Mutation of Rubie, a Novel Long Non-Coding RNA Located Upstream of Bmp4, Causes Vestibular Malformation in Mice

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Abstract

Background: The vestibular apparatus of the vertebrate inner ear uses three fluid-filled semicircular canals to sense angular acceleration of the head. Malformation of these canals disrupts the sense of balance and frequently causes circling behavior in mice. The Epistatic circler (Ecl) is a complex mutant derived from wildtype SWR/J and C57L/J mice. Ecl circling has been shown to result from the epistatic interaction of an SWR-derived locus on chromosome 14 and a C57L-derived locus on chromosome 4, but the causative genes have not been previously identified.

Methodology/Principal Findings: We developed a mouse chromosome substitution strain (CSS-14) that carries an SWR/J chromosome 14 on a C57BL/10J genetic background and, like Ecl, exhibits circling behavior due to lateral semicircular canal malformation. We utilized CSS-14 to identify the chromosome 14 Ecl gene by positional cloning. Our candidate interval is located upstream of bone morphogenetic protein 4 (Bmp4) and contains an inner ear-specific, long non-coding RNA that we have designated Rubie (RNA upstream of Bmp4 expressed in inner ear). Rubie is spliced and polyadenylated, and is expressed in developing semicircular canals. However, we discovered that the SWR/J allele of Rubie is disrupted by an intronic endogenous retrovirus that causes aberrant splicing and premature polyadenylation of the transcript. Rubie lies in the conserved gene desert upstream of Bmp4, within a region previously shown to be important for inner ear expression of Bmp4. We found that the expression patterns of Bmp4 and Rubie are nearly identical in developing inner ears.

Conclusions/Significance: Based on these results and previous studies showing that Bmp4 is essential for proper vestibular development, we propose that Rubie is the gene mutated in Ecl mice, that it is involved in regulating inner ear expression of Bmp4, and that aberrant Bmp4 expression contributes to the Ecl phenotype.

Introduction

The vestibular apparatus of the vertebrate inner ear is an intricate structure responsible for sensing motion and position of the head. Within the vestibular system, angular head movements are detected by three fluid-filled semicircular canals oriented in nearly orthogonal planes. Fluid movement is generated by changes in head position and detected by complex sensory structures, known as cristae ampullares, located at the base of each canal. This precise inner ear architecture is crucial for proper vestibular function and even small structural changes can profoundly impact the ability to detect and transmit sensory information. Vestibular abnormalities can lead to debilitating dizziness and imbalance in humans [1,2] and frequently cause circling behavior in mice [3,4]. Inner ear morphogenesis is a highly regulated process that depends upon precise spatiotemporal patterns of gene expression to generate three-dimensional structures [5,6]. Identifying these genes and elucidating their functions is key to better understanding this intricate process, and mouse models of vestibular dysfunction have proven remarkably useful in this regard [3,4].

The Epistatic circler (Ecl) is a complex mouse mutant originally discovered in a multi-generation intercross population derived from wildtype SWR/J and C57L/J mice [7]. In Ecl mice, circling and hyperactivity result from bilateral malformation of the lateral semicircular canal (LSC), while the anterior and posterior canals are unaffected [8]. Circling is caused by the epistatic interaction of a recessive, SWR-derived gene on chromosome 14 and a dominant, C57L-derived gene on chromosome 4. Additional C57L alleles at modifier loci on chromosomes 3 and 13 increase circling risk [9]. While the broad chromosomal locations of these four Ecl loci have been defined, none of the underlying genes have yet been identified.

Here we describe the development of a mouse chromosome substitution strain that genetically resembles Ecl, and exhibits similar behavioral and structural abnormalities. We utilized this new mouse strain to identify a likely causative gene at the
chromosome 14 Ecl locus by positional cloning. Our candidate interval lies upstream of bone morphogenetic protein 4 (Bmp4), a gene known to be essential for proper vestibular development [10,11,12,13,14]. While Bmp4 is excluded as an Ecl candidate, we discovered a long, non-coding RNA (ncRNA) within our interval that is co-expressed with Bmp4 in developing semicircular canals. We demonstrate that expression of this novel ncRNA is disrupted by an SWR-specific endogenous retrovirus and propose a role for it in regulating Bmp4 expression.

