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X-inactivation analysis of embryonic lethality in *Ocrl*$^{wt/-}$;*Inpp5b*$^{-/-}$ mice

David J. Bernard · Robert L. Nussbaum

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Abstract Mutations in the human *OCRL* gene, which encodes a phosphatidylinositol(4,5)bisphosphate 5-phosphatase, result in the X-linked oculocerebrorenal syndrome of Lowe. Mice with a targeted disruption of *Ocrl* have no phenotypic abnormalities. Targeted disruption of its closest paralog, *Inpp5b*, causes male infertility in the 129S6 background. Mice with disruptions of both genes are lost in utero prior to 9.5-10.5 dpc, indicating that there is a functional overlap between the two paralogs early in development. We analyzed the pattern of X-inactivation in four tissues of distinct embryonic origin from *Ocrl*$^{wt/-}$;*Inpp5b*$^{-/-}$ females to explore the timing and tissue distribution of the functional overlap. X-inactivation was strongly skewed against the disrupted *Ocrl*$^{-}$ allele being on the active X chromosome in all four tissues tested, indicating that there is early selection against cell lineages lacking both *Ocrl* and *Inpp5b*. Extraembryonic tissue was also involved in the lethality because there were never any live-born *Ocrl*$^{wt/-}$;*Inpp5b*$^{-/-}$ females when the functional *Ocrl*^{wt} allele was on the paternal X chromosome, which is preferentially inactivated in trophoblast-derived extraembryonic tissues. Live-born *Ocrl*$^{wt/-}$;*Inpp5b*$^{-/-}$ females were found when the functional *Ocrl*^{wt} allele was maternal, although in fewer numbers than expected. The importance of the extraembryonic tissues in the early embryonic lethality of embryos lacking both *Ocrl* and *Inpp5b* is reinforced by the successful isolation of a viable 40,XX *Ocrl*$^{-/-}$;*Inpp5b*$^{-/-}$ embryonic stem cell from the inner cell mass of a 3.5-dpc blastocyst prior to implantation. These results indicate a functional overlap of *Ocrl* and *Inpp5b* in most cell lineages, especially in extraembryonic tissues.

D. J. Bernard · R. L. Nussbaum
Inborn Errors and Cell Biology, Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA

Present Address:
D. J. Bernard
Molecular Pathogenesis Section, Genome Technology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA

R. L. Nussbaum (✉)
Division of Medical Genetics, Department of Medicine, University of California, San Francisco, 513 Parnassus Ave, HSE 901E, Box 0794, San Francisco, CA 94143, USA
e-mail: robert.nussbaum@ucsf.edu

R. L. Nussbaum
Institute for Human Genetics, University of California, San Francisco, CA 94143, USA

Introduction

The oculocerebrorenal syndrome of Lowe (OCRL; OMIM #309000) is a rare X-linked disorder characterized by congenital cataracts, mental retardation, behavioral abnormalities, and a proximal renal tubular dysfunction that includes low-molecular-weight proteinuria and variable aminoaciduria, phosphaturia, and bicarbonaturia (Suchy and Nussbaum 2009). The disorder is caused by mutations in a gene encoding a phosphatidylinositol(4,5)bisphosphate 5-phosphatase [PI(4,5) 5-phosphatase] that was originally named *OCRL* because it was identified by positional cloning from Lowe syndrome patients (Attree et al. 1992). An attempt to develop a mouse model for Lowe syndrome failed when it was found that mice with targeted disruption of the *Ocrl* gene failed to have the phenotype of the Lowe syndrome, or any other discernible phenotype (Janne et al.

1998). We hypothesized that $Ocrl^{-}$ mice might be protected from disease by a compensating enzymatic activity. *Ocrl* is highly homologous to an autosomal paralog, *Inpp5b* (Jefferson and Majerus 1995; Zhang et al. 1995), which has a similar enzymatic function (Ross et al. 1991), and we hypothesized that *Inpp5b* might serve to compensate for loss of *Ocrl* in mice. Mice with targeted disruptions of *Inpp5b* have a mild phenotype of male infertility in the inbred 129S6 background but no signs of Lowe syndrome (Hellsten et al. 2001, 2002), while mice deficient in both enzyme activities appear to die during embryogenesis, prior to E9.5–10.5, indicating that the two genes do encode proteins with overlapping function (Janne et al. 1998).

In this report, we used mouse crosses, X chromosome inactivation (XCI) analysis, and cell culture experiments to further characterize the timing of the embryonic lethality in doubly deficient embryos and to determine which subsets of embryonic tissues require that one or the other of these two PI(4,5) 5-phosphatases be present. We show that the functional overlap between these two enzymes occurs in all three embryonic layers as well as in the trophoblast-derived extraembryonic tissues during the critical period of implantation and early embryonic development between E3.5 and E9.5.

