Mice Carrying a Hypomorphic Evi1 Allele Are Embryonic Viable but Exhibit Severe Congenital Heart Defects

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Abstract

The ecotropic viral integration site 1 (Evi1) oncogenic transcription factor is one of a number of alternative transcripts encoded by the Mds1 and Evi1 complex locus (Meocom). Overexpression of Evi1 has been observed in a number of myeloid disorders and is associated with poor patient survival. It is also amplified and/or overexpressed in many epithelial cancers including nasopharyngeal carcinoma, ovarian carcinoma, ependymomas, and lung and colorectal cancers. Two murine knockout models have also demonstrated Evi1’s critical role in the maintenance of hematopoietic stem cell renewal with its absence resulting in the death of mutant embryos due to hematopoietic failure. Here we characterize a novel mouse model (designated Evi1fl3) in which Evi1 exon 3, which carries the ATG start, is flanked by loxP sites. Unexpectedly, we found that germine deletion of exon3 produces a hypomorphic allele due to the use of an alternative ATG start site located in exon 4, resulting in a minor Evi1 N-terminal truncation and a block in expression of the Mds1-Evi1 fusion transcript. Evi1fl3/fl3 mutant embryos showed only a mild non-lethal hematopoietic phenotype and bone marrow failure was only observed in adult Vav-iCre/+; Evi1fl3/fl3 mice in which exon 3 was specifically deleted in the hematopoietic system. Evi1fl3/fl3 knockout pups are born in normal numbers but die during the perinatal period from congenital heart defects. Database searches identified 143 genes with similar mutant heart phenotypes as those observed in Evi1fl3/fl3 mutant pups. Interestingly, 42 of these congenital heart defect genes contain known Evi1-binding sites, and expression of 18 of these genes are also effected by Evi1 siRNA knockdown. These results show a potential functional involvement of Evi1 target genes in heart development and indicate that Evi1 is part of a transcriptional program that regulates cardiac development in addition to the development of blood.

Introduction

The complexity of an organism is defined not only by the number of its genes, but also how expression of these genes is controlled. This also includes several post-transcriptional events that control protein production, including alternative splicing, translational repression, microRNA-induced mRNA degradation, and the regulated generation of distinct gene products through the alternative use of translational initiation sites. These various mechanisms provide a tremendous diversity of protein sequence, structure and function [1,2]. Much improvement has been made in defining the molecular basis of these regulations. However, it remains a major challenge to integrate this knowledge into a complete understanding of the resulting physiological functions, in normal and pathological conditions.

The MDS1 and EVI1 complex locus (MECOM) contains several transcription start sites and alternative splice options. It produces multiple transcripts coding for nuclear transcription factors. One of its major gene products is ecotropic viral integration site 1 (EVI1), an oncogenic zinc finger transcription factor (TF) whose overexpression in myeloid disorders such as acute and chronic myeloid leukemia (AML and CML), and myelodysplastic syndrome (MDS) has been extensively studied and correlated with poor patient survival [3–5]. Amplification and/or overexpression of EVI1 have also been observed in multiple epithelial cancers, including nasopharyngeal carcinoma, ovariun...
carcinoma, ependymomas, and lung and colorectal cancers [6–11]. In addition, EVI1 controls several aspects of embryonic development including hematopoiesis where it has been shown to be important for hematopoietic stem cell (HSC) renewal [12] and angiogenesis [13]. The most oncogenic human MECOM isoform, EVI1, encodes a 1051 amino acid protein containing two zinc finger domains, a central transcriptional repression domain and an acidic C-terminal region [5,14,15]. The seven zinc finger domains located in the N-terminus are known to bind to a GATA-like consensus motif [13,16–19], while the three zinc finger domains in the C-terminus bind to an ETS-like motif [16,20]. Additional alternative splicing of MECOM in human and mouse produces, amongst others, two major isoforms, EVI1 and MDS1-EVI1 [5,14,15,21]. MDS1-EVI1 is a larger MECOM variant. Although MDS1 was originally described as a distinct gene, it is now recognized to be an alternative transcription start site and part of the MECOM locus. MDS1-EVI1 contains a 188 amino acid extension at its N-terminus, adding the so-called PR domain, which is a derivative of the SET domain [5,14,15,22]. Several lines of evidence suggest that the form of EVI1 lacking the PR domain and MDS1-EVI1 display opposite functions. The shorter isoform (EVI1) acts as an aggressive oncogene while expression of the longer isoform (MDS1-EVI1) is linked to good prognosis in cancer [23–25]. MDS1-EVI1 was also recently described as a regulator of long term HSC repopulating activity [21]. Another important MECOM isoform, called EVI1Δex3, resembles EVI1 but lacks zinc fingers motifs 6 and 7, which prevents its binding to GATA-like sites. Additional alternative splicing lead to the deletion of 9aa in the repressor domain of EVI1, MDS1-EVI1, or EVI1Δex3 [14,26–28], thus producing additional isoforms. The exact physiological roles of these various MECOM products remain to be characterized. Two mouse knockout models have been previously reported that target MECOM. The first one was produced by deletion of Evi1 exon 7 [13,29] while the second represents a conditional deletion of exon 4 [12]. For both alleles, homozygous Evi1−/− mice resulted in the deletion of both Evi1 and MDS1-EVI1.
Mds1-Evi1 transcripts. Both phenotypes showed embryonic lethality and impairment of hematopoiesis due to the loss of HSC renewal ability.