Results

Generation of a Chromosome Substitution Strain that Exhibits Circling Behavior

We developed a mouse chromosome substitution strain carrying an SWR-J (SWR) chromosome 14 on a C57BL/10J (B10) genetic background (C57BL/10J-Chr14SWR-J, referred to here as CSS-14). While heterosomic CSS-14 mice (Chr14B10/SWR on a B10/ B10 background) showed no behavioral abnormalities, a subset of their intercross progeny displayed hyperactivity and bidirectional circling that was evident before weaning and persisted throughout life. We recognized that the strain combination of CSS-14 resembled that of Ecl, and hypothesized that the same epistatic interaction was responsible for circling in both mutants. All CSS-14 mice are homozygous for C57-derived alleles at chromosomes 3, 4, and 13, and genotyping of intercross progeny confirmed that CSS-14 circlers always inherit two SWR alleles at the recessive Ecl locus on chromosome 14 (Chr14SWR/SWR). This inheritance pattern strongly suggests that we unintentionally recapitulated Ecl in the construction of CSS-14. As previously hypothesized for Ecl, CSS-14 circling is not fully penetrant [7,9]; we observed circling in 54% of Chr14SWR/SWR mice.

CSS-14 Circlers Exhibit Malformation of the Lateral Semicircular Canal

Circling behavior can be caused by a wide range of vestibular abnormalities. In Ecl mice, circling results from bilateral malformation of the LSC, with defects including complete truncation, severe distortion, and narrowing of the canals [8]. To determine whether CSS-14 circlers exhibit similar structural defects, we visualized inner ears from Chr14B10/SWR and Chr14SWR/SWR pups at postnatal day 0 (P0) using a standard paintfill technique. Chr14B10/SWR inner ears displayed normal morphology with intact LSCs (Figure 1A). In contrast, truncation or severe distortion of the LSC was observed in six of twelve Chr14SWR/SWR ears (Figure 1B–D); the anterior and posterior semicircular canals, as well as the cochlea, appeared normal in all Chr14SWR/SWR ears examined. These results confirm that CSS-14 and Ecl circling are caused by the same type of structural malformation and further support the conclusion that both mouse models result from the same genetic defect.

Semicircular canals develop from two epithelial outpocketings of the otic vesicle. Anterior and posterior canals develop from a single vertical pouch and the LSC develops from a horizontal pouch. Following fusion and resorption of epithelial tissue at the center of the pouch, a tube-shaped canal remains along its perimeter [15]. Analyzing pouch morphology during morphogenesis can provide important information about the etiology of postnatally-observed vestibular malformation. Because Ecl inner ears had not previously been studied during embryogenesis, we used paintfilling to visualize canal pouches of CSS-14 embryos at E12.25. Chr14B10/SWR embryos exhibited normal lateral pouches (Figure 1E), while those of Chr14SWR/SWR embryos frequently lacked the distal rim necessary for proper LSC formation (Figure 1F). This observation is consistent with defects in either pouch outgrowth or fusion [11,16].

Positional Cloning of the Chromosome 14 Ecl Gene

Next, we utilized CSS-14 mice to identify the chromosome 14 Ecl gene by positional cloning. Offspring of (Chr14SWR/SWR × Chr14B10/SWR) matings were phenotyped as circling or non-circling and genotyped at markers across chromosome 14. The resulting candidate gene interval, defined as the region of SWR homozygosity common to all CSS-14 circlers, spanned 445 Kb from D14Kar16 to D14Kar40 (Figure 2). This region, extending from 135 Kb to 600 Kb upstream of (telomeric to) Bmp4, contains five annotated genes (Cdkn3, Csth, Gmfb, Cgfr1, Samd4) and genes for four spliced ESTs (Figure 2). None of the annotated genes in this interval are known to be involved in inner ear development.