Materials and methods

Mouse breeding

All mouse experiments were approved by the National Human Genome Research Institute Animal Care and Use Committee and followed the National Institutes of Health guidelines. The mouse strains carrying disrupted *Ocrl* and *Inpp5b* were previously published (Hellsten et al. 2001; Janne et al. 1998). The *Ocrl* mutant strain had a mixed genetic background from the 129S4 (J1 ES cell line) founder mice outcrossed to C57Bl/6J. The *Inpp5b* mutant strain had a mixed background from 129S6 (TC1 ES cell line) founder mice bred to NIH Black Swiss in order to maintain fertility in this line. B6.Cg-$Pgk1^{a}$/J mice were from the Jackson Laboratory (stock number 000827). Thus, the test and control mice, with differential *Pgk1* alleles marking the two X chromosomes, were on a mixed genetic background of 129, C57Bl/6, and NIH Black Swiss.

Allele-specific marking of the X chromosome

The disrupted *Ocrl* allele was originally engineered in mice carrying the X-linked phosphoglycerate kinase 1 (*Pgk1*) *b* allele. To differentially mark the two X chromosomes in an $Ocrl^{wt/-}$ female for allele-specific expression analysis, we carried out a four-step breeding scheme (Fig. 1) to ultimately generate female mice heterozygous for disruption of *Ocrl* in which the *Pgk1 a* allele was always in coupling phase with the disrupted *Ocrl* allele and the *Pgk1 b* allele was in coupling with the wild-type *Ocrl* allele, all in the background of homozygous disrupted for *Inpp5b*.

Genotyping

Ocrl and *Inpp5b* mice were genotyped either by Southern blotting (Janne et al. 1998) or PCR as follows:

Ocrl wild-type: The forward primer 5′-CCCTTTTC ATCTGTTAGGAGAAATC-3′ overlaps the junction of intron 18 and the 5′ end of exon 19. The reverse primer 5′-GCATGGTTAAACGCACTATGTGG-3′ is located in intron 19 which is deleted in the *Ocrl* knockout (KO) line. These primers yield a product 227 bp in length using an annealing temperature (T_A) of 58°C.

Ocrl knockout: The *Ocrl* KO forward primer has the sequence 5′-GCCCTTTGATTCTAATCCCTTTTCATC-3′ and is located in the intron just prior to exon 19. The reverse primer is located in the PGK promoter, which is part of the targeting construct and has a sequence of 5′-TCTGAGCCCAGAAAGCGAA-3′. At $T_A = 58$°C, these primers yield a product of about 480 bp.

Inpp5b wild-type: Primers flank the Neo selection cassette in the targeted allele but will not amplify through the *Neo* gene with the 1-min extension time. The forward primer, 5′-CTTGTGGCTGGGAGACCT-3′, is located in exon 14 and the reverse primer, 5′-AGAGAAATGCAGA CGGAATG -3′, is located in intron 14. At $T_A = 52$°C, these primers yield a product of 234 bp.

Inpp5b knockout: The reverse primer is the same as the *Ocrl* KO reverse primer, 5′-TCTGAGCCCAGAAA GCGAA-3′. The forward primer is the same as the wild-type primer, 5′-CTTGTGGCTGGGAGACCT-3′. Together these primers yield a 500-bp product at $T_A = 52$°C.

Pgk1 alleles: The *Pgk1* alleles were genotyped by a method previously described (Shanmugam et al. 1996). Briefly, a fragment of the *Pgk1* gene was amplified by PCR using forward primer 5′-TTCTCCTCTTCCTCATCTCC-3′ and reverse primer 5′-TAAAGGACACATTTGGTCGC-3′. The PCR product is then digested at a polymorphic site with the restriction endonuclease Tth111I. The $Pgk1^{a}$ allele is resistant to Tth111I digestion while the $Pgk1^{b}$ allele is cleaved into two fragments of 272 and 72 bp.

DNA extraction

Mouse tail biopsies were incubated with proteinase K (Invitrogen) at 50°C overnight. DNA was purified using either a high-salt method (Thomas et al. 1992) or phenol/chloroform extraction and ethanol precipitation.