In this study, we analyzed a new conditional mutant allele of Mecom that was produced by flanking Evi1 exon 3, also Mds1-Evi1 exon 4, with loxP sites. The removal of Evi1 exon 3 is predicted to generate a frame shift mutation that would block the translation of Mds1-Evi1 protein. As Evi1 and Evi1d324 both have a translational initiation site located in exon3, it was also predicted that their protein expression would be blocked. However, Evi1 and Evi1d324 proteins are produced in Evi1d324ex3 tissues, likely due to an alternative translation start site located in exon 4. Thus, only the Mds1-Evi1 isoform is fully disrupted in Evi1d324ex3 mice. Evi1d324ex3 mice do not die in utero and display a different phenotype compared to exon 4 and 7 knockout mice. The analysis of this new hypomorphic exon 3 Evi1 allele has uncovered novel physiological functions for MECOM in the formation of the circulatory system and provided a better understanding of the function of the various MECOM transcripts.

**Experimental Procedures**

**Animals**

The Institute of Molecular and Cell Biology Animal Care and Use Committee approved all animal protocols used in this study. The Evi1 exon 3 floxed allele, Evi1^{ex3flox/del}[21], was maintained in a pure C57BL/6 background. After crossing to a b-actin-Cre deleter strain to generate the Evi1^{d324ex3} null allele, Evi1^{d324ex3} bearing mice were a mixture of strains 129/Sv and C57BL/6. They were made congenic on a C57BL/6 background over the course of the study, with no observed change in the experimental results. Mice were genotyped by PCR using primers F1 (5’- GGAGGTTGT-AAGCTTGAATTCC-3’), F2 (5’-GAAGAGCTCTTGCTGTTCATG-3’), and R7 (5’- CAGCTTAGACCTCAGCTAAC-3’).
F2 and R7 were used to discriminate between the Evi1fl3 (375 bp) and wild type (269 bp) alleles. F1 and R7 were used to detect the Evi1d ex3 allele (125 bp) (Fig. S1A,B in File S1). Vav-iCre was genotyped using Cre-F (5'-GCCTGCATTACCGGGT-GATGCAACGA-3') and Cre-R (5'-GTGGCAGATGGCGCG-GCAACACCATT-3') primers (700 bp amplicon). Blood was obtained by retro-orbital bleeding for adult mice, and by decapitation for embryos. Blood counts were performed with a Hemavet 950 device.

Quantitative real time RT-PCR (qRT-PCR)

RNA was isolated from mouse tissues using Trizol and an RNeasy Mini Kit (Qiagen), and 0.5–2 μg were used for cDNA synthesis (SuperScript III First-Strand Synthesis; Invitrogen) with oligo(dT). qPCR was performed with the ABI-Prism 7500 (Applied Biosystems), SYBR green Master Mix, and primers designed with Primer Express Software v2.0 (Applied Biosystems). A primer list is provided in File S1. We used the 2−ΔΔCt method [30] to calculate the fold change of expression. Relative expression was normalized to Tubg1 mRNA levels.

