Genetic Etiology of Renal Agenesis: Fine Mapping of \textit{Renag1} and Identification of \textit{Kit} as the Candidate Functional Gene

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Abstract

Congenital anomalies of the kidney and urogenital tract (CAKUT) occur in approximately 0.5% of live births and represent the most frequent cause of end-stage renal disease in neonates and children. The genetic basis of CAKUT is not well defined. To understand more fully the genetic basis of one type of CAKUT, unilateral renal agenesis (URA), we are studying inbred ACI rats, which spontaneously exhibit URA and associated urogenital anomalies at an incidence of approximately 10%. URA is inherited as an incompletely dominant trait with incomplete penetrance in crosses between ACI and Brown Norway (BN) rats and a single responsible genetic locus, designated \textit{Renag1}, was previously mapped to rat chromosome 14 (RNO14). The goals of this study were to fine map \textit{Renag1}, identify the causal genetic variant responsible for URA, confirm that the \textit{Renag1} variant is the sole determinant of URA in the ACI rat, and define the embryologic basis of URA in this rat model. Data presented herein localize \textit{Renag1} to a 379 kilobase (kb) interval that contains a single protein coding gene, \textit{Kit} (v-kit Hardy-Zukerman 4 feline sarcoma viral oncogene homolog); identify an endogenous retrovirus-derived long terminal repeat located within \textit{Kit} intron 1 as the probable causal variant; demonstrate aberrant development of the nephric duct in the anticipated number of ACI rat embryos; and demonstrate expression of \textit{Kit} and \textit{Kit} ligand (\textit{Kitlg}) in the nephric duct. Congenic rats that harbor ACI alleles at \textit{Renag1} on the BN genetic background exhibit the same spectrum of urogenital anomalies as ACI rats, indicating that \textit{Renag1} is necessary and sufficient to elicit URA and associated urogenital anomalies.
These data reveal the first genetic link between Kit and URA and illustrate the value of the ACI rat as a model for defining the mechanisms and cell types in which Kit functions during urogenital development.

Introduction

Congenital anomalies of the kidney and urogenital tract (CAKUT) occur in approximately 0.5% of live births and together represent the most common class of developmental abnormalities in humans [1–4]. CAKUT is comprised of an assortment of interrelated phenotypes including bilateral renal agenesis (BRA), unilateral renal agenesis (URA), renal hypodysplasia, hydronephrosis, megaureter and pelviureteric junction obstructions. Together, these anomalies are the most frequent cause of end-stage renal disease in neonates and children [5,6]. The genetic bases of CAKUT are heterogeneous and only partially defined. Familial forms of CAKUT generally exhibit an autosomal dominant pattern of inheritance with incomplete penetrance [1,7]. Mutations in over 30 different genes have thus far been observed in association with CAKUT [1,4,8,9]. Roles for many of these CAKUT associated genes in urogenital development were first demonstrated in studies of genetically modified mouse models and the genes were subsequently implicated in the genesis of CAKUT by identification of mutations in families with multiple affected members. Other CAKUT associated genes were first identified in genetic studies of developmental syndromes that include anomalies in urogenital organs as associated phenotypes. Data from multiple genetic linkage and genome wide association studies, many of which were focused on vesicoureteral reflux as the phenotype of interest, further establish the heterogeneous genetic bases of CAKUT [6,10–16].

A solitary kidney resulting from URA or renal aplasia is a common CAKUT. A study in which 132,686 asymptomatic school children in China were evaluated by ultrasound and all suspected renal abnormalities were confirmed by radiography revealed a 0.08% incidence of solitary kidney and a 0.1% incidence of unilateral renal hypoplasia [17]. A similar study of 2920 asymptomatic 3 year olds in Japan revealed a 0.1% incidence of solitary kidney and a 0.07% incidence of unilateral renal hypoplasia [18]. A high incidence of solitary kidney has also been observed in adult populations examined postmortem. A solitary kidney was observed at an incidence of 0.09% in a large series of autopsies reported by the Armed Forces Institute of Pathology [19]. Similarly, a 0.18% incidence of solitary kidney was observed in a series of 13,775 consecutive autopsies performed at Vanderbilt University between 1928 and 1986 [20]. Multiple reports, only a few of which are cited here, indicate the occurrence of multiple cases of BRA and/or URA within families [21–24]. Moreover, the incidence of URA in first-degree relatives of individuals with BRA has been shown to significantly exceed the incidence of URA in the general population, strongly suggesting a genetic basis for familial renal agenesis. For example, Carter et al. observed BRA or URA in 7 of 199 (3.5%) siblings of individuals with BRA, whereas Roodhooft et al. observed asymptomatic URA in 3 of 71 (4.2%) parents and 2 of 40 (5.0%) siblings of cases of BRA [25,26]. It is becoming increasingly clear that the presence of a solitary kidney increases the risk of chronic kidney disease and hypertension and adversely impacts survival both in animal models and humans [5,27–30].

In order to understand more fully the genetic basis of renal agenesis, we are studying inbred ACI rats, which spontaneously exhibit URA and associated urogenital anomalies at an incidence of approximately 10%. The occurrence of URA in ACI rats was first reported in 1953 by Morgan and has been confirmed in multiple subsequent studies [31–38]. In addition to URA,
female ACI rats generally exhibit an absent uterine horn ipsilateral to the missing kidney, whereas the ipsilateral vas deferens and epididymis of URA-affected males are frequently missing or incompletely developed [31–38]. Interestingly, the urogenital anomalies in ACI rats exhibit a pronounced right side bias. URA and associated urogenital anomalies are inherited as an incompletely dominant trait with incomplete penetrance in crosses between ACI and Brown Norway (BN) rats [39,40]. We mapped to a 14.4 megabase (Mb) interval on rat chromosome 14 (RNO14) a locus, designated Renag1, that appears to act as the sole genetic determinant of URA in reciprocal intercrosses between ACI and BN rats [39,40]. The goals of this study were to fine map Renag1, identify the causal genetic variant responsible for URA, confirm that the Renag1 variant is the sole determinant of URA in the ACI rat, and define the embryologic basis of URA in this rat model. Data presented herein localize Renag1 to a 379 kilobase (kb) interval that contains a single protein coding gene, Kit (v-kit Hardy-Zukerman 4 feline sarcoma viral oncogene homolog); identify the probable causal variant within Kit intron 1; demonstrate aberrant development of the nephric duct at day 11.5 of embryonic development; and demonstrate expression of Kit and Kit ligand (Kitlg) in the nephric duct.

Results
Fine mapping of Renag1
A population comprised of 4994 (BNxACI) F2 rats was generated and evaluated for renal abnormalities as a prerequisite for fine mapping Renag1. Of these F2 animals, 351 (7.0%) exhibited a renal abnormality, frequently in association with an abnormality in or absence of another urogenital organ(s) (Fig. 1A). The incidence of urogenital abnormalities observed in this F2 population was approximately half of that observed in ACI rats. These data are consistent with the published observation that URA and related urogenital abnormalities segregate as an incompletely dominant and incompletely penetrant trait in progeny generated by crossing ACI rats, which exhibit these developmental anomalies, and BN rats, which do not [40]. URA was observed in 238 of the 4994 F2 rats (4.8% incidence) and was the most frequently observed renal abnormality (67.8% of observed anomalies) in the F2 population (Fig. 1B). The other renal abnormalities observed in the F2 population were unilateral renal hypoplasia (URH), which was observed in 66 rats (1.3% incidence in F2 population, 18.8% of observed abnormalities), and an enlarged, fluid filled, vestigial kidney and ureter indicative of hydroureteronephrosis (HUN), which was observed in 47 rats (0.9% incidence in F2 population; 13.4% of renal abnormalities) (Figs. 1B and 2).