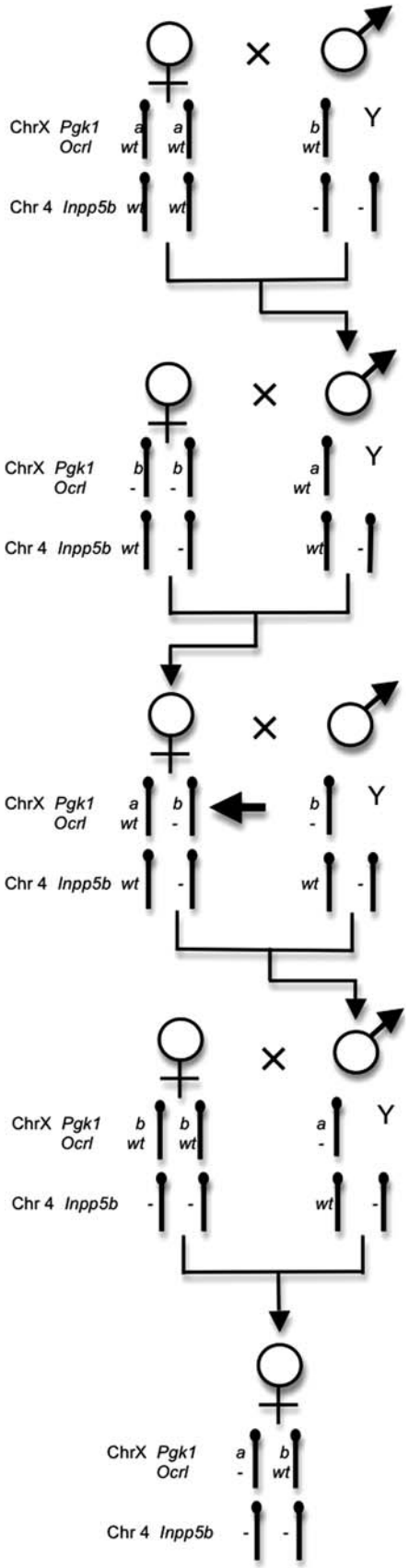


Fig. 1 Breeding scheme used to generate female mice heterozygous for the previously described disrupted allele of *Ocrl* and for the two *Pgk1* alleles, *a* and *b*, putting the *Pgk1 a* allele in coupling phase with the disrupted *Ocrl* allele. The mice are also homozygous for the previously described disrupted allele of *Inpp5b*. Arrow indicates meioses in which recombination brought the *Pgk1 a* allele into coupling phase with the disrupted *Ocrl* allele. The final breeding step results in fewer $Pgk1^{a/b}$;$Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females than expected

RNA extraction

Tissues were homogenized using a 6-ml Dounce homogenizer and Trizol (Invitrogen), following the manufacturer's protocol. RNA concentration was determined by OD_{260} UV absorption on a Beckman DU640 spectrophotometer. DNA contamination was eliminated by treatment with Amplification Grade DNase (Invitrogen).

RT-PCR

Reverse transcriptase PCR was performed using the Superscript II RT-PCR kit (Invitrogen) according to manufacturer's instructions. The gene-specific primer *Pgk1* RT (5′-GTAAAGGCCATTCCACCACCAA-3′) was used to synthesize the first strand followed by amplification using the nested reverse primer *Pgk1*-R2 (5′-TTTAGCGCCT CCCAAGATAGC-3′) and forward primer *Pgk1*-F296 (5′-GTGTGGGCCCAGAAGTCG-3′). Products were separated from primers and dNTPs on 1% SeaPlaque low-melt agarose (FMC Bioproducts). Bands were visualized using ethidium bromide and excised from the gel using a clean razor blade. Agarose was removed by treatment with ß agarase (New England BioLabs). The final product was ethanol precipitated and resuspended in 25 µl of TE, and the concentration determined by densitometry of ethidium bromide gel electrophoretograms using NIH Image (http://rsb.info.nih.gov/nih-image/Default.html).

Single-nucleotide polymerase extension reaction (SNuPE)

A modification of the SNuPE reaction was employed (Singer-Sam et al. 1992). Briefly, a slightly longer 25-bp PAGE-purified SNuPE primer, 5′-GATGCTTTCCGAG CCTCACTGTCCA-3′, was designed which sits one base 5′ of the single base polymorphism at position 489 in the *Pgk1* mRNA (GenBank ID# gi66792290). Both nonradioactive dCTP (0.2 µM final concentration) and 2 µCi 32P ddATP (Amersham) were added to a single tube containing either 10 or 7 ng of the purified RT-PCR product, 0.25 µM PAGE-purified *Pgk1* SNuPE primer, 2 units Thermosequenase polymerase (Amersham), and 1 × Thermosequenase reaction buffer (Amersham) in a final volume of

20 µl. One cycle of 95°C for 2 min, 60°C for 20 s, 72°C for 15 s was performed in a PTC-100 thermocycler (MJ Research Inc.). $Pgk1^b$ alleles incorporated the chain terminating 32P dideoxyATP for a 26-bp product. $Pgk1^a$ alleles added the dCTP followed by the chain terminating 32P dideoxyATP for a product 27 bp in length. Samples were diluted with an equal volume of loading buffer (90% formamide, 0.5 × TBE, 0.025% bromophenol blue, 0.025% xylene cyanole), denatured for 3-5 min at 95°C, and immediately plunged into ice prior to loading 14 µl per lane in a 31 cm × 38.5 cm × 0.4 mm-thick denaturing 18% polyacrylamide [Accugel 19:1 acrylamide:bisacrylamide (National Diagnostics)] gel. Samples were run overnight on an S2 sequencing apparatus (Life Technologies) at a constant 4-6 W at room temperature until the leading dye ran off the gel. Oligonucleotide-sizing markers (Amersham Pharmacia Biotech) were labeled with γ32P ATP as per the manufacturer's protocol, diluted in an equal volume of loading buffer, denatured at 90°C for 3 min, plunged into ice, and loaded at 3 µl per lane on the denaturing PAGE gel. The gel was dried (Bio-Rad model 583 gel dryer), exposed to a phosphoimager cassette, and analyzed on a Typhoon 8600 phosphoimager (Amersham Pharmacia Biotech). SNuPE reactions were performed in triplicate on tissues derived from 10-11 mice in the test population and 7 mice in the control populations. The control mice ranged in age from 10 to 16 weeks old (mean ± SD = 12.0 ± 2.3 weeks), while the test mice were 8–28 weeks old (mean ± SD 17.4 ± 9.1 weeks).