HSC characterization

Hematopoietic cells were extracted from the fetal liver or bone marrow. Flow cytometric analyses and cell sorting were performed using a LSR II, a fluorescence-activated cell sorter (FACS) Vantage, or a FACS Aria as previously described [32]. Antibodies were purchased from BD Biosciences: PE-conjugated anti-Gr1 (RB6-8C5), Mac-1 (M1/70), Ter119 (TER-119), CD4 (RM4-5), CD3 (145-2C11), CD8 (53–6.7), B220 (RA3-6B2), IL7Ra (SB/199), PE-Cy7-conjugated anti-c-Kit (2B8), APC-conjugated anti-Sca-1 (E13-161.7) and FITC-conjugated CD34 (RAM34). Colony forming unit-culture (CFU-C) assays, using fetal liver cells or bone marrow cells, were performed as previously described [32]. Briefly, fetal liver or bone marrow cells were cultured in 35-mm dishes in triplicate in Methocult M3231 methylcellulose medium (StemCell Tec., Vancouver, BC, Canada) supplemented with 20 ng/mL recombinant mouse IL-3, 100 ng/mL mouse SCF, 200 ng/mL mouse G-CSF and 10 ng/mL mouse EPO. Colonies were counted on day 10.

Figure 3. Profound depletion of hematopoietic cells in adult mice carrying an Evi1 exon3 deletion. (A) Kaplan-Meyer survival curves indicate significant lethality in Vav-iCre; Evi1fl3/fl3 mice, with a median survival of 7.7 weeks (Log rank test, Chi square p value <0.0001). (B) Hemograms for 6 to 9 week-old Vav-iCre; Evi1fl3/fl3 mice. These adult mice displayed leukopenia, severe anemia and thrombocytopenia. Mean ± SEM is indicated. *p<0.05, **p<0.01, ***p<0.001, unpaired t-test. (C) Flow cytometric profiles of bone marrow cells from Vav-iCre;Evi1fl3/fl3 and littermate control mice (Evi1fl3/+ or Evi1fl3/fl3). HSC and progenitor cell subpopulations were detected by a combination of markers (KSL: c-Kit+, S: Sca-1+, L: lineage ). We found a significant reduction of cells in Evi1-deleted samples, p = 0.00011 and p = 0.0024, for KSL and KL, respectively (unpaired t-test). (D) Colony forming counts for cells from bone marrow of Vav-iCre;Evi1fl3/fl3 and littermate control mice (Evi1fl3/+ or Evi1fl3/fl3). N = 3 for each group, p = 0.0019 (unpaired t-test). No BFU-E and CFU-Mix colonies were identified.

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Histology
Mice received a complete necropsy after which their tissues were fixed in 10% neutral buffered formalin overnight and embedded in paraffin. Embryos were fixed and embedded whole before sectioning. Sections of 5 μm were stained with Hematoxylin and Eosin or Giemsa.

Magnetic Resonance Imaging and 3D reconstruction
Embryos were harvested at E15.5, euthanized and fixed in 4% paraformaldehyde (PFA) with 2 mM Gd-DTPA (gadolinium-diethylenetriaminepentaacetate) as a contrast agent. Multi-embryo imaging was conducted as previously described [33]. The raw MR data were reconstructed as described previously [34]. The files were analyzed using Amira 5.3.3 software.

In situ hybridization in embryos
Evi1 mRNA in situ hybridization was carried out using a full length Evi1 cDNA probe [35] using standard protocols. Probes were labeled using a DIG RNA Labeling Kit (Roche Applied Science, Tokyo, Japan). Detection was via an anti-DIG antibody coupled to alkaline phosphatase (Roche, Tokyo, Japan) followed by staining with BCIP-NBT (Bromo-4-chloro-3-indolyl Phosphate/Nitro Blue Tetrazolium) (Nacalai, Tokyo, Japan) as previously described [36].

Results

Deletion of Evi1 exon 3 results in postnatal lethality
Mice homozygous for an Evi1 exon 3 deletion (designated Evi1<sup>dex3/dex3</sup>) have recently been generated and used to access the function of Mecom in hematopoiesis ex vivo [18]. Deletion of exon3 is predicted to prematurely abrogate the expression of Mds1-Evi1 due to the presence of an out-of-frame stop codon in exon 4 (Fig. 1A). Exon 3 also encodes the ATG translation start site for Evi1 and Evi1<sup>dex3/dex3</sup> is thus predicted to be a Mecom null allele (Fig. S1A in File S1). We therefore expected that similar to other Evi1 knockout mice [12,13,29], deletion of...
exon 3 would lead to embryonic lethality between E10.5 and E16 due to defects in HSC self-renewal and subsequent hematopoietic failure. Surprisingly, this was not the case. Homozygous Evi1<sup>Δex3</sup> knockout mice (Fig. S1B,C in File S1) were born with a normal Mendelian ratio (Fig. 1B). They were indistinguishable from their control littermates, there were no gross morphological defects and they were normal in size (Fig. S1D in File S1). The presence of grossly visible milk-filled stomachs a few hours after birth also attested to their ability to feed, which was confirmed by histology (Fig. S1E in File S1). However, several hours to a few days after birth, Evi1<sup>Δex3</sup> mice became weak, lost weight and eventually died, with no Evi1<sup>Δex3</sup> animals surviving longer than three days (Fig. 1C,D). These results suggest that Evi1<sup>fl3</sup> might encode a hypomorphic allele rather than a null allele.