URA occurred on the right side in 200 of the 238 (84%) affected F2 rats, which constitutes a statistically significant right side bias (p = 2.2 x 10^{-16})(Fig. 1C). This observation is consistent with published reports on URA in ACI rats [35,38,40]. Interestingly, no statistically significant right or left side bias was evident in F2 animals that exhibited URH or HUN. A subset of the male F2 rats that exhibited URA also exhibited additional urogenital anomalies, including absent or incompletely developed ipsilateral adrenal gland, testis, vas deferens and/or epididymis. All of the females that exhibited URA lacked all or part of the ipsilateral uterine horn, 6 of the affected females lacked the ipsilateral ovary, and 5 lacked the ipsilateral ovary and adrenal gland.

Each of the 351 F2 rats that exhibited a renal abnormality was genotyped at polymorphic microsatellite markers distributed across the Renag1 region on RNO14 to define more precisely the location of Renag1. The ACI allele of Renag1 has been demonstrated to act in an incompletely dominant and incompletely penetrant manner to elicit URA; therefore, homozygosity for the BN allele at a marker excludes that marker from Renag1 [40]. These analyses localized Renag1 to the 2.0 Mb interval on rat chromosome 14p11 defined by markers D14Uwm7 (34.25
Mb) and D14Uwm12 (36.25 Mb) (Fig. 3). This region contains 12 annotated protein-coding genes and is orthologous to human chromosome 4q12 and mouse chromosome 5 (Fig. 4A).

Many of the male F2 rats that were generated in the experiment described above were genotyped at select markers on RNO14 prior to being euthanized. Those male F2 rats that harbored a recombination within the Renag1 region were mated to BN females and the resulting progeny were evaluated for renal abnormalities. Five such recombinant chromosomes were capable of eliciting renal abnormalities in progeny that inherited the recombinant chromosome (Fig. 4B). In addition, one of the progeny generated by backcrossing male F2-4747 to BN rats exhibited
URA and was determined to harbor a recombinant chromosome different than that harbored by F2-4747. Together, the genetic evaluation of progeny that inherited one of these six recombinant chromosomes and exhibited a renal abnormality further mapped the location of Renag1 to the 379 kb interval on RNO14 defined by markers D14Uwm8 (34.73 Mb) and D14Uwm9 (35.11 Mb). This interval harbors a single annotated protein-coding gene, Kit.

Confirmation of Renag1 mapping to RNO14 through generation and evaluation of congenic rat strains

Two congenic rat strains, each of which harbors ACI alleles across Renag1 on the BN genetic background, were generated as described below and evaluated with respect to urogenital anomalies. The BN.ACI-(D14Uwm4-D14Rat39) congenic strain (Con1) was generated from the recombinant chromosome that originated in rat F2-3766, whereas the BN.ACI-(D14Uwm1-D14Uwm5) congenic strain (Con2) was generated from the chromosome that originated in rat F2-3840 (Fig. 4B). Both Con1 and Con2 rats exhibited virtually the identical spectrum of urogenital abnormalities, including URA, URH and HUN, observed in ACI rats and (BNxACI)F2 rats. The overall incidence of urogenital abnormalities was 13.4, 20.6 and 11.7% in ACI, Con1 and Con2 rats, respectively (Fig. 5A). Interestingly, the incidence of urogenital anomalies in Con1 rats was significantly higher than in ACI (p = 0.0469) or Con2 (p = 0.0059) rats. URA was the most commonly observed urogenital anomaly in each rat strain, comprising 82.8, 79.6 and 77.1% of total urogenital abnormalities in ACI, Con1 and Con2 rats, respectively (Fig. 5B). URH comprised between 6.9 and 14.3% of total abnormalities in the three rat strains. These data indicate that homozygosity of ACI alleles at Renag1, when harbored on the genetic background of the BN strain, is sufficient to confer the full incidence and spectrum of urogenital abnormalities observed in ACI rats.

As expected from previous studies, URA in ACI rats was observed more frequently on the right side than the left; 91.7% right vs. 8.3% left (p < 0.0034, Fig. 5C). Interestingly, no right
Side bias was observed upon evaluation of URA in Con1 rats \((p = 0.2452)\), Con2 rats \((p = 1.000)\) or the combined congenic population \((p = 0.3747)\). This observation indicates the right side bias observed in ACI and (BNxACI)F2 rats exhibiting URA is determined by a locus distinct from Renag1. The right-left distributions of URH and HUN were not analyzed statistically because of the low incidence of these anomalies in ACI, Con1 and Con2 rats. The incidence of renal abnormalities was similar in male and female ACI, Con1 and Con2 rats (data not shown).

**Cosegregation of urogenital anomalies and white spotting phenotype**

It became apparent during generation of the two congenic strains described above that the congenic intervals from the ACI strain that were being introgressed onto the BN genetic background harbored a genetic variant that confers a white spotting phenotype. Those progeny that inherited the recombinant chromosome at each generation of back crossing exhibited small
patches of white on the abdomen. When heterozygous siblings were intercrossed, those progeny that were homozygous for the recombinant chromosomes (i.e., ACI alleles) exhibited a greater degree of abdominal white spotting. All individuals from each of the two congenic strains exhibited a varying degree of white spotting on the abdomen between the rear legs and extending cranially (Fig. 6). By contrast, BN rats only rarely exhibited any white spotting, and when present it was small and located on the chest, not the abdomen. Interestingly, the extent of the abdominal white spotting in the congenic animals was generally not as extensive as that exhibited by ACI rats. In addition, only the distal segment of each rear foot of the Con1 and Con2 rats was unpigmented, whereas the rear feet were entirely unpigmented in ACI rats. Together, these data indicate that a genetic variant inherited from the ACI donor strain and residing within the \textit{Renag1} region on RNO14 acts in an incompletely dominant but completely penetrant manner to confer the unique pattern of white spotting observed in rats from the two congenic strains.

The \textit{Hooded} locus, at which different alleles confer varying patterns of white spotting, has been mapped to intron 1 of \textit{Kit} [41–43]. Moreover, associations between different \textit{Kit} alleles and a wide variety of white spotting phenotypes have been noted in multiple species. ACI rats harbor the Irish allele, \textit{hi}, at \textit{Hooded}, whereas BN rats harbor the Self allele, \textit{H}, at \textit{Hooded}, which explains the presence of abdominal white spotting in Con1 and Con2 rats. To further establish cosegregation of \textit{Renag1} and \textit{Hooded}, we analyzed unpublished data from previously
described reciprocal intercrosses between ACI and Copenhagen (COP) rats that were performed to define the genetic bases of susceptibility to induction of mammary and pituitary tumors by administered estrogens [44–46]. Genome wide interval mapping analyses of the F2 progeny from these intercrosses linked URA to the Renag1 region on RNO14 (data not shown). Forty F2 progeny from these intercrosses exhibited URA and each of these 40 affected F2 progeny harbored at least one ACI allele at Renag1, strongly supporting a genetic model in which an ACI allele at Renag1 is both necessary and sufficient to confer URA in crosses.

Fig 5. BN.ACI-Renag1 congenic rats exhibit the same spectrum and incidence of urogenital anomalies as ACI rats. The Con1 and Con2 congenic rat strains were generated as described in Materials and Methods. Each strain is homozygous for ACI alleles across the Renag1 interval. A. The incidence of all grossly discernable urogenital anomalies in ACI (n = 216), Con1 (n = 238) and Con2 (n = 298) rats is illustrated. Numeral 1 indicates statistically significant difference relative to ACI. B. The frequency of unilateral renal agenesis (URA), unilateral renal hypoplasia (URH) and hydroureteronephrosis (HUN) in ACI, Con1 and Con2 rats is illustrated as a percent of total urogenital anomalies. C. The frequency of URA in ACI, Con1 and Con2 rats observed on the left (L) versus right (R) sides is illustrated. The asterisk indicates a statistically significant right side bias.

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Fig 6. Abdominal white spotting is linked to the Renag1 congenic interval. The ventral aspects of representative ACI, BN, Con1 and Con2 rats are illustrated. ACI rats harbor the Irish allele at the Hooded locus and exhibit abdominal white spotting. BN rats harbor the Self allele at Hooded and are fully pigmented on the abdomen. Con1 and Con2 rats harbor the ACI derived Irish allele at Hooded and exhibit varying amounts of abdominal white spotting and a clear demarcation of pigmentation on the rear feet (noted by arrows).