Phosphoimager analysis

Densitometry was performed using the Typhoon 8600 Phosphoimager using manufacturer-supplied software (Amersham Pharmacia Biotech). Background correction was calculated using the local average function. For each sample, each of the two individual alleles was measured separately and the sum of the two alleles was defined as 100% expression. Percent expression for a single allele in a sample was calculated as the densitometry value of the single allele divided by the sum of both alleles in that sample multiplied by 100%. Triplicate reactions were performed for each sample, averaged, and used to calculate standard deviation using Microsoft Excel spreadsheet software. Probability calculations reported are from *t*-test analysis, two-tailed, equal variance from the Microsoft Excel software.

Isolation of ES cell line

An embryonic stem (ES) cell line, lacking both *Ocrl* and *Inpp5b*, was generated as published (Hogan et al. 1994). Briefly, 3.5-day post coitum (dpc) blastocysts were harvested from naturally mated mice, individually plated into one well of a 24-well plate containing mouse fibroblast feeder layers, and cultured for 5 days. The inner cell mass-derived cells were scraped from the plate, trypsinized, and dissociated with a finely drawn glass capillary. The cells were replated and grown until ES cell-like colonies appeared. DNA was isolated from the individual colonies and subjected to karyotyping (Heng and Tsui 1993; Schweizer 1980, 1981) and genotyping by PCR and Southern blot.

Statistical analysis

Tests of significance in mouse-breeding experiments were either by the χ^2 test or by exact calculation from the binomial distribution. Tests of significance on SNuPE measurements were by Student's *t* test.

Results

We sought to generate $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females, in which X-inactivation would theoretically produce functional mosaicism, with some cells deficient in both Ocrl and Inpp5b and the rest deficient only in Inpp5b. We then sought to determine whether the tissues of $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ mice would actually demonstrate such mosaicism or would show complete skewing of X-inactivation with exclusive inactivation of the X chromosome carrying the mutant *Ocrl* allele.

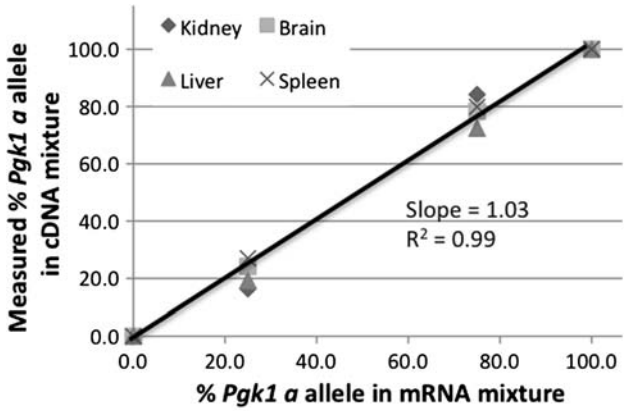
We mated $Ocrl^{-/y}$;$Inpp5b^{wt/-}$ males to $Ocrl^{wt/wt}$;$Inpp5b^{-/-}$ females. In this cross, the functional *Ocrl* allele in $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females is maternal in origin. Since the X chromosome of paternal origin is exclusively and nonrandomly inactivated in the trophoblast-derived extraembryonic tissues of $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ embryos as they develop (Takagi and Sasaki 1975), the extraembryonic tissue lineage of $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ zygotes in which the $Ocrl^{wt}$ allele is maternal is not mosaic and retains *Ocrl* function.

We succeeded in generating mice with the $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ genotype. No obvious phenotype was apparent among the live-born $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females. Our success in generating mice of the correct genotype from this cross indicates that $Ocrl^{-}$;$Inpp5b^{-}$ sperm are capable of fertilization. However, instead of the expected 1:1 ratio of $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ to $Ocrl^{wt/-}$;$Inpp5^{wt/-}$ females, only 36 females of the $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ genotype were produced compared to 82 $Ocrl^{wt/-}$;$Inpp5b^{wt/-}$ females, a significantly different ratio approaching 1:2 ($P = 2.3 \times 10^{-5}$, χ^2 test) (Table 1). Since the X chromosome of maternal origin is exclusively and nonrandomly activated in the trophoblast-derived extraembryonic tissues of $Ocrl^{wt/-}$;$Inpp5b^{-/-}$

Table 1 Females resulting from $Ocrl^{wt/wt}$;$Inpp5b^{-/-}$ female × $Ocrl^{-/y}$;$Inpp5b^{wt/-}$male cross