**Evi1<sup>fl3</sup> encodes a hypomorphic allele**

To determine whether Evi1<sup>fl3</sup> encodes a hypomorphic allele we used 5' RACE to confirm that exon3 was deleted from all Mecom transcripts expressed in Evi1<sup>Δex3</sup> embryos. We also performed RT-qPCR to quantify the level of the Mecom transcripts expressed in Evi1<sup>Δex3</sup> embryos using primers located in exons 2 and 3, 3 and 4 or 13 and 14. No significant amplification was detected in Evi1<sup>Δex3</sup> embryos using the two first sets of primers (Fig. 1E), confirming that exon3 was deleted from all Mecom transcripts in Evi1<sup>Δex3</sup> animals. Transcripts encoding Evi1 exons 13 and 14 were, however, produced at normal levels, confirming that stable Evi1 transcripts are expressed in Evi1<sup>Δex3</sup> embryos. Western blot analyses showed that proteins with a similar size to Evi1, Evi1<sup>Δ105</sup>, and Evi1<sup>Δ324</sup> were also expressed in Evi1<sup>Δex3</sup> embryos. Evi1<sup>Δ105</sup> is a splice variant present in mouse but not in human tissues [37]. Deletion of exon3 thus did not appear to affect Evi1 protein translation as would have been expected by removal of exon 3. We therefore decided to look for alternative ATG translation start sites that might be located downstream of exon 3. We found a potential ATG start site in exon 4, which contains a Kozak sequence [38] and is in frame with the rest of the protein. This start site is well conserved in higher vertebrates and provides a better Kozak sequence than the start site in exon 3 (Fig. 1G, S2). The use of this alternative start site would remove 42 amino acids from the N-terminus of Evi1 including the first zinc finger motif of the proximal Evi1 zinc finger domain (Fig. S2 in File S1). Evi1Δ105, an isoform specifically present in mice [37] and Evi1Δ324 would be similarly affected since they share the same transcription start site as Evi1.

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**Figure 5. Cardiac malformations and failure in Evi1<sup>Δex3</sup> mice.** (A) Transverse sections and (B) 3D reconstruction (left-ventral oblique view) of hearts from Evi1<sup>Δex3</sup> or wild type littermate (+/+ E15.5 embryos analyzed by magnetic resonance imaging (MRI). The aorta (Ao), right ventricle (RV), left ventricle (LV), ventricular septum (VS), trachea (Tr), aortic arch (AoA) and ductus arteriosus (DA) are indicated. Ventricular septal defect (VSD), interrupted aortic arch (IAA) and common arterial trunk (CAT) were observed in Evi1<sup>Δex3</sup> hearts. (C) List of the congenital heart defects identified in fifteen E15.5 embryos of various different genotypes by MRI and 3D reconstruction. (D) Hematoxylin and eosin staining of 5 μm sections of a sick Evi1<sup>Δex3</sup> pup. Subcutaneous and other tissue edema (white spaces) was present, consistent with heart failure.