Importantly, the coding sequence of Bmp4 was excluded from our candidate interval by two independent crossovers (Figure 2). Prior to genetic exclusion, Bmp4 was a strong Ecl candidate because it is expressed in developing semicircular canals and known to play a crucial role in inner ear development [10,11,12,13,14]. In mice, homozygous deletion of Bmp4 in the inner ear results in agenesis of all three semicircular canals [10], while heterozygous deletion causes LSC defects indistinguishable from those of CSS-14 circlers [10,11]. With Bmp4 excluded as a candidate, we examined the genes and spliced ESTs located within our interval more closely.

Rubie is a Long, Non-Coding RNA Expressed in Developing Semicircular Canals

One of the four ESTs in our candidate interval, BY232928, was originally identified in a RIKEN inner ear cDNA library and is not expressed in other tissues (data not shown). BY232928 lies upstream of Bmp4, within a genomic region previously shown to be important for Bmp4 expression in the developing inner ear [17]. While genomic databases display 388 bp divided among three exons, our resequencing of the BY232928 RIKEN clone revealed a 1404 bp cDNA insert comprised of five exons (Figure 3A; Table S1). We refer to the gene encoding this EST and its RNA product as Rubie (RNA upstream of Bmp4 expressed in inner ear). We confirmed expression of Rubie in embryonic (E15.5) and postnatal (P6) inner ears by RT-PCR (Figure 3B) and performed in situ hybridization in sectioned E11.5 embryos (Figure 3C,D). Rubie was detected in the three presumptive sensory cristae that give rise to semicircular canals, and at the tip of the developing cochlea. This expression pattern is strikingly similar to that of Bmp4 (Figure 3E,F) [10,18].

Rubie is a spliced, polyadenylated transcript that we believe functions as an mRNA-like, long ncRNA. Long ncRNAs are abundantly expressed in eukaryotes and recent studies suggest that many are involved in regulation of gene expression and development. However, only a small subset of these have been functionally characterized [19,20,21]. Long ncRNAs lack obvious predictive features and, therefore, are typically defined by their failure to exhibit protein-coding characteristics [22]. They generally contain only short (<100 amino acids) open reading frames (ORFs) and are less likely than protein-coding RNAs to be conserved across species or have significant homology to known proteins [22,23]. Rubie has ten potential ORFs, the longest of which begins at the fifth ATG and could encode 120 amino acids (Figure 4A), but similar to all preceding ATGs, lacks consensus features for translational initiation. Furthermore, this ORF is only present in mouse strains of the C57 lineage; it is disrupted by a termination codon in other strains. Rubie exons are poorly
conserved across species (Figure 4B) and BLAST analysis revealed no significant homology to known proteins. We were unable to detect a protein product by in vitro transcription/translation (not shown). An SWR-Specific Endogenous Retrovirus Disrupts Rubie Splicing and Expression

While C57-lineage mice are frequently crossed to other inbred strains, Ecl has never been recapitulated by crosses that do not involve SWR. Therefore, we reasoned that the SWR allele of the causative chromosome 14 gene would likely be unique among inbred strains. We sequenced Rubie in SWR, B10, and thirteen additional mouse strains. Twelve single nucleotide polymorphisms distinguished SWR and B10, but none were unique to SWR (Figure S1). Sequencing of Rubie introns in four inbred strains (B10, SWR, 129S6/SvEvTac, and FVB/NJ) identified numerous polymorphisms, but again none were SWR-specific. However, we were consistently unable to amplify a portion of intron 1 from SWR genomic DNA, despite robust amplification in other strains (Figure S2). Using long-range PCR, we identified a large insertion in the SWR allele of Rubie (Figure 5A,B) attributable to a 5542 bp ETnII-β endogenous retrovirus (ERV) [24] (Figure S2). Analysis of additional inbred strains indicated that this insertion is found only in SWR (Figure 5A); even the closely-related FVB/NJ strain does not carry this ERV insertion in Rubie.