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between ACI and COP rats, similar to that observed in crosses between ACI and BN rats. Mapping by exclusion from Renag1 of markers at which homozygosity for COP alleles were observed in URA affected F2 rats localized the causal genetic variant to the 48.55 Mb interval on RNO14 defined by markers D14Rat78 (17.42 Mb) and D14Rat88 (65.96 Mb) (data not shown). In addition, genome wide interval mapping analyses of the F2 progeny from these intercrosses between ACI (Irish allele, h', at Hooded) and COP rats, which harbor the Hooded allele, h, at Hooded, localized Hooded to RNO14. Fine mapping by inclusion within Hooded of markers at which homozygosity for COP alleles (h is recessive to h') was observed in those rats that exhibited the Hooded phenotype (white extending from abdomen upward on both flanks) further localized Hooded to the 20.72 Mb interval of RNO14 defined by markers D14Arb6 (22.00 Mb) and D14Rat15 (42.72 Mb)(data not shown). Together, these data indicate that Renag1 cosegregates with allelic variants at Hooded that likely impact Kit expression in a cell type specific and/or temporal manner and thereby influence melanoblast migration during embryonic development resulting in variation in coat color.

Sequence analyses of Renag1

In addition to our published and current data linking URA to the Renag1 locus on RNO14 in multiple crosses between ACI rats and BN or COP rats, URA has been linked to a single marker on RNO14, D14Rat65 (36.41 Mb), in a study of (F344 x ACI)F2 progeny evaluated in relation to prostate cancer susceptibility [47]. Based on the linkage and exclusion mapping data, we generated and/or evaluated available whole genome sequence data from the ACI, BN, COP and F344 rat strains to identify genetic variants within the 379 kb Renag1 minimal interval that are unique to the ACI strain; i.e., variants where ACI rats differ from BN, COP and F344 rats. No such variants were identified when all available whole genome sequences for ACI (2 sequences), BN (2 sequences), COP and F344 rats were mapped onto Rat Genome Assembly 5.0 (Rnor_5.0) and compared (Table A in S1 File). The Renag1 candidate gene, Kit, was also evaluated by standard PCR based sequencing methods (prior to the availability of whole genome sequences other than the BN reference sequence). The GenBank accession numbers for these Kit sequences are listed in Table B in S1 File. Five SNPs were identified upon comparison of Kit cDNA generated from mRNA prepared from ACI and BN rats. Four of these SNPs were synonymous variants within the Kit coding region: 1) nucleotide 34,947,632, exon 2, codon 51, glycine; 2) nucleotide 34,945,425, exon 3, codon 130, asparagine; 3) nucleotide 34,945,200, exon 3, codon 205, arginine; and 4) nucleotide 34,904,134, exon 21, codon 706, leucine. The exons harboring these SNPs were sequenced using DNA isolated from ACI, BN, COP and F344 rats, indicating that each of these four nucleotide variants present in ACI rats are also present in F344 and COP rats. These data are consistent with available whole genome sequence data for these four rat strains, with exception of the SNP at nucleotide 34,945,200, where one of two ACI sequences and the F344 sequence are instead identical to the reference BN sequence. The fifth SNP variant resides within the Kit 3’ untranslated region (UTR). Interestingly, the data generated by sequencing cDNA and exon 21 indicated that this variant was unique to the ACI rat strain. However, examination of available whole genome sequences at this position (nucleotide 34,903,999) indicated that one of the two available ACI sequences was consistent with data generated by sequencing cDNA and exon 21 whereas the other was identical to the reference BN sequence. In addition, the whole genome sequence for COP at this position differed from that observed upon sequencing COP cDNA and exon 21, and instead was identical to that observed upon sequencing cDNA and exon 21 from ACI rats. In spite of these inconsistencies between the whole genome and PCR based sequences, these data indicate that the five nucleotide
variants residing within Kit, if they were to impact Kit mRNA stability or translation, are most probably not functionally associated with URA and associated urogenital anomalies.

One genetic variant within the 379 kb Renag1 interval that was not apparent upon evaluation of whole genome sequences mapped onto the Rnor_5.0 genome assembly is the rat strain specific presence of sequences related to a class I endogenous retrovirus (ERV) within intron 1 of Kit (at position 34,957,384 in Rnor_5.0). The ERV-related sequences were first identified by Kuramoto et al. as the causal variants residing within the Hooded locus, at which the Hooded (h) and Irish (hi) alleles each confer distinct coat color phenotypes resulting from variation in the extent of melanoblast migration during embryogenesis [43]. These investigators demonstrated that BN, F344 and ACI rats each harbor a distinct allele at the Hooded locus: 1) BN rats harbor the Self allele (H) at Hooded and lack ERV-related sequences at the specific location in intron 1 of Kit; 2) F344 rats carry the Hooded allele (h) and harbor a 7098 bp ERV-related element, including both 5’ and 3’ long terminal repeats (LTR), within intron 1 of Kit; and 3) ACI rats carry the Irish allele (hi) and harbor a 584 bp ERV-related element consisting of a single copy of the viral LTR. We have confirmed and extended the data of Kuramoto et al. by performing PCR based analyses across the site in Kit intron 1 into which the ERV-related elements would be inserted (Fig. 7). These data confirmed the lack of an integrated ERV in BN rats, illustrated the presence of the ERV in COP rats, which like F344 rats harbor the h (Hooded) allele at Hooded, and confirmed the presence of a single ERV LTR in ACI, Con1 and Con2 rats, which harbor the hi (Irish) allele at the Hooded locus (Fig. 7B). We further confirmed the insertion of the ERV related sequences by determining the nucleotide sequences across the insertion junction (Fig. 7C). Together, these analyses of sequence variants within the Renag1 locus for BN, ACI, COP and F344 rats strongly suggest that the ERV-related LTR responsible for the Irish coat color phenotype in ACI rats is also the causal variant for URA and associated urogenital anomalies.

Expression of Pax2, Kit and Kitlg in the nephric duct

Pax2 is required for normal development of the nephric duct and is frequently used as a marker for identifying the nephric duct throughout the course of embryonic development. Therefore, expression of Pax2 in the developing embryo was examined in order to understand better the developmental basis of urogenital anomalies in ACI rats. Evaluation of Pax2 expression in e11.5 ACI embryos by in situ hybridization revealed strong labeling of the nephric duct and mesonephric tubules (Fig. 8). This pattern of Pax2 expression was similar to that reported for e9.5 mouse embryos [48,49]. The majority of ACI embryos exhibited symmetrical Pax2 expression in both the right and left nephric ducts (Fig. 8A, left panel). However, some embryos exhibited a clear unilateral absence of Pax2 expression consistent with premature termination of the nephric duct (Fig. 8A, right panel). In a subset of these embryos, a pattern of intermittent Pax2 expression was observed in the caudal segment of the nephric duct adjacent to the point of apparent truncation (data not shown). Select embryos were embedded in methacrylate polymer, sectioned and evaluated histologically. The sections selected for imaging were confirmed to be taken from a similar plane based on symmetry with respect to outline and position of nephric ducts, neural tube, caudal dorsal aortae and central gastrointestinal tract. These analyses confirmed the unilateral absence of Pax2 expression in the expected anatomic location for the nephric duct (Fig. 8B). Additionally, no cell condensate in the expected location of the nephric duct was discernable upon histological examination, further associating lack of detectable Pax2 expression with abnormal development of the nephric duct. Examination of 75 e11.5 ACI embryos revealed 6 embryos (8% incidence) with asymmetrical nephric duct Pax2 expression (Fig. 8C). The absence of discernable Pax2 staining was on the right side in 4 of these...
embryos and on the left side in two. Thus, the total incidence and right side bias of abnormal \textit{Pax2} expression in the nephric duct closely resembled the incidence and asymmetry of urogenital anomalies exhibited by ACI rats.