Evi1<sup>fl3</sup> is critical for heart and blood development.
These results support the notion that Evi1<sup>ex3</sup> encodes a hypomorphic allele that results from the expression of an N-terminally truncated Evi1 protein initiated in exon 4. Evi1<sup>ex3</sup> newborn pups have a milder hematopoietic phenotype than that observed in Evi1<sup>ex4</sup> embryos. The embryonic lethality in Evi1 exon 4 knockout mice has been ascribed to defective HSC self-renewal and subsequent hematopoietic failure [12]. To determine whether Evi1<sup>ex3</sup> embryos have similar defects, we counted the number of two immunophenotypically defined HSC populations, c-Kit<sup>+</sup>, Sca-1<sup>+</sup>, lineage-(KSL) and c-Kit<sup>+</sup>, lineage-, CD34<sup>+</sup> (KL-CD34<sup>+</sup>) cells from E14.5 wild-type, Evi1<sup>ex3/+</sup> and Evi1<sup>ex3/ex3</sup> fetal livers (Fig. 2A). The number of KSL HSCs and KL-CD34<sup>+</sup> progenitor cells was significantly reduced in Evi1<sup>ex3/ex3</sup> fetal livers as compared to wild type livers, while Evi1<sup>ex3/+</sup> fetal livers presented an intermediate phenotype (Fig. 2A). In addition, there was a slight reduction in the number of B220<sup>+</sup> B-lymphocytes (Fig. 2B) and colony-forming cells (Fig. 2C) in E14.4 Evi1<sup>ex3/ex3</sup> and Evi1<sup>ex3/ex3</sup> fetal livers. These results show that deletion of Evi1 exon 3 leads to a reduction in the number of HSC and progenitor cells, but this deletion does not affect the differentiation of progenitors once they are formed. This hematopoietic phenotype is milder than that described for Evi1<sup>ex4/ex4</sup> mice [12] as the HSC counts were reduced by only 76% versus 93% for Evi1<sup>ex4/ex4</sup> mice. Blood counts from Evi1<sup>ex3/ex3</sup> newborn animals (Fig. 2D) also showed that erythropoiesis was normal in Evi1<sup>ex3/ex3</sup> newborn animals. Mild leucopenia was however detected, which equally affected all hematopoietic compartments. Hypoproliferative thrombocytopenia was the most prominent phenotype linked to the Evi1 exon 3 deletion. Histological analyses showed that 31% of the Evi1<sup>ex3/ex3</sup> pups had grossly visible focal hemorrhages in various tissues at birth (4 out of 13 pups) (Fig. 2E), while no control animals were seen with hemorrhagic lesions (0 out of 8 controls). These hemorrhages were unlikely to be the cause of embryonic lethality, however, because other genetically engineered mouse models with much lower platelet counts have been shown to survive to adulthood [39].

Spontaneous lethal bone marrow failure in the hematopoietic compartment of Evi1<sup>ex3/ex3</sup> animals

To further characterize the hematopoietic phenotype linked to the Evi1 exon 3 deletion, we crossed Evi1<sup>ex3/ex3</sup> animals with Vav-iCre transgenic mice [40]. Vav-iCre is expressed in all hematopoietic, but few other cell types, and as expected Vav-iCre/+; Evi1<sup>ex3/ex3</sup> animals displayed a selective loss of Evi1 exon 3 in the hematopoietic compartment (Fig. S3A in File S1). These mice did not die during prenatal development but instead died between 2.8 and 24.8 weeks of age (N = 37), with a median survival of 6.3 weeks (Fig. 3A). Heterozygous deletion of exon 3 did not affect the mortality rate compared to control mice (Fig. 3A). Most mice became weak and lost weight before dying (Fig. S3B in File S1). Hemograms were subsequently performed on Vav-iCre/+; Evi1<sup>ex3/ex3</sup> weak animals and corresponding littermate controls +/-; Evi1<sup>ex3/ex3</sup>. The hematopoietic phenotype was dramatic, with severe thrombocytopenia, anemia and leucopenia in this condi-

Figure 6. Expression of Mecom mRNA in cardiac structures of wild type embryos. (A–D) Whole mount mRNA in situ hybridization to show Mecom expression. A–C) Expression during subsequent stages of heart tube formation E8.5 (black brackets). D) At E9.5 Evi1 is expressed in the endothelial cells and in the endocardium of the heart and in the mesenchyme of the aortic arches. Expression also includes a population of migrating neural crest cells (white arrowhead). E–J) E10.5 Sagittal sections (from right to left) showing Evi1 in the aortic arches (a), mesenchyme of the secondary heart field (black arrowheads), outflow and atrio-ventricular canal endocardium including the cushions. doi:10.1371/journal.pone.0089397.g006
Figure 7. Evi1 regulates the expression of other CHD genes during embryonic heart development. (A) The number of CHD genes represented in Evi1 ChIP-Seq data (Evi1 bound genes) or in the list of genes regulated by Mecom. An enriched number of CHD genes were found bound or regulated by Mecom (50 out of 143 genes), *p* = 0.0453 and *p* = 0.0276, respectively. These genes represent potential Mecom target genes in heart development. (B) Mecom regulates the expression of 23 CHD genes, which contain Evi1-binding sites specifically in heart. Heart and head (neural crest) tissues were harvested from WT and Evi1<sup>dex3/dex3</sup> embryos of somite number 9 to 18. RT-qPCR assays were performed. Genes considered to be mis-regulated in Evi1<sup>dex3/dex3</sup> hearts were increased or decreased in expression by at least three fold in average for all samples of the same time-point. These graphs are representative of two to five independent experiments.