Gamete genotype		Live-born genotype	# Females observed	# Females expected
Egg	Sperm			
$Ocrl^{wt}$;$Inpp5b^{-}$	$Ocrl^{-}$;$Inpp5b^{wt}$	$Ocrl^{wt/-}$;$Inpp5b^{wt/-}$	82	59
$Ocrl^{wt}$;$Inpp5b^{-}$	$Ocrl^{-}$;$Inpp5b^{-}$	$Ocrl^{wt/-}$;$Inpp5b^{-/-}$	36*	59
		Total	118	118

* $P = 0.000023$ (χ^2 test)



	Measured %*Pgk1 a* allele in cDNA			
%*Pgk1 a* allele in mRNA mixture	Kidney	Brain	Liver	Spleen
100	100±0	100±0	100±0	100±0
75	84.2±1.2	78.4±4.3	72.4±2.1	79.9±2.7
25	16.4±1.8	24.2±6.6	19.3±4.3	27.0±2.4
0	0.0±0.0	0.0±0.0	0.2±0.2	0.0±0.0

Fig. 2 Calibration of SNuPE assay using various mixtures of $Pgk1^{a/a}$ and $Pgk1^{b/b}$ DNA consisting of 100% $Pgk1^{a/a}$, 75% $Pgk1^{a/a}$, 75% $Pgk1^{b/b}$, or 100% $Pgk1^{b/b}$. The actual numbers with standard deviations are given in the table at the bottom of the figure

embryos as they develop (Takagi and Sasaki 1975), these tissues were nonmosaic and contained a functioning $Ocrl^{wt}$ allele on the active maternal X chromosome. The 50% underrepresentation of $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females therefore must represent an abnormality in development of the embryo proper or the embryonic mesoderm-derived extraembryonic tissues.

In order to determine the X-inactivation pattern in tissues of $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females, we took advantage of an expressed single-nucleotide polymorphism in the coding region of the X-linked housekeeping gene phosphoglycerate kinase (*Pgk1*) (Boer et al. 1990). This gene undergoes X-inactivation and the expressed polymorphism is therefore useful for determining which of the two X chromosomes is active in $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females heterozygous for the *Pgk1* polymorphism by analyzing the *Pgk1* mRNA. We mated B6.Cg-$Pgk1^{a}$/J females to male $Ocrl^{-/y}$;$Inpp5b^{wt/-}$ mice that were hemizygous for the more common $Pgk1^{b}$ allele in order to introduce the $Pgk1^{a}$ allele from Danish feral mice (Nielsen and Chapman 1977) into the strains carrying a disrupted *Ocrl* allele (Fig. 1). The *Pgk1* and *Ocrl* loci recombined 22% of the time to bring the *a* allele into coupling phase with the targeted mutant $Ocrl^{-}$ allele. Once recombinant males were obtained that carried the $Ocrl^{-}$ allele in phase with $Pgk1^{a}$, the phase was maintained by carrying this recombinant chromosome in the males to avoid the potential for meiotic recombination in females. The more common domestic mouse *Pgk1 b* allele was kept in phase with the *Ocrl* wild-type allele.

We designed a modified single-nucleotide polymerase extension (SNuPE) assay of the expressed *Pgk1* SNP that differentially marked the two X chromosomes to measure allele-specific expression of the *Pgk1* gene. A 25-bp primer was annealed directly adjacent to the site of the SNP that distinguishes the $Pgk1^{a}$ allele from the $Pgk1^{b}$ allele. dCTP and the chain-terminating radioactive dideoxyATP were the only nucleotides added to the single-cycle polymerase extension reaction. The $Pgk1^{b}$ allele extension product incorporates just the chain-terminating dideoxyATP, resulting in a 26-base product, while the $Pgk1^{a}$ allele extension product incorporates a dCTP followed by the chain-terminating dideoxyATP, resulting in a 27-base product.

The SNUPE assay was calibrated by mixing cDNA made from mRNA from C57BL/6J ($Pgk1^{b}$ allele) and the congenic strain B6.Cg-$Pgk1^{a}$/J in fixed ratios to mimic 100, 75, 25, and 0% of each allele. These results demonstrated the quantitative accuracy of the SNuPE reaction and densitometry analysis (Fig. 2), with a slope in the linear regression very close to 1 and R^2 for the regression of 0.99.