Expression of \textit{Kit} and \textit{Kitlg} was similarly examined to begin to define the roles of these genes in urogenital development. \textit{Kit} and \textit{Kitlg} were observed to be expressed in the nephric duct of e11.5 ACI embryos evaluated by whole mount \textit{in situ} hybridization (Fig. 9A). To confirm the localization of these mRNAs within and around the nephric duct, select embryos were embedded in methacrylate resin, sectioned, counter stained with nuclear fast red and evaluated.
**Fig 8. Embryologic basis of urogenital anomalies in ACI rats.** e11.5 ACI rat embryos were stained by ISH to visualize mRNA expression (purple) of the nephric duct epithelium marker, paired box gene 2 (Pax2). The embryos were stained intact and the caudal portion was dissected after staining and positioned for optimal imaging. A. Representative images of each embryo's caudal region revealed two different Pax2 staining patterns: the normal pattern, where Pax2 staining is present and extends to the cloaca in both nephric ducts, and the abnormal pattern, where Pax2 staining in one nephric duct inappropriately terminates cranial to the hindlimb (indicated by white arrowheads). Note that in the same embryo, staining in the contralateral nephric duct extends past the caudal aspect of the hindlimb to the cloaca. B. Some whole-mount stained embryos were cut into transverse sections to reveal histological architecture. In most embryos, Pax2 staining was identified in the bilaterally symmetrical nephric duct cell condensates. However, some embryos featured an atypical unilateral Pax2 staining pattern. There was no histologically discernible cell condensate in the expected nephric duct region on the unstained side of the embryo (dashed circle). Abbreviations used are: ND, nephric duct; MT, mesonephric tubule; and NT, neural tube. Both images are the same magnification. C. Incidence of normal Pax2 staining in both nephric ducts and abnormal (i.e., absent) staining in left and right nephric ducts of 75 rat embryos.

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**Fig 9. Kit and Kitlg mRNA are expressed in the nephric duct of ACI rat embryos.** e11.5 ACI rat embryos were stained by ISH to visualize mRNA expression (purple) of the nephric duct epithelium marker Pax2, Kit (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) or Kitlg (kit ligand). A. Lateral view of representative stained embryos. B. Some stained embryos were embedded in methacrylate and cut into transverse sections to reveal histological architecture. Abbreviations used are: C, cloaca; G, gut endoderm; FL, forelimb bud; HL, hindlimb bud; MT, mesonephric tubule; ND, nephric duct; and NT, neural tube. Images within each panel are of the same magnification and are representative of at least seven independent embryos.

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Histologic examination of embryo cross sections confirmed expression of both Kit and Kitlg mRNA in the nephric ducts (Fig. 9B). Together, these data indicate that URA and associated urogenital anomalies in the ACI rat are a manifestation of failed development of the nephric duct and strongly suggest a functional role of the Kit receptor and its cognate ligand in urogenital development in the rat.

Discussion
The studies described herein localized the genetic variant responsible for URA and associated urogenital anomalies in the ACI rat to a 379 kb interval of RNO14 that harbors a single protein coding gene, Kit, as well as three predicted genes that may yield non-coding RNAs, LOC102553597, LOC102553643 and LOC102553856. Kit encodes a transmembrane receptor tyrosine kinase that is activated by its cognate ligand Kitlg, also known as stem cell factor. Also presented herein are studies that indicated that Kit and Kitlg are expressed in the nephric duct of e11.5 ACI rat embryos. Published studies indicate that Kit is similarly expressed in the nephric duct of the developing mouse embryo [50,51]. These data suggest that perturbations in Kit signaling may contribute to urogenital abnormalities.

It has been suggested that failed development of the nephric duct gives rise to URA and associated urogenital anomalies in male and female ACI rats [35,36,40]. This study provides the first illustration of unilateral truncation of the nephric duct in a subset of ACI e11.5 embryos, as evidenced by in situ analyses of Pax2 expression and histological evaluation. Roles for Kit signaling in migration, proliferation and/or survival of a variety of stem and progenitor cell types have been well documented [52]. Based on these data, we propose a model in which the causal Renag1 genetic variant results in cell type specific and/or temporal alterations in Kit expression, and that this in turn impacts migration, proliferation and/or survival of a cell population(s) that is required for full and appropriately timed caudal extension of the nephric duct. Truncation of the nephric duct would account for the observed spectrum of aberrations in the ipsilateral urogenital tissues that are derived from the mesonephric duct, including vas deferens, seminal vesicle and epididymis, as well as the observed aberrations in the ipsilateral Mullerian duct derived tissues, such as the uterine horn. However, truncation of the nephric duct would not explain the absence of adrenal glands and gonads occasionally observed in ACI rats, suggesting that the Renag1 causal variant may impact Kit signaling within multiple cell populations in the intermediate mesoderm. This model supports the hypothesis of Mackie and Stephens and is consistent with data from multiple studies that demonstrate that a specific gene mutation or environmental factor can exert pleiotropic actions on urogenital development resulting in a spectrum of distinct but related urogenital abnormalities [53–57].

To the best of our knowledge, this is the first study to suggest a causal role for a Kit allele in the etiology of URA and associated urogenital anomalies in any species. However, published reports provide circumstantial evidence linking Kit to aberrant urogenital development in humans. For example, the absence of the right kidney was noted in the child with piebaldism who was the subject of one of the first studies to associate that autosomal dominant trait to a loss of function mutation in Kit [58,59]. Second, agenesis/absence of the right kidney was noted in 2 of 12 cases of congenital abnormalities observed in offspring of women who were treated for chronic myelogenous leukemia during pregnancy with imatinib, a small molecule inhibitor of Kit and select other tyrosine kinases, including Pdgfra and Ret, which also play roles in renal development [60]. However, it is important to note that neither renal agenesis nor any of the other urogenital anomalies observed in ACI rats have been noted in studies of White Spotting (Ws) rats, which harbor a 12 nucleotide deletion in the Kit coding region that generates a dominant negative Kit protein, or in mice that harbor mutant Kit alleles [61–63].
The data from this study and our previous study indicate that the causal Renag1 variant acts in an incompletely dominant and incompletely penetrant manner to confer development of urogenital anomalies. Interestingly, the same variant appears to act in an incompletely dominant but fully penetrant manner to confer the abdominal white spotting phenotype. We do not believe that these observations are contradictory. Instead, we interpret these data to suggest that the causal Renag1 variant affects expression of Kit, which would then result in downstream actions on migration, proliferation and/or survival of specific populations of cells contributing to nephric duct development. If the number of cells required for the proper development of the nephric duct were not to achieve a critical threshold, duct elongation would fail at variable points along the cranial-caudal axis, resulting in the observed spectrum of anatomic abnormalities exhibited by rats harboring an ACI allele of Renag1. By contrast, variation in melanoblast migration resulting from actions of Renag1 (i.e., h' allele at Hooded) on Kit expression would simply result in variation in the extent to which white spotting occurs.

A solitary ERV-derived LTR located within intron 1 of Kit is the sole variant within the Renag1 locus that is unique to ACI rats. By contrast, BN rats lack ERV related sequences at the specified location in intron 1, whereas COP and F344 rats harbor the complete ERV at this location. This solitary LTR confers the Irish coat color trait upon ACI rats by means of its actions on Kit expression and melanoblast migration [43]. We hypothesize this LTR also confers the propensity for aberrant urogenital development upon ACI rats through its actions on Kit expression in an as yet unknown population(s) of cells. Additional studies are required to fully validate this hypothesis. The genomes of mammals harbor many thousands of full or partial copies of ERVs. It is well established that ERV LTRs can influence expression of nearby genes, and multiple examples are known in which LTRs have been exapted during evolution to function as cell type specific promoters or enhancers [64,65]. Also known are multiple examples in which ERVs and/or their LTRs are responsible for Mendelian or quantitative genetic traits [66–69].