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### Table 1. List of 23 congenital heart defect (CHD) genes whose expression is disrupted in Evi1<sup>ex3</sup>/<sup>ex3</sup> developing hearts.

| mouse gene symbol | human gene symbol | common arterial trunk (MP:0002633) | Ventricular septal defect (MP:00010402) | double outlet right ventricle (MP:0000284) | overriding aorta (MP:0000273) | interrupted aortic arch (MP:0004157) | EVII target gene by ChIP-Seq | Regulated by EVI1 (microarray in SKOV3 cells) | Evit<sup>ex3</sup>/<sup>ex3</sup> affects gene expression in embryos hearts |
|------------------|------------------|----------------------------------|-------------------------------------|------------------------------------------|---------------------------------|----------------------------------|-----------------------------|-----------------------------------------------|-----------------------------------------------|
| Adam9            | ADAM9            | yes                              | yes                                 | yes                                      | yes                             | yes                              | yes                         | up-regulated                        | up-regulated                        |
| Bmp4             | BMP4             | yes                              | yes                                 | yes                                      | yes                             | yes                              | yes                         | up-regulated                        | up-regulated                        |
| Bmpr2            | BMPR2            | yes                              |                                     |                                         | yes                             | yes                              | yes                         | up-regulated                        | up-regulated                        |
| Cav1             | CAV1             | yes                              |                                     |                                         | yes                             |                                  | yes                         | down-regulated                      | up-regulated                        |
| Chd7             | CHD7             | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Cited2           | CITED2           | yes                              | yes                                 | yes                                      | yes                             | yes                              | yes                         | up-regulated                        | up-regulated                        |
| Crkl             | CRKL             | yes                              | yes                                 | yes                                      | yes                             | yes                              | yes                         | up-regulated                        | up-regulated                        |
| Cxcr4            | CXCR4            | yes                              |                                     |                                         | yes                             |                                  | yes                         | down-regulated                      | up-regulated                        |
| Egln1            | EGLN1            | yes                              |                                     |                                         | yes                             |                                  | yes                         | down-regulated                      | up-regulated                        |
| Flna             | FLNA             | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Foxp1            | FOXP1            | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Gata6            | GATA6            | yes                              |                                     |                                         | yes                             |                                  | yes                         | down-regulated                      | up-regulated                        |
| Hey1             | HEY1             | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Jag1             | JAG1             | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Jun               | JUN              | yes                              |                                     |                                         | yes                             |                                  | yes                         | down-regulated                      | down-regulated                      |
| Nf1              | NF1              | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Nrp2             | NRP2             | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Psen1            | PSEN1            | yes                              |                                     |                                         | yes                             |                                  | yes                         | down-regulated                      | up-regulated                        |
| Rarg             | RARG             | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Rxra             | RXRA             | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Sox4             | SOX4             | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Tgfb2            | TGFBR2           | yes                              |                                     |                                         | yes                             |                                  | yes                         | up-regulated                        | up-regulated                        |
| Thbs1            | THBS1            | yes                              |                                     |                                         | yes                             |                                  | yes                         | down-regulated                      | down-regulated                      |

These genes were previously found targeted by Evi1 in ChIP-Seq and microarray experiments [16], indicating they may be directly regulated by Evi1. doi:10.1371/journal.pone.0089397.t001
Table 2. Overview of Major Reported Expression Domains.

| Gene | Reported Expression Domains | References |
|------|----------------------------|------------|
| Mecom | AA, CC/HT, End+CsN, NC, SHF |            |
| Adam9 | End+Csn, Myo                  | [56,57]    |
| Bmpr4 | AA, Myo, NC, OFT, SHF         | [58,59,60] |
| Bmpr2 | AA, End, Myo, NC              | [58,59,60] |
| Cav1  | End                          | [61]       |
| Chd7  | AA                           | [62]       |
| Cited2| AA, CC/HT, End+Csn, Myo, OFT | [63,64]   |
| Crk1  | AA, NC                       | [65]       |
| Cxcr4 | AA, Myo                      | [66]       |
| Flna  | AA, End+Csn, NC, OFT         | [67]       |
| Foxp1 | End+Csn, Myo, OFT            | [68]       |
| Gata6 | End+Csn, Myo, OFT, NC        | [69,70]    |
| Hey1  | AA, End, OFT                 | [71,72,73] |
| Jag1  | AA, End, OFT                 | [71,74]    |
| Jun   | AA, End+Csn, OFT, SHF        | [75]       |
| Nf1   | AA, End+Csn, Myo, NC         | [76]       |
| Nrp2  | NC                           | [77,78]    |
| Psen1 | AA, End+Csn, Myo, NC, OFT    | [79,80]    |
| Rarg  | AA                           | [81]       |
| Roxa  | AA, End+Csn, Myo, NC, OFT    | [82]       |
| Sox4  | End+Csn, Myo                 | [68,83]    |
| Tfgb2 | AA, CC/HT, End+Csn, Myo, NC  | [84,85,86] |
| Thbs1 | End, Myo                     | [87]       |

Key

AA = Aortic Arch and Aortic Arch Arteries.
CC/HT = Cardiac Crescent/Heart Tube.
End = Endocardium (+Csn – including Cushions).
Myo = Myocardium.
NC = Neural Crest (Cardiac).
OFT = Outflow Tract.
SHF = Secondary Heart Field.

