tph1* $P = 0.03$ for *Tph2* interaction $P = 0.09$	$P = 0.68$ for *Tph1* $P < 0.001$ for *Tph2* interaction $P = 0.44$	$P = 0.33$ for *Tph1* $P = 0.12$ for *Tph2* interaction $P = 0.10$
Female WT ($N = 15$)	3.10 ± 0.13	16.6 ± 0.1	3.10 ± 0.13
Female *Tph1* KO ($N = 9$)	2.87 ± 0.13	16.8 ± 0.2	2.87 ± 0.13
Female *Tph2* KO ($N = 14$)	3.06 ± 0.21	16.6 ± 0.1	3.06 ± 0.21
Female *Tph1*/*Tph2* DKO ($N = 6$)	2.54 ± 0.23	16.6 ± 0.3	2.54 ± 0.23
Statistics	$P = 0.06$ for *Tph1* $P = 0.35$ for *Tph2* interaction $P = 0.46$	$P = 0.66$ for *Tph1* $P = 0.44$ for *Tph2* interaction $P = 0.42$	$P = 0.08$ for *Tph1* $P = 0.67$ for *Tph2* interaction $P = 0.52$

Abbreviations: DKO, double knockout; KO, knockout; MicroCT, microcomputed tomography; WT, wild type. Male and female mice were examined at 33 and 65 weeks of age, respectively.

in the DKO study (**Table 3**), but it was normal in all other *Tph2* KO cohorts. Midshaft femur total area was normal in all *Tph2* KO mice but slightly decreased (3% in male mice and 5% in female mice) in *Tph1*/*Tph2* DKO mice. Female *Tph2* KO mice had reduced midshaft femur cortical thickness in both the *Tph2* KO (**Table 4**) and DKO studies (**Figure 4**), as did female *Tph1*/*Tph2* DKO mice. Femur cortical thickness was normal in male *Tph2* KO mice.

Table 4 MicroCT bone data for male and female *Tph2* KO mice

Parameter	*Male mice WT = 12, KO = 13*	*Statistics*	*Female mice WT = 11, KO = 11*	*Statistics*
LV5 Tb BV/TV (%)	WT: 20.2 ± 0.9 KO: 24.0 ± 0.5	Δ = 19% *P* = 0.004	WT: 12.8 ± 0.9 KO: 13.0 ± 1.2	Δ = 1% *P* = 0.90
LV5 Tb number (1/*N*)	WT: 4.84 ± 0.19 KO: 5.67 ± 0.18	Δ = 17% *P* = 0.001	WT: 2.75 ± 0.16 KO: 2.97 ± 0.30	Δ = 8% *P* = 0.54
LV5 Tb thickness (μm)	WT: 44.5 ± 0.7 KO: 44.6 ± 1.0	Δ = NC *P* = 0.99	WT: 46.1 ± 2.7 KO: 42.6 ± 2.6	Δ = −8% *P* = 0.31
DFM Tb BV/TV (%)	WT: 5.4 ± 0.8 KO: 7.3 ± 0.8	Δ = 35% *P* = 0.11	Not measured	
DFM Tb number (1/*N*)	WT: 3.19 ± 0.28 KO: 3.77 ± 0.19	Δ = 18% *P* = 0.09	Not measured	
DFM Tb thickness (μm)	WT: 44.7 ± 1.3 KO: 44.3 ± 2.0	Δ = −1% *P* = 0.89	Not measured	
Femur length (mm)	WT: 16.3 ± 0.1 KO: 16.0 ± 0.1	Δ = −2% *P* = 0.03	WT: 16.5 ± 0.1 KO: 16.7 ± 0.2	Δ = 1% *P* = 0.47
Femur total area (mm2)	WT: 1.96 ± 0.08 KO: 1.97 ± 0.06	Δ = NC *P* = 0.95	WT: 2.24 ± 0.09 KO: 2.21 ± 0.05	Δ = −1% *P* = 0.78
Femur Ct.Th (μm)	WT: 237 ± 3 KO: 245 ± 6	Δ = 3% *P* = 0.43	WT: 266 ± 5 KO: 232 ± 7	Δ = −13% *P* < 0.001

Abbreviations: BV/TV, bone volume/total volume; DFM, distal femur metaphysis; Ct.Th, cortical bone thickness; KO, knockout; MicroCT, microcomputed tomography; NC, no change; Tb, trabecular bone; WT, wild type. Male and female mice were examined at 21 and 83 weeks of age, respectively.