Intronic ETnII-β insertions have been previously shown to disrupt transcript processing in two ways: by aberrantly splicing ERV sequence into the transcript, or by coupling aberrant splicing with premature polyadenylation within the ERV [25,26]. These effects have generally been shown to occur when the ERV is inserted in the sense orientation with respect to the affected gene, as is the case with Rubie. To determine whether Rubie processing is affected by this intronic SWR insertion, we isolated RNA from inner ears of B10, SWR and CSS-14 mice at postnatal day 6 (P6). PCR amplification spanning exons 1 and 2, 1 and 4, or 2 and 4, reproducibly failed to detect Rubie expression in SWR mice and...
Figure 2. Positional cloning of the chromosome 14 Ecl gene. CSS-14 offspring were phenotyped as circling or non-circling and genotyped at SSLP markers across chromosome 14. Due to incomplete penetrance of the circling phenotype, only circlers were used to narrow the candidate interval. Blue bars depict regions of SWR homozygosity in CSS-14 circlers; each bar represents multiple circling mice with the same genotype. The Ecl candidate gene interval (red bar) was defined as the region of SWR homozygosity common to all CSS-14 circlers. This interval spans 445 Kb from D14Kar16 to D14Kar40 and includes five annotated genes and four spliced ESTs (bottom panel). The coding sequence of Bmp4 was excluded from the candidate interval by two independent crossovers.

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Figure 3. Sequence and expression analysis of Rubie. (A) Rubie (EST BY232928) sequence displayed in genomic databases is derived from a partially-sequenced RIKEN cDNA clone and includes 388 bp, divided among three exons. Resequencing of the RIKEN clone revealed a full-length insert of 1404 bp, divided among five exons. (B) Expression of Rubie in embryonic (E15.5) and postnatal (P6) mouse inner ears was confirmed by RT-PCR. Expression was not observed in P6 hindbrain. The bottom PCR band represents use of an alternative splice site between exons 1 and 2 of Rubie. (C) Rubie expression was detected in the three sensory cristae (anterior crista, AC; lateral crista, LC; posterior crista, not shown) and the developing cochlea (Co) of sectioned E11.5 embryos by in situ hybridization. (D) No signal was detected using a Rubie sense control probe. Rubie (E) and Bmp4 (F) exhibited similar expression patterns in the developing inner ear.

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CSS-14 mice with the Chr7^SWR/ SWR genotype (Figure 5C and not shown). However, splicing of Rubie exon 1 to a previously-characterized ETnII-β splice acceptor [26] was detected in both SWR and CSS-14 (Figure 5D), suggesting that Rubie expression is disrupted by aberrant splicing coupled with premature polyadenylation. Based on these results, we conclude that the ERV insertion in intron 1 of Rubie significantly reduces expression of the SWR allele.

**Discussion**

Based on our findings that Rubie expression is restricted to the inner ear, that it is expressed in the sensory cristae that give rise to the semicircular canals, that it is the only compelling candidate gene in the chromosome 14 Ecl candidate interval and that the insertional mutation is specific to SWR, we conclude that this mutation is likely responsible for the circling phenotype of CSS-14.

Rubie is a long ncRNA expressed in the cristae of the developing inner ear in a pattern identical to Bmp4, the transcript of a gene located less than 200 kb downstream of (centromeric to) Rubie. If its coding sequence had not been excluded through positional cloning, Bmp4 would have been an ideal candidate for the chromosome 14 Ecl gene. The proximity of Rubie and Bmp4 suggests to us that Rubie might be involved in the control of Bmp4 gene expression.

Bmp4, like other BMPs, regulates many developmental processes and displays numerous spatiotemporal-specific expression patterns throughout development. These complex expression patterns are controlled by multiple tissue-specific, cis-regulatory elements, which are often located far from the coding regions and promoters of the genes they regulate [27]. BAC transgenic studies have been used to map tissue-specific regulatory elements surrounding Bmp3, Gdf6, Bmp2, and Bmp4 [17,28,29,30]. While global Bmp4 deletion causes embryonic lethality, it has been suggested that genetic variation within cis-regulatory elements might affect tissue-specific Bmp4 expression and result in tissue- or organ-restricted developmental defects [17].