The ACI rat is unique in that it is the only genetically defined rat model for studying the molecular and cellular bases of URA and associated urogenital anomalies. URA (i.e., solitary kidney) also segregates in heterogeneous stock (HS) rats, an outbred population derived from 8 inbred rat strains, including ACI [70,71]. Linkage of the solitary kidney phenotype to multiple loci was observed in a comprehensive QTL mapping study of HS rats, including the Renag1 region on RNO14, suggesting the existence of modifiers of Renag1 and/or other genes that independently contribute to urogenital anomalies in the HS rat population [71]. Moreover, a white spotting phenotype was mapped to the same region of RNO14 as the solitary kidney phenotype in the QTL mapping study of HS rats, suggesting that the Irish allele at the Hooded locus and Renag1 cosegregate in the outbred HS rat population. The unilateral urogenital anomalies (UUA) rat represents another unique model for studying the etiology of urogenital abnormalities [72]. Although the spectrum of urogenital anomalies exhibited by UUA rats is similar to that of ACI rats, the urogenital anomalies occur exclusively on the left side in UUA rats, in contrast to the strong right side bias observed for ACI rats.

This study demonstrated that a genetic variant in intron 1 of Kit is both necessary and sufficient to disrupt normal urogenital development in the ACI rat. The presented data strongly suggest that this variant ERV derived LTR exerts its actions through Kit expression and downstream signaling. If Kit is the gene through which the Renag1 variant exerts its actions, then the incidence of URA and associated urogenital anomalies may be significantly higher in individuals with piebaldism than in the general population. Moreover, the spectrum of urogenital anomalies exhibited by ACI rats resembles the anomalies associated with specific genetic syndromes in humans. For example, the combination of right side preponderance of URA and an ipsilateral anomaly of the uterus exhibited by ACI rats resembles the phenotypic profile that is
characteristic of Herlyn-Werner-Wunderlich (HWW) Syndrome [73–77]. Similarly, the anomalies exhibited by male ACI rats resemble those in humans with URA combined with congenital absence of the vas deferens [78–80]. An evaluation of the association of Kit and these genetic syndromes in humans appears warranted.

Materials and Methods

Housing and care of animals

The Institutional Animal Care and Use Committees of the University of Nebraska Medical Center (protocol 04-064-08) and the University of Wisconsin-Madison (protocol M02422) approved this entire study including all procedures involving live animals. ACI/SegHsd and BN/SsNHsd rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed under controlled temperature, humidity and 12h light/12h dark conditions in animal facilities that were accredited by the American Association for Accreditation of Laboratory Animal Care and operated in accordance with the standards outlined in Guide for the Care and Use of Laboratory Animals. Euthanasia prior to phenotypic evaluation was by asphyxiation with carbon dioxide.

Fine mapping of Renag1

BN females were mated to ACI males to generate F1 progeny. F1 siblings were mated to generate F2 progeny. F2 progeny were euthanized at approximately 21 days of age and evaluated to ascertain the presence or absence of urogenital anomalies. Those animals that exhibited a urogenital anomaly were genotyped at a panel of microsatellite markers distributed across the Renag1 locus on RNO14 as described previously [40,45,46,81]. Because an ACI allele at Renag1 is required for URA and associated urogenital anomalies, homozygosity for the BN allele at a marker was interpreted to exclude that marker from the Renag1 locus [40]. As fine mapping was in progress, a large number of male F2 rats were genotyped at RNO14 markers and those F2 rats that were determined to harbor recombinations within the Renag1 locus were mated to BN females. The presence or absence of URA and associated anomalies was then evaluated in the resulting progeny in order to determine whether the specific recombinant ACI segment of RNO14 possessed the ability to elicit URA. Additional polymorphic microsatellite markers within the Renag1 region were developed as needed. Pertinent information for these markers is included as Table C in S1 File.

Generation and evaluation of Renag1 congeneric strains

The BN.ACI-(D14Uwm4-D14Rat39) congeneric strain (Con1, Rat Genome Database id 8663453) was generated from the recombinant chromosome that originated in rat F2-3766, and the BN.ACI-(D14Uwm1-D14Uwm5) congeneric strain (Con2, Rat Genome Database id 8663455) was generated from the chromosome that originated in rat F2-3840 using a selective breeding protocol adapted from that published previously [82–84]. The lineage carrying the recombinant chromosome from rat F2-3892 was lost during progeny testing, preventing development of a congeneric strain that harbored that chromosome. Male [(BNxACI)F1 x BN] rats generated during progeny testing and known to harbor recombinant chromosomes capable of conferring URA were mated to female BN rats. The male N3 progeny from these matings were genotyped to identify those that were heterozygous across the Renag1 region, the heterozygous males were backcrossed to BN females, and this process was repeated through multiple rounds of backcrossing. Rigorous negative selection to eliminate ACI alleles at markers on autosomes other than RNO14 was initiated at the N5 generation. The markers used for positive and
negative selection during backcrossing are listed in Table D in S1 File. N₆ progeny were evaluated in order to confirm inheritance of the propensity to exhibit urogenital anomalies together with ACI alleles at Renag1. After 7 generations of backcrossing for Con1 and 6 generations of backcrossing for Con2, a female rat from each strain harboring ACI alleles across Renag1 was mated to a male BN rat to generate male progeny that were heterozygous at Renag1 and carried the Y chromosome from the BN strain. Male rats from these matings were used in subsequent rounds of backcrossing to BN females. N₉ siblings harboring the same recombinant chromosome were mated to generate founders for each congenic strain that were homozygous for ACI alleles at Renag1 and homozygous for BN alleles at all background markers. Con1 and Con2 rats were generally evaluated for urogenital anomalies at approximately 21 days of age. Breeders from each congenic strain were evaluated upon retirement.

Evaluation of linkage of Renag1 and Hooded to RNO14 in reciprocal intercrosses between ACI and COP rats

Data on urogenital anomalies and coat color were collected as secondary phenotypes in previously described reciprocal intercrosses between ACI and COP rats that were performed to identify genetic determinants of susceptibility to estrogen-induced mammary and pituitary tumors [44–46]. These data were subjected to interval mapping and fine mapping analyses as described previously [40,44–46].

Sequence evaluation of Renag1 locus

Sequencing of the Kit candidate gene from ACI, BN, COP and F344 rats was performed using PCR amplified complementary DNA and/or genomic DNA as templates, standard Sanger sequencing methodologies, and an ABI 3730 capillary sequencing instrument. Sequence comparisons for the Renag1 interval from the same four rat strains were performed using whole genome sequence data from multiple sources available through the Rat Genome Database [85,86].

Evaluation of site of ERV insertion in ACI, BN, COP, Con1 and Con2 rats

The sequences of the PCR primers used to amplify across the site of the ERV in Kit intron 1 were the same as those described by Kuramoto et al. and are presented in Table E in S1 File [43]. PCR products were analyzed on 1.2% agarose gels run in Tris acetate EDTA buffer. For sequencing, the PCR products were extracted from the gel using Qiagen QIAquick gel extraction reagents. Sequencing was performed in the University of Wisconsin Biotechnology Center on an ABI 3730XL capillary sequencer. The primers used for sequencing were the same as used for PCR amplification of genomic DNA template, plus additional nested primers designed to achieve full coverage of insertion junctions (Table E in S1 File).