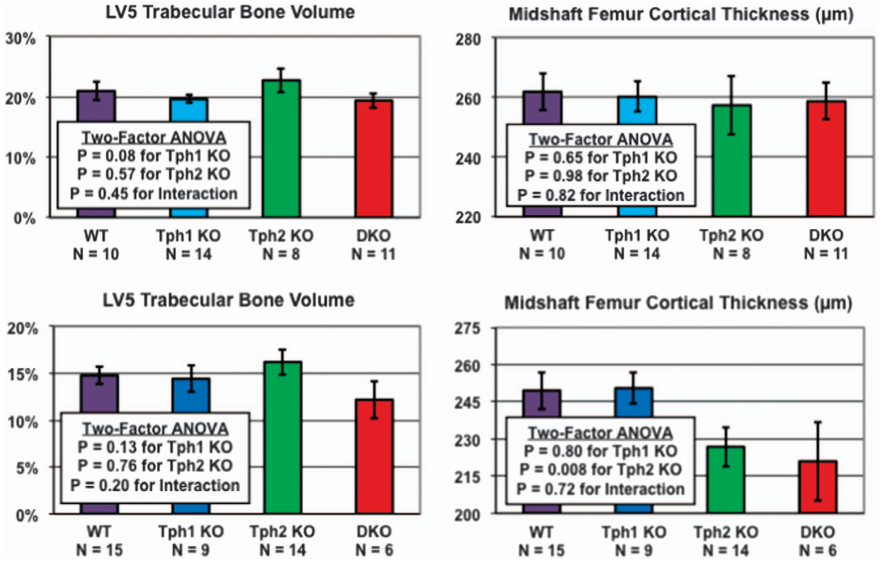

Figure 4 MicroCT bone data for *Tph1* KO, *Tph2* KO and *Tph1/Tph2* DKO mice. Male (top) and female (bottom) mice were examined at 21 and 83 weeks of age, respectively.

In the *Tph2* KO study, spine bone mineral density (BMD) was elevated in male and female KO mice, but body and femur BMD were normal (**Table 1**). MicroCT analyses (**Table 4**) showed normal LV5 trabecular bone parameters in female mice, but elevated trabecular bone volume/total volume (BV/TV) and trabecular number in male mice. Similar trends were observed for distal femur trabecular bone in male mice, but these differences did not reach statistical significance. In the DKO study, LV5 trabecular bone BV/TV was slightly increased (8% in male mice and 9% in female mice), but these elevations did not reach statistical significance (**Figure 3**). LV5 trabecular number was elevated 23% in male, but not female, *Tph2* KO mice in the DKO study (**Table 3**). Trabecular thickness was consistently normal in *Tph2* KO mice. No skeletal phenotypes were observed in male and female *Tph1* KO mice examined as part of the DKO cohort (**Figure 4**, **Tables 2 and 3**).

Discussion

Contrary to published data showing reduced spine trabecular bone mass in *Tph2* KO mice,[11,13] our KO mice had moderately elevated vertebral body trabecular bone. Previous work did not examine cortical bone, and thus our observation of moderately reduced femoral cortical bone thickness in female, but not male, *Tph2* KO mice is novel. Our observation of greatly reduced body fat in all four cohorts of *Tph2* KO mice confirms previous findings.[11]

Previous studies[11,13] examined *Tph2* KO mice at 4 to 16 weeks of age, whereas Lexicon's mice were examined at 18 through 83 weeks of age. *Tph2* KO mice have a transient growth retardation starting a few days after birth and lasting through 4 months of age[18,19] and limited data from Lexicon's KO mouse screening analyses[16] support this finding. Low trabecular bone

mass in studies examining growing mice is possibly related to their small size. Body length and bone width were normal in one study,[11] with neither study reporting body weight.