Distant non-coding regulatory elements frequently reside within conserved gene deserts found adjacent to BMPs and other developmentally-regulated genes [27,31]. The conserved gene desert surrounding Bmp4 spans approximately 1 Mb, with 370 Kb lying upstream of (telomeric to) the Bmp4 transcriptional start site. Chandler and colleagues demonstrated that an unidentified regulatory element important for inner ear expression of Bmp4 lies in this gene desert, between 28 and 199 Kb upstream of the gene [17]. Rubie is an inner ear-specific, long ncRNA in that region. The expression patterns of Rubie and Bmp4 are nearly identical in developing inner ears and the anatomical phenotype of mice haploinsufficient for Bmp4 is indistinguishable from that of Ecl mice and CSS-14 circlers [10,11].

Taken together, our results suggest that Rubie might be involved in the quantitative or temporal regulation of Bmp4 expression in the inner ear. Perturbation of Rubie expression resulting from the SWR mutation might cause either a modest alteration in Bmp4 expression, analogous to Bmp4 heterozygosity, or abnormal timing of Bmp4 expression. Either mechanism could disrupt the most sensitive aspect of vestibular development, the formation of the semicircular canals, that it is the only compelling candidate gene in the chromosome 14 Ecl candidate interval and that the insertional mutation is specific to SWR, we conclude that this mutation is likely responsible for the circling phenotype of CSS-14 circlers [10,11].

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In humans, the gene desert upstream of BMP4 has been shown to express unspliced, but no known spliced ESTs and no Rubie-like RNA has yet been identified. This may simply be an ascertainment issue, as there has been relatively little characterization of human EST expression during early development. Because long ncRNAs are typically not well-conserved across species, it is possible that a human long ncRNA with little sequence homology to Rubie may serve a similar function. Alternatively, tissue-specific regulation of Bmp4 expression may be achieved via different mechanisms in human and mouse.
Incomplete and varies with genetic background [10,11]. Vervoort will be necessary to determine the precise role of within the Rubie regulation (e.g., that a classical DNA enhancer element Bmp4 but remains possible that transcription of SWR ERV insertion does not interrupt these conserved sequences, introns that might contain DNA regulatory elements. The note that there are blocks of highly conserved sequence within that would otherwise inhibit activity of an inner ear enhancer. We Bmp4 facilitate might be necessary to open chromatin, in order to directly expression in the inner ear acts in cis [17].

We do not yet have a model for how Rubie might regulate Bmp4 expression, and previous studies of long ncRNAs offer little insight. Chandler’s BAC transgenic experiment, examining a Bmp4 reporter on a wildtype genetic background that should express full-length Rubie, suggests that the element regulating Bmp4 expression in the inner ears acts in cis [17]. Rubie transcription might be necessary to open chromatin, in order to directly facilitate Bmp4 expression or to relieve the effects of an insulator that would otherwise inhibit activity of an inner ear enhancer. We note that there are blocks of highly conserved sequence within Rubie introns that might contain DNA regulatory elements. The SWR ERV insertion does not interrupt these conserved sequences, but remains possible that transcription of Rubie is not required for Bmp4 regulation (e.g., that a classical DNA enhancer element within the Rubie gene is perturbed by the ERV). Further studies will be necessary to determine the precise role of Rubie in Bmp4 regulation.

Penetrance of the Bmp4 heterozygote circling phenotype is incomplete and varies with genetic background [10,11]. Vervoort and colleagues recently reported a chromosome 4 locus, in close proximity to the dominant Ecl locus, that modifies the circling phenotype of Bmp4 heterozygous mice [11]. This finding indirectly supports the idea that Rubie transcription regulates Bmp4 expression. While we have not identified the chromosome 4 Ecl gene, our results demonstrate that Rubie expression is disrupted in both CSS-14 circles and non-circling SWR mice, suggesting that the chromosome 4 gene is likely not involved in regulation of Rubie expression or splicing. It remains to be determined whether the chromosome 4 Ecl gene influences BMP signaling or operates through a parallel pathway also necessary for canal morphogenesis [32].