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Evi1 controls the expression of genes that regulate heart development

How might Evi1 act to control heart development? Because Evi1 is a transcription factor that can both activate or repress its target genes [16], we hypothesized that it might be part of the transcriptional program that controls heart development. To

with the profound HSC depletion seen in Evi1 exon 4 conditional knockout at E10.5–16.5 [12], but it occurs at a much later stage, in Evi1 exon 3 deleted adult mice.

Evi1 is Critical for Heart and Blood Development

Congenital heart defects in Evi1<sup>ex3/ex3</sup> newborn mice

Since it was unlikely that the perinatal lethality observed in Evi1<sup>ex3/ex3</sup> mice was caused by the hematopoietic defects we looked for other possible causes. We used magnetic resonance imaging (MRI) to visualize organ formation in six Evi1<sup>ex3/ex3</sup>, three Evi1<sup>ex2/ex3</sup>, and six E15.5 control littersmates, as previously described [41]. Structural abnormalities were observed in the hearts of all six Evi1<sup>ex3/ex3</sup> embryos (Fig. 5A,B), while small benign bilateral cysts were observed in the jugular lymphatic sacks of two Evi1<sup>ex3/ex3</sup> embryos (Fig. S4 in File S1). No defects were observed in wild type or heterozygous mutant animals. Evi1<sup>ex3/ex3</sup> embryos displayed several congenital heart defects (Fig. 5C).

All six Evi1<sup>ex3/ex3</sup> embryos had ventricular septal defects (VSD) - failure to form the septum between the ventricles of the heart (Fig. 5B,C).

Common arterial trunk (CAT), where two great arteries fail to separate and leave the heart as one common vessel, was also observed in 3 out of 6 Evi1<sup>ex3/ex3</sup> embryos. Double outlet right ventricle (DORV), where both the aorta and pulmonary trunk leave one ventricle, was also observed in half of the Evi1<sup>ex3/ex3</sup> embryos (Fig. 5B,C). In addition, overriding aorta (aorta originating just above the VSD) was seen in one Evi1<sup>ex3/ex3</sup> embryo. Finally, aortic arch formation impairments were found in 4 out of 6 Evi1<sup>ex3/ex3</sup> embryos (Fig. 5B,C). These impairments were manifested as an interrupted aortic arch (IAA), with a complete discontinuation between the ascending and descending parts of the aorta. These type of congenital heart defects are known to be viable in utero but lethal during the neonatal phase of life for other mouse knockouts [42], and thus likely represent the major cause of the perinatal lethality seen in Evi1<sup>ex3/ex3</sup> pups. Consistent with this, heart failure was sometimes accompanied by oedema and congested lungs in Evi1<sup>ex3/ex3</sup> pups (Fig. 5D).
Evil Is Critical for Heart and Blood Development

Our results demonstrate that deletion of Evil exon 3 produces a hypomorphic allele compared to previous studies involving Evil exons 4 and 7, where their removal produced complete null alleles [12,29]. Deletion of exon 3 indeed does not affect Evil, Evil18105 [37] and Evil18324 protein production but does block the generation of Mds1-Evil protein production. All Evil isoforms expressed in these mice are expected to carry a 42 amino acid truncation at the N-terminus that constitutes nearly 4% of the protein. Such truncated proteins would be predicted to lack one zinc finger motif out of the seven present in the proximal DNA-binding site. It is not completely clear if and how this truncation affects Evil transcriptional activity or function. Several findings suggest that translation from Evil exon4 ATG start site produces a functional protein. First, the exon4 contains the best Kozak sequence with highest cross-species conservation. Thus, it is possible that the exon4 translation start site may be naturally produced in vivo. Secondly, a previous study has suggested that Evil protein initiated from exon 4 is oncogenic and able to give rise to leukemic clones in mice [52]. Retroviral insertional mutagenesis screens in mice have identified Evil isoform as a targeted mutant gene in myeloid leukemia [53,54]. Sequencing of the retroviral insertion sites from these tumors has shown that the majority of insertions are located upstream of Evil coding sequence, where they serve to upregulate the expression of oncogenic Evil but block the expression of Mds1-Evil. The genomic region located between exons 3 and 4 is only 4 kb compared to the rest of the Evil upstream region which is 90 kb in size, thus providing 23 times less chance to contain a retrovirus insertion by random chance. However, retroviral insertions located between exon 3 and 4 have been described in tumors, which would serve to activate Evil translation from the alternative translation start site located in exon 4 [52].