Besides aging effects in *Tph2* KO mice, there is no obvious explanation for differences in bone phenotypes of *Tph1* and *Tph2* KO mice observed by other groups[11–13] and Lexicon. Normal bone mass in male (33 weeks) and female (65 weeks) *Tph1* KO mice reported here confirms previous findings in nine mouse cohorts independently examined by Lexicon and the Max Delbrück Center for Molecular Medicine.[14] Novartis also observed normal bone mass in *Tph1* KO mice (Michaela Kneissel, personal communication). We encourage additional laboratories to examine bones from *Tph1* and *Tph2* KO mice, with particular emphasis on effects of age for *Tph2* KO mice. In contrast to differences in bone phenotypes of *Tph1* KO mice, our *Tph1* KO mice had reduced body fat with normal LBM, confirming leanness in a recent report.[20]

Lexicon has extensive experience examining skeletal phenotypes resulting from disrupting mouse genes[16] and Lexicon's expertise in serotonin actions and metabolism resulted in successful clinical trials examining TPH1 inhibitors for irritable bowel[21,22] and carcinoid syndromes.[23] Medicinal chemistry and preclinical pharmacology data for these TPH inhibitors have been published.[24–27]

A limitation of this report is the lack of mechanistic studies designed to explain the observed trabecular and cortical bone phenotypes in *Tph2* KO mice. High trabecular bone BV/TV resulting from elevated trabecular number rather than greater trabecular thickness is consistent with reduced bone resorption rather than increased bone formation. Low cortical bone