Evaluation of Pax2, Kit and Kitlg expression by in situ hybridization

Embryos were collected from ACI rats on day e11.5. Tissue collection, storage, and in situ hybridization (ISH) were performed as described previously [87,88]. The primers used to generate PCR-amplified probe templates from whole embryo rat cDNA were designed using Primer3 [89]. Primer sequences are presented in Table F in S1 File. A T7 RNA polymerase recognition sequence was incorporated onto the reverse primer for use in generating labeled RNA probes. The Primer Blast Program was used to ensure specificity of PCR primers for the target sequence [90]. Selected primer sequences uniquely match the target sequence and no other sequence in the rat reference genome. We used the MegaBLAST program to ensure specificity of
the riboprobe sequence [91]. The riboprobe sequence was considered specific for its target when, using an EXPECT threshold of 0.01 and a word size of 128, it did not align with other members of the rat RefSeq RNA database. Embryos were processed together in a single tube for ISH and color development to allow for qualitative comparisons among them. BM Purple was used as alkaline phosphatase chromagen for digoxigenin- and fluorescein labeled riboprobe detection. The staining pattern for each riboprobe was assessed in at least seven litter-independent embryos. Embryos were stained intact. For some embryos, the caudal portion was dissected after staining and positioned for optimal imaging. Some whole-mount ISH stained samples were post-fixed in paraformaldehyde, embedded in JB-4 plus methacrylate polymer (Electron Microscopy Sciences, Hatfield, PA) and sectioned to a thickness of 5 μm with a rotary microtome. The sections were mounted on glass slides and counterstained with nuclear fast red.

Statistical analyses of data

All categorical data were evaluated using Fisher’s exact test in R [92]. \( p \) values \( \leq 0.05 \) were considered statistically significant.

Supporting Information

S1 File. Contains supporting information Tables A-F. Table A, Renag1 Variants. Genetic variants within the Renag1 minimal interval. Table B, Kit GenBank. GenBank accession numbers for Kit sequences. Table C, Uwm Markers. Additional polymorphic microsatellite markers developed for genotyping within the Renag1 region of RNO14. Table D, Congenic Markers. Markers used for positive and negative selection during generation of Renag1 congenic strains. Table E, ERV Primers. Sequences of primers used to amplify and/or sequence across the site of the ERV insertion in Kit intron 1. Table F, ISH Oligos. Primers used to generate PCR-amplified probe templates for in situ hybridization.

(XLSX)

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Author Contributions

Conceived and designed the experiments: JDS RS CMV. Performed the experiments: NBS TWC KLD QEH SGK CML KLW MB IJN VG EC NH RS CMV JDS. Analyzed the data: NBS TWC KLD QEH SGK CML KLW RS CMV JDS. Wrote the paper: JDS RS CMV.

References

1. Sanna-Cherchi S, Caridi G, Weng PL, Scolari F, Perfumo F, et al. (2007) Genetic approaches to human renal agenesis/hypoplasia and dysplasia. Pediatr Nephrol 22: 1675–1684. PMID: 17437132
2. Kerecuk L, Schreuder MF, Woolf AS (2008) Renal tract malformations: perspectives for nephrologists. Nat Clin Pract Nephrol 4: 312–325. doi: 10.1038/ncpneph0807 PMID: 18446149
3. Renkema KY, Winyard PJ, Skovorodkin IN, Levchenko E, Hindryckx A, et al. (2011) Novel perspectives for investigating congenital anomalies of the kidney and urinary tract (CAKUT). Nephrol Dial Transplant 26: 3848–3851. doi: 10.1093/ndt/gfr655 PMID: 22121240

4. Hwang DY, Dworschak GC, Kohl S, Saisawat P, Vivante A, et al. (2014) Mutations in 12 known dominant disease-causing genes clarify many congenital anomalies of the kidney and urinary tract. Kidney Int 85: 1429–1433. doi: 10.1038/ki.2013.508 PMID: 24429398

5. Sanna-Cherchi S, Ravani P, Corbani V, Parodi S, Haupt R, et al. (2009) Renal outcome in patients with congenital anomalies of the kidney and urinary tract. Kidney Int 76: 528–533. doi: 10.1038/ki.2009.220 PMID: 19536081

6. Sanna-Cherchi S, Sampogna RV, Papeta N, Burgess KE, Nees SN, et al. (2013) Mutations in DSTYK and dominant urinary tract malformations. N Engl J Med 369: 621–629. doi: 10.1056/NEJMoa1214479 PMID: 23862974

7. Pohl M, Bhatnagar V, Mendoza SA, Nigam SK (2002) Toward an etiological classification of developmental disorders of the kidney and upper urinary tract. Kidney Int 61: 10–19. PMID: 11786080

8. Weber S, Moriniere V, Knuppel T, Charbit M, Dusek J, et al. (2006) Prevalence of mutations in renal developmental genes in children with renal hypodysplasia: results of the ESCAPE study. J Am Soc Nephrol 17: 2864–2870. PMID: 16971658

9. Saisawat P, Tasic V, Vega-Warmer V, Kehinde EO, Gunther B, et al. (2012) Identification of two novel CAKUT-causing genes by massively parallel exon resequencing of candidate genes in patients with unilateral renal agenesis. Kidney Int 81: 196–200. doi: 10.1038/ki.2013.315 PMID: 21900877

10. Feather SA, Malcolm S, Woolf AS, Wright V, Blaydon D, et al. (2000) Primary, nonsyndromic vesicoureteric reflux emphasizes high genetic heterogeneity and shows linkage and association with various genes already implicated in urinary tract development. Mol Genet Genomic Med 2: 7–29. doi: 10.1002/mgg3.22 PMID: 24498626

11. Conte ML, Bertoli-Avella AM, de Graaf BM, Punzo F, Lama G, et al. (2008) A genome search for primary vesicoureteral reflux and its nephropathy is genetically heterogeneous, with a locus on chromosome 1. Am J Hum Genet 66: 1420–1425. PMID: 19739767

12. Weng PL, Sanna-Cherchi S, Hensle T, Shapiro E, Werzberger A, et al. (2009) A recessive gene for primary vesicoureteral reflux maps to chromosome 12p11-q13. J Am Soc Nephrol 20: 1633–1640. doi: 10.1681/ASN.2008111199 PMID: 19443636

13. Ashraf S, Hoskins BE, Chaib H, Hoefele J, Pasch A, et al. (2010) Mapping of a new locus for congenital abnormalities of the kidney and urinary tract on chromosome 8q24. Nephrol Dial Transplant 25: 1496–1501. doi: 10.1093/ndt/gfp650 PMID: 20007758

14. Briggs CE, Guo CY, Schoettler C, Rosoklija I, Silva A, et al. (2010) A genome scan in affected sib-pairs with familial vesicoureteral reflux identifies a locus on chromosome 5. Eur J Hum Genet 18: 245–250. doi: 10.1038/ejhg.2009.142 PMID: 19690587

15. Marchini GS, Onal B, Guo CY, Rowe CK, Kunkel L, et al. (2012) Genome gender diversity in affected sib-pairs with familial vesico-ureteric reflux identified by single nucleotide polymorphism linkage analysis. BJU Int 109: 1709–1714. doi: 10.1111/j.1464-410X.2011.10634.x PMID: 21981614

16. Darlow JM, Dobson MG, Darlay R, Molony CM, Hunziker M, et al. (2014) A new genome scan for primary nonsyndromic vesico-ureteric reflux emphasises high genetic heterogeneity and shows linkage and association with various genes already implicated in urinatary tract development. Mol Genet Genomic Med 2: 7–29. doi: 10.1002/mgg3.22 PMID: 24498626

17. Schimke RN, King CR (1980) Hereditary urogenital adysplasia. Clin Genet 18: 417–420. PMID: 7449179

18. Arfeen S, Rosborough D, Luger AM, Nolph KD (1993) Familial unilateral renal agenesis and focal and segmental glomerulosclerosis. Am J Kidney Dis 21: 663–668. PMID: 8503422

19. Doray B, Bass B, Reinartz I, Stoll C (1999) Hereditary renal adenysplasia in a three generations family. Genet Couns 10: 251–257. PMID: 10546096

20. Schwaderer AL, Bates CM, McHugh KM, McBride KL (2007) Renal anomalies in family members of infants with bilateral renal agenesis/adysplasia. Pediatr Nephrol 22: 52–56. PMID: 16977473
25. Carter CO, Evans K, Pescia G (1979) A family study of renal agenesis. J Med Genet 16: 176–188. PMID: 469895

26. Roodhooft AM, Birnholz JC, Holmes LB (1984) Familial nature of congenital absence and severe dysgenesis of both kidneys. N Engl J Med 310: 1341–1345. PMID: 6717505

27. Hostetter TH, Olson JL, Renneke HG, Venkatachalam MA, Brenner BM (1981) Hyperfiltration in remnant nephrons: a potentially adverse response to renal ablation. Am J Physiol 241: F85–93. PMID: 7246778

28. Westland R, van Wijk JA, Schreuder MF (2012) The reason why mother nature provided us with two kidneys: the risks of a congenital solitary functioning kidney. Nephrol Dial Transplant 27: 2603–2604. doi:10.1093/ndt/gfr625 PMID: 22058171

29. Westland R, Kurvers RA, van Wijk JA, Schreuder MF (2013) Risk factors for renal injury in children with a solitary functioning kidney. Pediatrics 131: e478–485. doi:10.1542/peds.2012-2088 PMID: 23319536

30. Wang X, Johnson AC, Williams JM, White T, Chade AR, et al. (2014) Nephron Deficiency and Predisposition to Renal Injury in a Novel One-Kidney Genetic Model. J Am Soc Nephrol.