Materials and Methods

Animal Care

Ethics Statement. Mouse breeding and experimentation was carried out in accordance with protocols approved by the Children’s Hospital Boston and Duke University Institutional Animal Care and Use Committees (protocol A091-11-04), in accord with national and international guidelines. Animals: CSS-14 strain development was performed on a fee-for-service basis by Charles River Laboratories (CRL). All other mice were housed in the barrier facilities at Children’s Hospital Boston or Duke University.

CSS-14 Strain Development

CSS-14 was generated by marker-assisted backcrossing at CRL. Generations N2 through N5 were genotyped using a 99 marker genome-wide microsatellite panel, which included D14Mit126, D14Mit60, D14Mit157, and D14Mit228. At each generation, optimal breeders were selected from among all offspring carrying a non-recombiant SWR/J chromosome 14, and backcrossed to C57BL/10J. Additional backcrossing was carried out in our colony prior to experimentation. Genome-wide SNP genotyping was performed by The Mutation Mapping and Developmental Analysis Project at Brigham and Women’s Hospital. Analysis of 455 informative SNPs confirmed that the CSS-14 genetic background was 100% C57BL/10J-derived and that the introgressed SWR/J chromosome 14 encompassed a minimal interval of 92.8 Mb, extending from dbSNP rs3710916 (8.8 Mb) to rs6179144 (101.6 Mb).

CSS-14 Genotyping

Genomic DNA was prepared from tail biopsies using the QIAGEN DNeasy Blood & Tissue Kit according to the manufacturer’s recommendations. SSLP markers found to be polymorphic between C57BL/10J and SWR/J (shown in Figure 2) were used for strain maintenance and positional cloning. Primer sequences of newly-developed SSLP markers, located between D14Mit126, D14Mit60, D14Mit157, and D14Mit228, were used for strain maintenance and positional cloning. Primer sequences of newly-developed SSLP markers, located between D14Mit126 and D14Mit60, are provided in Table S2. All markers were resolved by gel electrophoresis in 4% agarose.

Paintfill Analysis of Inner Ears

E12.25 embryos and hemisected P0 heads were fixed overnight at 4°C in Bodian’s Fixative (5% glacial acetic acid, 5% formalin, 75% ethanol, 15% water). Following fixation, samples were washed for 10 minutes in 100% ethanol, dehydrated overnight in 100% ethanol at room temperature, then cleared overnight at room temperature with methyl salicylate. White latex paint diluted to 0.025% in methyl salicylate was injected with a pulled glass pipette. Inner ear morphology was assessed using conventional light microscopy. In embryos, the precise stage of canal development was determined by analyzing the unaffected anterior and posterior canals, which develop earlier than the LSC [15].

Figure 5. Disruption of Rubie by an SWR-specific endogenous retrovirus. (A) Long-range PCR spanning a previously-mapped 27 bp region of Rubie intron 1 (shown in Figure S2) yielded the expected 300 bp product in B10, while a larger product of approximately 6 Kb was generated in SWR mice and CSS-14 mice with the Chr14SWR/Chr14SWR genotype. Sequence analysis of this larger product revealed insertion of a 5542 bp endogenous retrovirus in the SWR allele. All other inbred strains produced the shorter PCR product, indicating that this insertion is unique to SWR. (B) The B10 and SWR alleles of Rubie are depicted schematically. (C) Rubie expression in the inner ears of B10, SWR, and CSS-14 mice at P6 was assessed in cDNA using PCR primers spanning exons 1 and 2 of Rubie. Rubie expression was observed in B10 and Chr14B10/Chr14SWR mice, but not in SWR or Chr14SWR/Chr14SWR mice. (D) Hemi-nested PCR using forward primers in exon 1 of Rubie and a reverse primer in the ERV demonstrated that aberrant splicing to the ERV occurs in both SWR inbreds and CSS-14 mice.

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Rubie Sequence Analysis
RIKEN Mouse FANTOM clone F930028D17 was purchased through DNAFORM Clone Distribution and sequenced using standard T3 and T7 primers. Rubie exons were amplified from genomic DNA using the primers listed in Table S2, purified with the QIAGEN Gel Extraction Kit according to the manufacturer’s instructions, and sequenced. Inbred strain genomic DNA samples were isolated from mice in our colony or purchased from The Jackson Laboratory.