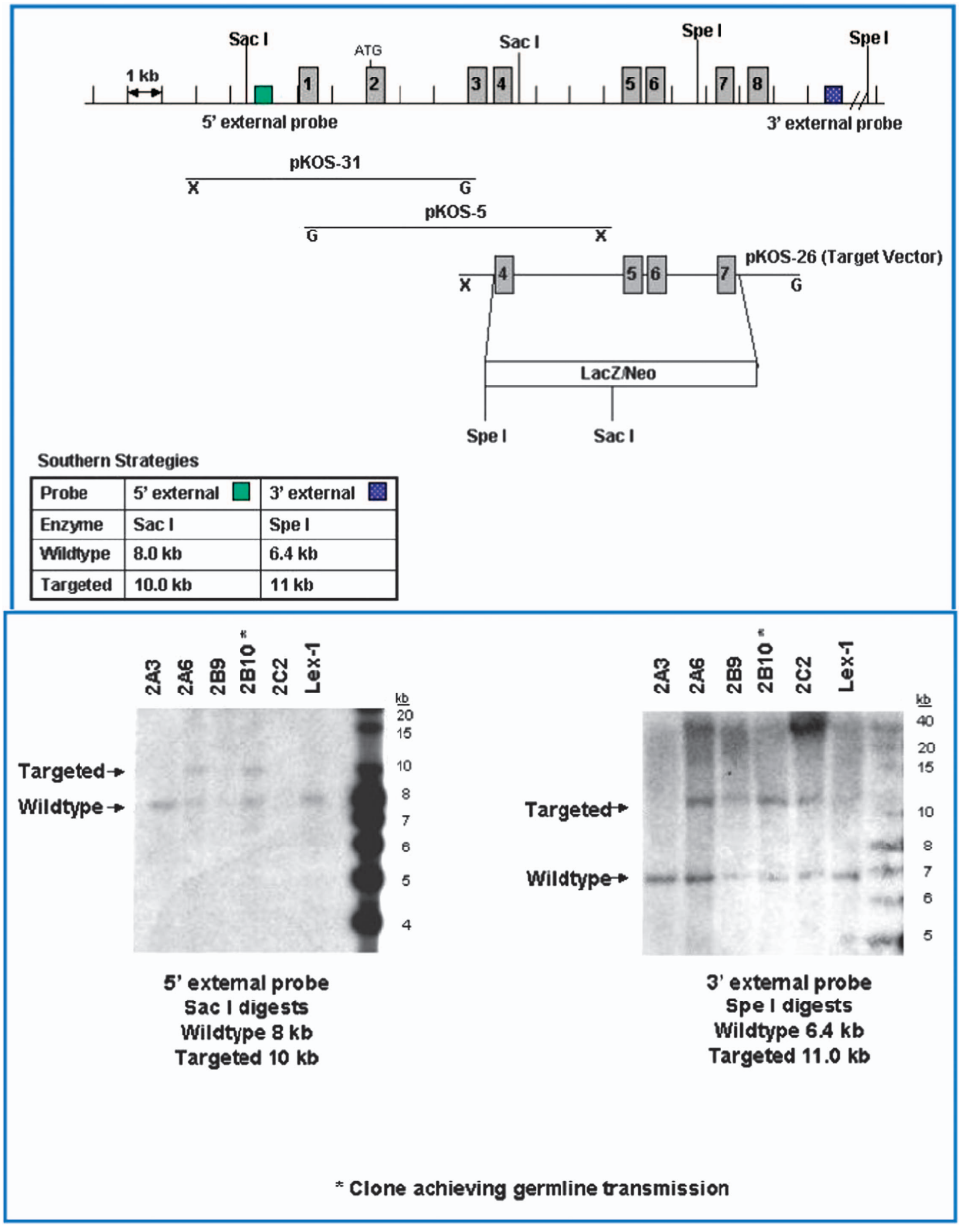

Figure 5 *Tph1* (exons 4 through 7) KO strategy.

thickness with a normal diameter results from elevated endocortical bone resorption.

From our extensive experience examining KO mice,[16] we classify the elevated spine bone mass and reduced cortical bone thickness observed in *Tph2* KO mice as moderate skeletal phenotypes. Such phenotypes can appear and disappear in various cohorts and skeletal sites and often show random sexual dimorphism when few cohorts are examined. Individual cohorts can show trends (without statistical significance) supporting phenotypes observed in other cohorts. The average sample size was 11 for the twelve mouse genotype groups reported here.

We have no explanation for the differences in bone phenotypes for *Tph1* and *Tph2* KO mice observed by Karsenty's laboratory and those reported here. The Karsenty group has produced several high-profile reports having substantial influence on skeletal biology research. In several cases, other groups have been unable to independently replicate these findings. These cases include studies involving brain serotonin metabolism,[28–30] bone phenotypes in mice with disrupted leptin signaling,[10,31–34] bone phenotypes in mice with KOs of β-adrenergic receptors[35–37] and actions of osteocalcin on glucose metabolism and diabetes.[38–40] Potential explanations for observations of distinct phenotypes in similar mouse models include, but are not limited to, environmental conditions, age, genetic backgrounds and diets. To help understand mechanisms behind these observed differences, Lexicon's *Tph1* (TF4141) and *Tph2* (TF2875) KO mice are available from Taconic Biosciences. We call upon all members of our scientific community to make available all mouse strains that have been used for their publications so that important observations can be independently verified.

Materials and Methods

Methods used to generate *Tph2* KO mice (excising exons 1 and 2) and determine 5-HIAA (urine) and serotonin (whole blood, brain and jejunum) levels have been reported previously.[24,41] Lexicon's previously published *Tph1* KO mice were generated by excising exon 3.[24] For this report, a distinct *Tph1* KO mouse line was generated by excising exons 4 through 7 (**Figure 5**). All mice were F2 hybrids of the C57BL/6J and 129SvEv parental strains. Wild-type control mice were littermates and cagemates of KO mice. Male and female mice in the *Tph2* KO study and, starting at 43 weeks of age, female mice in the *Tph1*/*Tph2* DKO study were fed purified high-fat diet with 45 percent of calories derived from fat (diet D12451 from Research Diets, New Brunswick, NJ, USA). Male mice and young female mice in the DKO study were fed standard rodent chow. BMD was determined with a PIXImus DEXA (InsideOutside Sales, Fitchburg, WI, USA). Body composition was measured by DEXA and QMR (Echo Medical Systems, Houston, TX, USA). Bone mass and architecture were determined using a Scanco μCT40 microCT (Wangen-Brüttisellen, Switzerland) using standard procedures. LV5 vertebral body trabecular bone parameters and midshaft femur cortical bone total area (diameter) and thickness were measured. Standard mouse metabolic cages were used to collect urine over 24-h intervals. All procedures involving mice were conducted following Lexicon Pharmaceuticals' Institutional Animal Care and Use Committee guidelines that are in compliance with state and federal laws and the standards described in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Statistical analyses involved Student's *t*-test (*Tph2* KO study) and two-factor ANOVA (*Tph1*/*Tph2* DKO study). Data are reported as means ± s.e.m.

Conflict of Interest

Lexicon Pharmaceuticals is currently examining a drug (telotristat etiprate) inhibiting TPH1 in a Phase III clinical trial for carcinoid syndrome. All authors were full-time employees of Lexicon Pharmaceuticals when the experiments were performed.

Acknowledgements

Lexicon's KO mouse phenotyping campaign involved hundreds of scientists.

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