We then performed the SNuPE assay on RNA isolated from four tissues, liver, spleen, kidney, and brain, from 11 female $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ mice 8–28 weeks old. As controls, we also examined the X-inactivation pattern in RNA from tissues from seven females between 10 and 16 weeks old that were wild type for both *Ocrl* and *Inpp5b* but heterozygous for the two *Pgk1* alleles. In the $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ mice, XCI was significantly skewed ($P << 0.0001$, two-sided Student's *t* test) from the expected 70:30 under the null hypothesis of random inactivation in X controlling element (Xce) $Xce^{a/c}$ heterozygotes (Fig. 3) (Johnston and Cattanach 1981). The X chromosomes carrying the $Pgk1^{a}$ and $Ocrl^{-}$ alleles were preferentially, but

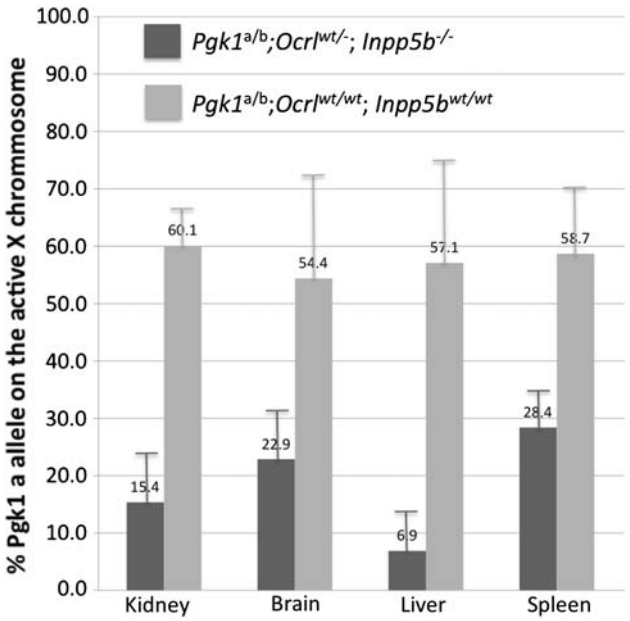


Fig. 3 X-inactivation analysis by SNuPE assay. Results are shown as mean + 1 standard deviation of the expression for the *Pgk1 a* allele in kidney, brain, liver, and spleen, expressed as % of total label incorporated into SNuPE extension products. Measurements were made in tissues from $Pgk1^{a/b}$;$Ocrl^{wt/-}$;$Inpp5b^{-/-}$ mice, where the *Pgk1 a* allele is in coupling phase with the disrupted *Ocrl* allele (dark shaded bars). X-inactivation analysis was performed in kidney, brain, spleen (11 individual mice), and liver (10 individual mice) from $Pgk1^{a/b}$;$Ocrl^{wt/-}$;$Inpp5b^{-/-}$ mice. These same four tissues were analyzed in seven control $Pgk1^{a/b}$;$Ocrl^{wt/wt}$;$Inpp5b^{wt/wt}$ mice (light shaded bars). *P* values for *Pgk1* allele *a* expression in $Pgk1^{a/b}$;$Ocrl^{wt/-}$;$Inpp5b^{-/-}$ mice compared to $Pgk1^{a/b}$;$Ocrl^{wt/wt}$;$Inpp5b^{wt/wt}$ mice (controls) are $P = 4.4 \times 10^{-9}$ in kidney, 1.6×10^{-4} in brain, 9.9×10^{-7} in liver, and 1.4×10^{-6} in spleen (Student's two-sided *t* test)

not exclusively, inactivated in $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ mice, despite carrying the somewhat stronger Xce^c on the X chromosome carrying the $Ocrl^-$ allele. Liver demonstrated the most severe skewing: less than 6.9 ± 6.5% (mean ± SD) of the X chromosomes that carried the mutant *Ocrl* were active ($P = 9.9 \times 10^{-7}$, Student's two-tailed *t* test). Kidney and brain had only 15.4 ± 8.6% and 22.9 ± 8.4%, respectively, of the mutant *Ocrl* chromosomes remaining active (for kidney tissue, $P = 4.4 \times 10^{-9}$; for brain tissue, $P = 1.6 \times 10^{-4}$, Student's *t* test). Spleen showed the least amount of skewing of the four tissues examined, with the mutant X chromosome active in 28.4 ± 6.1% of chromosomes ($P = 1.4 \times 10^{-6}$, Student's *t* test). The X-inactivation pattern in mRNA derived from the same four tissues from seven females that were wild type for both *Ocrl* and *Inpp5b* but heterozygous for the two *Pgk1* alleles demonstrated that the $Pgk1^a$ allele was on the active X chromosome 57.1 ± 18.8% SD of the time in liver, 60.1 ± 7.3% SD in kidney, 54.4 ± 18.8% SD in brain, and 58.7 ± 11.3% SD in spleen (Fig. 3). A fraction greater than 50% of cells with the $Pgk1^a$ on their active X chromosome is as expected given that an X chromosome carrying the *c* allele of the X controlling element (Xce^c), which is tightly linked to and in phase with the $Pgk1^a$ allele, is more likely to be active in a female heterozygous for the *a* and *c* alleles ($Xce^{a/c}$) (Cattanach and Papworth 1981; Krietsch et al. 1986). The more common Xce^a allele is in phase with the $Pgk1^b$ allele derived from the laboratory strain 129S4 (Courtier et al. 1995), the strain in which the disrupted *Ocrl* allele was originally made.