31. Morgan WC (1953) Inherited congenital kidney absence in an inbred strain of rats. Anat Rec 115: 635–639. PMID: 13040799

32. Deringer MK, Heston WE (1956) Abnormalities of urogenital system in strain A x C line 9935 rats. Proc Soc Exp Biol Med 91: 312–314. PMID: 13297786

33. Fujikura T (1970) Kidney malformations in fetuses of A x C line 9935 rats. Teratology 3: 245–249. PMID: 5471178

34. Cramer DV, Gill TJ 3rd (1975) Genetics of urogenital abnormalities in ACI inbred rats. Teratology 12: 27–32. PMID: 1162622

35. Marshall FF, Garcia-Bunuel R, Beisel DS (1978) Hydronephrosis, renal agenesis, and associated genitourinary anomalies in ACI rats. Urology 11: 58–61. PMID: 622762

36. Fujita K, Fujita HM, Ohtawara Y, Suzuki K, Tajima A, et al. (1979) Hydronephrosis in ACI/N rats. Lab Anim 13: 325–327. PMID: 529770

37. Marshall FF, Ewing LL, Zirkin BR, Cochran RC (1982) Testicular atrophy associated with agenesis of the epididymis in the ACI rat. J Urol 127: 155–158. PMID: 7057491

38. Solleveld HA, Boorman GA (1986) Spontaneous renal lesions in five rat strains. Toxicol Pathol 14: 168–174. PMID: 3764314

39. Lachel CM, Pennington KL, Murrin CR, Strecker TE, Shull JD (2003) Genetic characterization of renal agenesis in the ACI rat: mapping of Renag1 to RNO14. Abstracts, Cold Spring Harbor Laboratory Meeting on Rat Genomics and Models: 83.

40. Shull JD, Lachel CM, Strecker TE, Spady TJ, Tochacek M, et al. (2006) Genetic bases of renal agenesis in the ACI rat: mapping of Renag1 to chromosome 14. Mamm Genome 17: 751–759. PMID: 16845468

41. Serikawa T, Kuramoto T, Hilbert P, Mori M, Yamada J, et al. (1992) Rat gene mapping using PCR-analyzed microsatellites. Genetics 131: 701–721. PMID: 1628813

42. Torigoe D, Ichii O, Dang R, Ohashi T, Okano S, et al. (2011) High-resolution linkage mapping of the rat hooded locus. J Vet Med Sci 73: 707–710. PMID: 21224527

43. Kuramoto T, Nakashishi S, Ochiai M, Nakagama H, Voigt B, et al. (2012) Origins of albino and hooded rats: implications from molecular genetic analysis across modern laboratory rat strains. PLoS One 7: e43059. doi:10.1371/journal.pone.0043059 PMID: 22916206

44. Spady TJ, Pennington KL, McComb RD, Shull JD (1999) Genetic bases of estrogen-induced pituitary growth in an intercross between the ACI and Copenhagen rat strains: dominant mendelian inheritance of the ACI phenotype. Endocrinology 140: 2828–2835. PMID: 10342874

45. Gould KA, Tochacek M, Schaffer BS, Reindl TM, Murrin CR, et al. (2004) Genetic determination of susceptibility to estrogen-induced mammary cancer in the ACI rat: mapping of Emca1 and Emca2 to chromosomes 5 and 18. Genetics 168: 2113–2125. PMID: 15611180

46. Strecker TE, Spady TJ, Schaffer BS, Gould KA, Kaufman AE, et al. (2005) Genetic bases of estrogen-induced pituitary tumorigenesis: identification of genetic loci determining estrogen-induced pituitary growth in reciprocal crosses between the ACI and Copenhagen rat strains. Genetics 169: 2189–2197. PMID: 15687265

47. Yamashita S, Suzuki S, Nomoto T, Kondo Y, Wakazono K, et al. (2005) Linkage and microarray analyses of susceptibility genes in ACI/Seg rats: a model for prostate cancers in the aged. Cancer Res 65: 2610–2616. PMID: 15805257
48. Chia I, Grote D, Marcotte M, Batourina E, Mendelsohn C, et al. (2011) Nephric duct insertion is a crucial step in urinary tract maturation that is regulated by a Gata3-Raldh2-Ret molecular network in mice. Development 138: 2089–2097. doi:10.1242/dev.056838 PMID: 21521737

49. Soofi A, Levitan I, Dressler GR (2012) Two novel EGFP insertion alleles reveal unique aspects of Pax2 function in embryonic and adult kidneys. Dev Biol 365: 241–250. doi:10.1016/j.ydbio.2012.02.032 PMID: 22410172

50. Manova K, Bachvarova RF (1991) Expression of c-kit encoded at the W locus in developing embryonic germ cells and presumptive melanoblasts. Dev Biol 146: 312–324. PMID: 1713863

51. Bernex F, De Sepulveda P, Kress C, Elbaz C, Delouis C, et al. (1996) Spatial and temporal patterns of c-kit-expressing cells in WlacZ/+ and WlacZ/WlacZ mouse embryos. Development 122: 3023–3033. PMID: 8898216

52. Lennartsson J, Ronnstrand L (2012) Stem cell factor receptor/c-Kit: from basic science to clinical implications. Physiol Rev 92: 1619–1649. doi:10.1152/physrev.00046.2011 PMID: 23073628

53. Mackie GG, Stephens FD (1975) Duplex kidneys: a correlation of renal dysplasia with position of the ureteral orifice. J Urol 114: 274–280. PMID: 1171997

54. Mendelsohn C, Lohnes D, Decimo D, Lufkin T, LeMeur M, et al. (1994) Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development 120: 2749–2771. PMID: 7607068

55. Batourina E, Gim S, Bello N, Shy M, Clagett-Dame M, et al. (2001) Vitamin A controls epithelial/mesenchymal interactions through Ret expression. Nat Genet 27: 74–78. PMID: 11138002

56. Ichikawa I, Kuwayama F, Pope JCt, Stephens FD, Miyazaki Y (2002) Paradigm shift from classic anatomic theories to contemporary cell biological views of CAKUT. Kidney Int 61: 889–898. PMID: 11849443

57. Mendelsohn C (2009) Using mouse models to understand normal and abnormal urogenital tract development. Organogenesis 5: 306–314. PMID: 19568352

58. Yamamoto Y, Nishimoto H, Ikemoto S (1989) Interstitial deletion of the proximal long arm of chromosome 4 associated with father-child incompatibility within the Gc-system: probable reduced gene dosage effect and partial piebald trait. Am J Med Genet 32: 520–523. PMID: 2773996

59. Spritz RA, Droetto S, Fukushima Y (1992) Deletion of the KIT and PDGFRA genes in a patient with piebaldism. Am J Med Genet 44: 492–495. PMID: 1279971

60. Pye SM, Cortes J, Ault P, Hatfield A, Kantarjian H, et al. (2008) The effects of imatinib on pregnancy outcome. Blood 111: 5505–5508. doi:10.1182/blood-2007-10-114900 PMID: 18322153