The profound embryonic lethal disruption of HSC renewal seen in other studies [12,13] was not present in our Evilex3/ex3 mutant embryos and newborn pups. However, we did identify a dramatic perturbation of hematopoietic repopulation activity in Vav-iCre/+, Evil183/183 young adult mice. To our knowledge, there is no current genetically-modified mouse model that mimics spontaneous bone marrow failure as seen in the Vav-iCre/+, Evil183/183 mice. They therefore constitute the first model of spontaneous lethal bone marrow failure in the adult. Surprisingly, the hypomorphic deletion of Evil1 could delay the phenotype of hematopoietic failure and the appearance of bone marrow depletion. This is in line with a previous study [21] that specifically implicated Mds1-Evil in the regulation of long term HSC repopulating activity [53] and Evil in short term HSC renewal activity [12,29].

The delay in acquisition of the hematological phenotype in Evil183/183 knockout mice allowed the embryos to survive to the perinatal period and the congenital heart defects found in these mice to be observed. Our results are also consistent with those reported for Evil exon 7 knockout mice published in 1997, which reported that E10.5 Evil+/− mutant embryos displayed heart arrest and hematopoietic failure. Although their data based on only one histology section are not clear, Evil186/186 knockout embryos were reported to display arrested heart development with a looping defect of the posterior part of the heart and a poorly developed constriction between atria and ventricle [29], which is different from our findings. At the time of this previous study, the technologies to study embryonic cardiac development were based only on histological methods, which could not allow precise interpretations of the pathology. In our studies we used MRI and 3D modeling to clearly define the
pathology and heart developmental defects in Evi1 exon 3 knockout embryos.

We provide evidence that Mecom belongs to a transcriptional regulatory network that controls heart development. Mecom expression overlaps with the expression of multiple other factors required for heart development (Table 2). These factors can be Mecom targets, and their expression is deregulated expression in the Evi1 exon 3 knockout heart. Of particular interest may be factors in the Notch and TGFβ pathways as that Mecom or its homologues interact with these pathways [22]. In the endocardium for example, there is clear overlap of Mecom with the Notch ligand Jag1 and the TGFβ receptor Tgfb2.

The endocardium is major site of Mecom expression in the heart, and it is possible that Mecom regulates gene expression directly in this tissue. The cushions cells of the AVC originate from endocardium via an epithelial–mesenchymal transition, and they form the partition between the ventricles and the atria (atrio–ventricular canal and later valves). This partition provides the matrix for the growing ventricular and atrial septa [42,43]. Another possible site of Mecom action is in the neural crest cells. The spectrum of phenotypes seen in the Evi1 knockout embryos could also be attributed to defects in these cells causing disrupted remodelling of the aortic arches, and to a failure to septate the outflow tract [43]. Further studies (perhaps using a floxed-Evi1 null allele) and specific Cre lines can be used address if Mecom is required in a particular heart cell population, or in multiple populations to drive heart development.

Supporting Information

File S1 Figure S1, Targeting and knockout of Evi1 exon3. Figure S2, An alternative protein translation site.

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Table S1 List of 143 congenital heart defect genes with similar heart phenotypes as those observed in Evi1 exon 3 mice. All 143 genes linked to the Mammalian Phenotype identifications MP:00014042 (VSD), MP:0002633 (persistent truncus arteriosus, other name for CAT), MP:0000284 (DORV), MP:0001457 (IAA), MP:0000273 (overriding aorta) in the MGI database [88]. The genes found in previous Evi1 ChIP-Seq and microarray experiments [89] provide potential Mecom target genes in heart development.

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Author Contributions

Conceived and designed the experiments: EAB-C AP NAJ NGC. Performed the experiments: EAB-C DS BJ BQC GCC YZ EK FU. Analyzed the data: EAB-C DS JMW AW MB MO SA. Contributed reagents/materials/analysis tools: EAB-C DS SDV AW MB MO AP. Wrote the paper: EAB-C SDV AWJ NAJ NGC.

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