We also attempted a different breeding strategy to generate $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ mice in which we utilized $Ocrl^{-/-}$;$Inpp5b^{wt/-}$ females, deficient in *Ocrl* and heterozygous for the targeted mutant *Inpp5b*, and crossed them to $Ocrl^{wt/y}$;$Inpp5b^{wt/-}$ males, which expressed *Ocrl* and were heterozygous for the *Inpp5b*-targeted mutation. One-fourth of the resulting female zygotes would be expected to be $Ocrl^{wt/-}$;$Inpp5b^{-/-}$, with a functional $Ocrl^{wt}$ allele of paternal origin. In fact, we saw no live-born $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females among a total of 53 female offspring from this cross (Table 2). This result differs significantly from an expected approximately 13 females according to the expected Mendelian ratio of 1:2:1 ($P = 2.39 \times 10^{-7}$, exact binomial test). Since the X chromosome of paternal origin is exclusively and nonrandomly inactivated in the trophoblast-derived extraembryonic tissues of $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ embryos as they develop (Takagi and Sasaki 1975), we conclude that these extraembryonic tissue lineages of $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ zygotes in which the $Ocrl^{wt}$

Table 2 Females resulting from $Ocrl^{-/-}$;$Inpp5b^{wt/-}$ female × $Ocrl^{wt/y}$;$Inpp5b^{wt/-}$ male cross

Gamete genotype		Live-born genotype	# Females observed	# Females expected
Egg	Sperm			
$Ocrl^-$;$Inpp5b^{wt}$	$Ocrl^{wt}$;$Inpp5b^{wt}$	$Ocrl^{wt/-}$;$Inpp5b^{wt/wt}$	15	13.25
$Ocrl^-$;$Inpp5b^{wt}$ or $Ocrl^-$;$Inpp5b^-$	$Ocrl^{wt}$;$Inpp5b^-$ or $Ocrl^{wt}$;$Inpp5b^{wt}$	$Ocrl^{wt/-}$;$Inpp5b^{wt/-}$	38	26.5
$Ocrl^-$;$Inpp5b^-$	$Ocrl^{wt}$;$Inpp5b^-$	$Ocrl^{wt/-}$;$Inpp5b^{-/-}$	0*	13.25
		Total	53	53

* $P = 2.39 \times 10^{-7}$ (exact binomial test)

allele is paternal lacks both *Ocrl* and *Inpp5b* and is therefore unable to support embryonic development.

Despite the lack of females of the desired genotype from this cross, many $Ocrl^{-/-}$;$Inpp5b^{wt/-}$ mice were produced, indicating that $Ocrl^{-}$;$Inpp5b^{-}$ ova are generated, are capable of being fertilized, and can contribute to implantation and development of live mice, but only if the fertilizing sperm are functionally competent for *Inpp5b*.

We set up crosses between female $Ocrl^{-/-}$;$Inpp5b^{wt/-}$ and male $Ocrl^{-/y}$;$Inpp5b^{wt/-}$ and explanted 3.5-dpc blastocysts onto feeder layers to isolate embryonic stem cells. From these crosses we successfully isolated an embryonic stem cell clone from the inner cell mass of a $Ocrl^{-/-}$; $Inpp5b^{-/-}$ 3.5-dpc blastocyst. These cells had a 40,XX karyotype and could be propagated with repeated passages. The very fact that we could isolate such a cell line indicates that zygotes deficient in both enzyme activities can progress at least to the epiblast stage and can develop an inner cell mass. These cells could also be propagated in culture; any attempt, however, to induce these cells to differentiate, such as by removing them from feeder cells, resulted in rapid cell death (data not shown).

Discussion

In a previous report we demonstrated that $Ocrl^{-/-}$; $Inpp5b^{-/-}$ mice die prior to day E9.5 of embryogenesis (Janne et al. 1998). To explore this lethality, we attempted to generate $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females in order to examine the pattern of X-inactivation since the cells in these mice that inactivated the X carrying the wild-type *Ocrl* allele would be deficient in both Ocrl and Inpp5b. When the crosses were designed so that the wild-type *Ocrl* allele was paternal in origin, there was a total absence of the desired $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females at birth and the only embryos to survive were those rescued by the functional autosomal *Inpp5b*. In developing mouse embryos, X chromosome inactivation occurs through a random process in the embryo proper and in the primitive streak mesoderm-derived extraembryonic tissues such as the allantois, amnion, and mesoderm layer of the visceral yolk sac (Nagy 2003). However, X chromosome inactivation is imprinted by parent of origin in the trophoblast-derived extraembryonic tissues: the paternal X chromosome is preferentially inactivated in the extraembryonic tissues of the trophoblast, visceral endoderm, and parietal endoderm (Nagy 2003; Takagi and Sasaki 1975). We conclude that either the Ocrl PI(4,5)P_2 5-phosphatase or the Inpp5b PI(4,5)P_2 5-phosphatase must be present in the trophoblast-derived extraembryonic tissue of the developing mouse embryo in order for development to proceed, providing direct evidence that *Ocrl* and *Inpp5b* encode proteins with compensating and overlapping function in the placenta.