Rubie Expression Analysis
Total RNA was isolated from flash-frozen tissue using RNA STAT-60 (Tel-Test) according to the manufacturer’s recommendations. Isolated RNA was treated with DNase I (Invitrogen) to remove contaminating genomic DNA, then phenol-chloroform extracted. Random hexamer-primed cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed on the resulting cDNA using the Rubie and Actb primer pairs listed in Table S2. PCR products were resolved by agarose gel electrophoresis, purified with the QIAGEN Gel Extraction Kit, and sequenced.

In situ Hybridization
Non-radioactive in situ hybridization was performed on 10–12 μm cryosections of E11.5 embryo heads. Detailed probe preparation and hybridization protocols can be found at http://goodrich.med.harvard.edu/protocols.htm. Rubie probe template was amplified from RIKEN clone F930028D17 using primers in exons 2 and 5 (Table S2). Bmp4 probe was generated using template corresponding to the first 293 bp of exon 4.

Rubie Insertion Mapping and Sequence Analysis
The ERV insertion in intron 1 of Rubie was mapped by PCR analysis of SWR/J and C57BL/10J genomic DNA. The PCR reactions depicted in Figure S2 were performed using the primers listed in Table S2. For each PCR reaction, amplification in SWR/J and C57BL/10J was evaluated as “present” or “absent” by agarose gel electrophoresis. Following mapping, long-range PCR was performed using the Expand Long Template PCR System (Roche) according to the manufacturer’s instructions. PCR reactions were performed on inbred strain genomic DNA using primers Int-H-F1 and Int1-MapR7 (Table S2). C57BL/10J and SWR/J bands were purified with the QIAGEN Gel Extraction Kit. C57BL/10J PCR products were sequenced directly. SWR/J PCR products were cloned into the pCR-XL-TOPO vector (Invitrogen) and sequenced by primer walking.

Rubie Splicing Analysis
Hemi-nested PCR was performed on inner ear (P6) cDNA using nested forward primers in Rubie exon 1 and a reverse primer specific to ETnH-β endogenous retroviruses (Table S2). Inner nest PCR products were resolved by agarose gel electrophoresis, purified with the QIAGEN Gel Extraction Kit, and sequenced.

Supporting Information
Figure S1 Haaplotype analysis of Rubie exon sequence. Rubie’s five exons were sequenced in SWR/J, C57BL/10J, and thirteen other mouse strains. The twelve single nucleotide polymorphisms (SNPs) that distinguish SWR/J and C57BL/10J are shown schematically (asterisks) and detailed in the haplotype chart. For each SNP, the SWR/J allele is depicted in red and the C57BL/10J allele is shown in red. Gray boxes represent SNPs for which no sequence data is available.

Figure S2 Mapping and sequence analysis of the Rubie insertion. The retroviral insertion in intron 1 of Rubie was mapped using a PCR-based strategy. Horizionatal bars representing individual PCR products are shown in relation to their location in intron 1, and are flanked by names of the primers used to generate them. Green bars represent products that were easily amplified by conventional PCR from both C57BL/10J and SWR/J genomic DNA. Red bars represent products that were robustly amplified in C57BL/10J, but could not be amplified by conventional PCR in SWR/J. The vertical gray bar depicts the region in which an insertion or chromosomal rearrangement must occur. The sequence of this 27 bp region is shown above (underlined). Long-range PCR between primers H-F1 and MapR7 revealed that the SWR/J allele contains a 5542 bp endogenous retrovirus (blue sequence), flanked by a 6 bp target site duplication (red sequence), a hallmark of retrotransposition.

Table S1 Genomic Locations and Sequences of Rubie Exons.
Table S2 Sequences of Oligonucleotide Primers.

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Author Contributions
Conceived and designed the experiments: KAR VEA LVG NCA. Performed the experiments: KAR VEA AFT. Analyzed the data: KAR LVG NCA. Contributed reagents/materials/analysis tools: KAR VEA LVG NCA. Wrote the paper: KAR NCA.