61. Niwa Y, Kasugai T, Ohno K, Morimoto M, Yamazaki M, et al. (1991) Anemia and mast cell depletion in mutant rats that are homozygous at “white spotting (Ws)” locus. Blood 78: 1942–1946. PMID: 1912576

62. Tsujimura T, Hirota S, Nomura S, Niwa Y, Yamazaki M, et al. (1991) Characterization of Ws mutant allele of rats: a 12-base deletion in tyrosine kinase domain of c-kit gene. Blood 78: 1936–1941. PMID: 1912577

63. Schmidt-Ott KM, Chen X, Paragas N, Levinson RS, Mendelsohn CL, et al. (2006) c-kit delineates a distinct domain of progenitors in the developing kidney. Dev Biol 299: 238–249. PMID: 16942767

64. Cohen CJ, Lock WM, Mager DL (2009) Endogenous retroviral LTRs as promoters for human genes: a critical assessment. Gene 448: 105–114. doi:10.1016/j.gene.2009.06.020 PMID: 19577618

65. Lawrence MG, Stephens CR, Need EF, Lai J, Buchanan G, et al. (2012) Long terminal repeats act as androgen-responsive enhancers for the PSA-kallikrein locus. Endocrinology 153: 3199–3210. doi: 10.1210/en.2012-1267 PMID: 22597536

66. Bowes C, Li T, Frankel WN, Danciger M, Coffin JM, et al. (1993) Localization of a retroviral element within the rd gene coding for the beta subunit of cGMP phosphodiesterase. Proc Natl Acad Sci U S A 90: 2959–2959. PMID: 8385352

67. Bultman SJ, Klebig ML, Michaud EJ, Sweet HO, Davission MT, et al. (1994) Molecular analysis of reverse mutations from nonagouti (a) to black-and-tan (a(t)) and white-bellied agouti (Aw) reveals alternative forms of agouti transcripts. Genes Dev 8: 481–490. PMID: 8125260

68. Wang Y, Liska F, Gosele C, Sedova L, Kren V, et al. (2010) A novel active endogenous retrovirus family contributes to genome variability in rat inbred strains. Genome Res 20: 19–27. doi: 10.1101/gr.100073.109 PMID: 19887576

69. Bellone RR, Holl H, Setaluri V, Devi S, Maddodi N, et al. (2013) Evidence for a retroviral insertion in TRPM1 as the cause of congenital stationary night blindness and leopard complex spotting in the horse. PLoS One 8: e78280. doi: 10.1371/journal.pone.0078280 PMID: 24167615
70. Solberg Woods LC, Stelloh C, Regner KR, Schwabe T, Eisenhauer J, et al. (2010) Heterogeneous stock rats: a new model to study the genetics of renal phenotypes. Am J Physiol Renal Physiol 298: F1484–1491. doi: 10.1152/ajprenal.00002.2010 PMID: 20219828

71. Rat Genome Sequencing and Mapping Consortium, Baud A, Hermens R, Guryev V, et al. (2013) Combined sequence-based and genetic mapping analysis of complex traits in outbred rats. Nat Genet 45: 767–775. doi: 10.1038/ng.23708188

72. Arakas K, Suzuki K, Suzuki H (2009) The unilateral urogenital anomalies (UUA) rat: a new mutant strain associated with unilateral renal agenesis, cryptorchidism, and malformations of reproductive organs restricted to the left side. Comp Med 59: 249–256. PMID: 19619415

73. Gholoum S, Puligandla PS, Hui T, Su W, Quiros E, et al. (2006) Management and outcome of patients with combined vaginal septum, bifid uterus, and ipsilateral renal agenesis (Herlyn-Werner-Wunderlich syndrome). J Pediatr Surg 41: 987–992. PMID: 16677898

74. Vercellini P, Daguati R, Somigliana E, Vigano P, Lanzani A, et al. (2007) Asymmetric lateral distribution of obstructed hemivagina and renal agenesis in women with uterus didelphys: institutional case series and a systematic literature review. Fertil Steril 87: 719–724. PMID: 17430731

75. Acien P, Acien M, Sanchez-Ferrer ML (2009) Mullerian anomalies "without a classification": from the didelphys-unicollis uterus to the bicervical uterus with or without septate vagina. Fertil Steril 91: 2369–2375. doi: 10.1016/j.fertnstert.2008.01.079 PMID: 18367185

76. Acien P, Acien M (2010) Unilateral renal agenesis and female genital tract pathologies. Acta Obstet Gynecol Scand 89: 1424–1431. doi:10.3109/00016349.2010.512067 PMID: 20799917

77. Aveiro AC, Miranda V, Cabral AJ, Nunes S, Paulo F, et al. (2011) The unilateral urogenital anomalies (UUA) rat: a new mutant strain associated with unilateral renal agenesis, cryptorchidism, and malformations of reproductive organs restricted to the left side. Comp Med 59: 249–256. PMID: 19619415

78. Gholoum S, Puligandla PS, Hui T, Su W, Quiros E, et al. (2006) Management and outcome of patients with combined vaginal septum, bifid uterus, and ipsilateral renal agenesis (Herlyn-Werner-Wunderlich syndrome). J Pediatr Surg 41: 987–992. PMID: 16677898

79. Vercellini P, Daguati R, Somigliana E, Vigano P, Lanzani A, et al. (2007) Asymmetric lateral distribution of obstructed hemivagina and renal agenesis in women with uterus didelphys: institutional case series and a systematic literature review. Fertil Steril 87: 719–724. PMID: 17430731

80. Augarten A, Yahav Y, Kerem BS, Halle D, Laufer J, et al. (1994) Congenital bilateral absence of vas deferens in the absence of cystic fibrosis. Lancet 344: 1473–1474. PMID: 7968122

81. Kolettis PN, Sandlow JI (2002) Clinical and genetic features of patients with congenital unilateral absence of the vas deferens. Urology 60: 1073–1076. PMID: 12475673

82. Schaffer BS, Lachel CM, Pennington KL, Murrin CR, Strecker TE, et al. (2006) Genetic bases of estrogen-induced tumorigenesis in the rat: mapping of loci controlling susceptibility to mammary cancer in a Brown Norway x ACI intercross. Cancer Res 66: 7793–7800. PMID: 16885383

83. Schaffer BS, Leland-Wavrin KM, Kurz SG, Colletti JA, Seiler NL, et al. (2013) Mapping of three genetic determinants of susceptibility to estrogen-induced mammary cancer within the Emca8 locus on rat chromosome 5. Cancer Prev Res (Phila) 6: 59–69. doi: 10.1158/1940-6207.CAPR-12-0346-T PMID: 23151807

84. Kurz SG, Dennison KL, Samanas NB, Hickman MP, Eckert QA, et al. (2014) Ept7 influences estrogen action in the pituitary gland and body weight of rats. Mamm Genome 25: 244–252. doi: 10.1007/s00335-014-9504-4 PMID: 24484715

85. Colletti JA 2nd, Leland-Wavrin KM, Kurz SG, Hickman MP, Seiler NL, et al. (2014) Validation of six genetic determinants of susceptibility to estrogen-induced mammary cancer in the rat and assessment of their relevance to breast cancer risk in humans. G3 (Bethesda) 4: 1385–1394. doi: 10.1534/g3.114.011163 PMID: 24875630

86. Laulederkind SJ, Hayman GT, Wang SJ, Smith JR, Lowry TF, et al. (2013) The Rat Genome Database 2013—data, tools and users. Brief Bioinform 14: 520–526. doi: 10.1093/bib/bbt007 PMID: 23434633

87. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 365–386. PMID: 10547847
90. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, et al. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13: 134. doi: 10.1186/1471-2105-13-134 PMID: 22708584

91. Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7: 203–214. PMID: 10890397

92. R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available: http://www.R-project.org/. doi: 10.1002/cpp.1875 PMID: 25632406