When the functional *Ocrl* in an $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ zygote was maternal in origin, live-born $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ female offspring were obtained and displayed no overt phenotype. This lack of phenotype can be explained by the preferential use of the maternal X chromosome with a wild-type *Ocrl* allele as the active chromosome. However, approximately half the expected number of $Ocrl^{wt/-}$; v$Inpp5b^{-/-}$ female offspring were seen. Assuming that X chromosome inactivation in these females is random, we hypothesize that the approximately 45% of female embryos in which the X chromosome coding for functional *Ocrl* and carrying the somewhat weaker Xce^{a} allele associated with $Pgk1^{b}$ is inactivated early in embryogenesis, do not survive. Those females that did survive with one copy of functional *Ocrl* of maternal origin but are deficient in Inpp5b all showed highly skewed XCI in all four tissues tested: liver, kidney, brain, and spleen. These tissues are derived from all three of the primary embryonic layers: endoderm, mesoderm, and ectoderm. Liver demonstrated the most severely skewed XCI pattern of the four tissues tested, with the mutant X active in only 6.9 $\pm$ 6.5% SD of the cells. Mouse liver is a tissue with low to moderate *Ocrl* expression but high *Inpp5b* expression (Janne et al. 1998). The lack of Inpp5b in liver tissue of $Inpp5b^{-/-}$ mice, which normally has high levels of Inpp5b, may greatly reduce liver cell survival such that the remaining moderate *Ocrl* expression from the X active chromosome might be a strong survival factor and thus explain the severely skewed X active coding for functional *Ocrl*. Mouse spleen expresses *Ocrl* at low levels and *Inpp5b* at low to moderate levels (Janne et al. 1998). However, we demonstrated that spleen also undergoes XCI skewing but less severely than the other tissues tested, allowing the mutant X to be active in 28.4 $\pm$ 6.1% SD of the cells. *Ocrl* expression is high in mouse brain and moderate in kidney, while *Inpp5b* expression is moderate in brain but high in kidney. In our experiments, kidney demonstrates more severe skewing than brain, correlating with the level of *Inpp5b* expression. We conclude from our experiments that tissues with normally high levels of expression of *Inpp5b* demonstrate the most severely skewed XCI.

It should be noted, however, that the skewing in these four tissues was not absolute. There are two possibilities to explain this observation. One is that most or all of the cell types in each of these organs can tolerate a small percentage of cells that are deficient in both enzymes. The second possibility is that these organs are made of a number of different cell types and certain types of cells in these organs, such as cells of hematopoietic origin, may be less subject to a deficiency of both enzymes. Detailed

cytological methods would be needed to distinguish between these possibilities.

The XCI skewing in the control ($Ocrl^{wt/wt}$;$Inpp5b^{wt/wt}$) $Xce^{a/c}$ heterozygous mice used in this study ranged from approximately 40:60 to approximately 46:54 and was significantly less skewed than the 30:70 ratio found by others using $Xce^{a/c}$ mice (Johnston and Cattanach 1981). However, the parent of origin as well as the continued mixing of the genetic backgrounds from one generation to another can affect the *Xce* strength and degree of X chromosome skewing in mice heterozygous at the *Xce* locus (Chadwick and Willard 2005). We suspect that the highly mixed genetic background of the control mice used in our experiment, a mixture of 129S6, C57Bl/6J, and NIH Black Swiss, and the multiple generations of breeding used to produce them have influenced the XCI ratio as measured in our control population.

Of the two breeding schemes we used to generate $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ mice, as described in Tables 1 and 2, the first was successful and the second was not. However, both schemes were useful for demonstrating that doubly deficient $Ocrl^{-}$;$Inpp5b^{-}$ gametes either from males or females are functional and capable of participating in fertilization. In particular, homozygous $Inpp5b^{-/-}$ male mice in the inbred 129S6 demonstrate a fertility defect (Hellsten et al. 2001, 2002); the defect is complemented in a mixed genetic background that includes FVB/N (Nussbaum, unpublished observations). The breeding scheme shown in Table 1 shows that the doubly deficient male gametes lacking both *Ocrl* and *Inpp5b* function in a mixed genetic background are indeed capable of fertilizing ova, which develop into embryos and give rise to live births. Although we suspect that the XCI is the key to the loss of a portion of our $Ocrl^{wt/-}$;$Inpp5b^{-/-}$ females, we cannot entirely discount an effect from decreased production or fitness of doubly deficient male gametes.

By utilizing random X chromosome inactivation in embryos and nonrandom inactivation of the male X chromosome in trophoblast-derived extraembryonic tissues as an analytical tool, we have probed the basis for our previous observations on the prenatal lethality of doubly deficient $Ocrl^{-/-}$;$Inpp5b^{-/-}$ or $Ocrl^{-/y}$;$Inpp5b^{-/-}$ mice. We have shown that the two paralogs indeed have overlapping function in all three of the primary layers of the embryo and in the extraembryonic tissues and that the embryonic lethal effect of a double deficiency of *Ocrl* and *Inpp5b* occurs between 3.5 and 9.5 dpc.

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