Loss of Rearranged L-Myc Fusion (RLF) results in defects in heart development in the mouse

L. M. Bourke[a,b,*], G. del Monte-Nieto[c,d,*], J. E. Outhwaite[e], V. Bharti[a], P. M. Pollock[b], D. G. Simmons[e], A. Adam[c], S.S.J. Hur[c], G.J. Maghzal[c], E. Whitelaw[f], R. Stocker[c,g], C.M. Suter[c,d], R. P. Harvey[c,d,h#], S. K. Harten[a#]

[a]Epigenetics Laboratory, QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia, 4006

[b]School of Biomedical Sciences, Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland, Australia, 4001

[c]The Victor Chang Cardiac Research Institute, Sydney, NSW, Australia, 2010

[d]St. Vincent's Clinical School, University of New South Wales, Kensington, Australia 2052

[e]School of Biomedical Sciences, Faculty of Medicine and Biomedical Sciences, The University of Queensland, St. Lucia, Queensland, Australia, 4072

[f]Department of Genetics, La Trobe Institute for Molecular Science, Department of Genetics, La Trobe University, Bundoora, Victoria, Australia, 3086

[g]School of Medical Sciences, University of New South Wales, Kensington, Australia 2052[h]School of Biotechnology and Biomolecular Sciences, University of New South Wales, Kensington, Australia 2052

[*]These authors contributed equally to this work

[#]Co-corresponding Authors;

Sarah Harten: sarah.harten@qimrberghofer.edu.au;

Richard P. Harvey: r.harvey@victorchang.edu.au

Running title: *Rlf* mutants display defects in heart development

ABSTRACT

Recently we reported that Rearranged L-Myc Fusion, RLF, acts as an epigenetic modifier maintaining low levels of DNA methylation at CpG island shores and enhancers across the genome. Here we focus on the phenotype of *Rlf* null mutant mice generated via an ENU mutagenesis screen, to identify genes required for epigenetic regulation.

RLF is expressed in a range of fetal mouse tissues, including the fetal heart. Comprehensive timed-mating studies are consistent with our previously reported findings that *Rlf* homozygous mutant mice rarely survive to adulthood, with the majority dying shortly after birth. Histological analysis of two independent *Rlf* ENU mutant lines at E11.5-E14.5 showed heart defects resembling those present in humans with Left Ventricular Non-Compaction (LVNC). *In situ* hybridisation analysis localized expression of *Rlf* to the endocardium and epicardium of embryonic and postnatal hearts, and transiently to cardiomyocytes during heart looping and early chamber formation stages. RNA-seq analysis of *Rlf* mutant hearts highlighted defective NOTCH pathway signalling, recently describe as one cause of LVNC.

This study provides the first evidence that RLF is required for normal heart development in the mouse. The heart morphological defects present at high penetrance in *Rlf* mutants are consistent with features of LVNC in humans, and molecular analysis identified attenuated JAGGED 1 expression and NOTCH signalling as likely contributors to these defects. Our study highlights the importance of RLF-dependent epigenetic modifications to DNA for maintaining correct gene regulatory network and intercellular signalling interactions during heart chamber and septal development. Further investigations are needed to define the biochemical role of RLF in the developing heart, and whether RLF mutations are a cause of heart defects in humans.

KEYWORDS

Left Ventricular Non-compaction, NOTCH1 Signaling, cardiovascular development, RLF

INTRODUCTION

The genetic causes of congential heart disease (CHD) are not well established, as chromosomal abnormalities, Mendelian syndromes, or *de novo* mutations in genes known to cause CHD, account for only about one fifth of cases [1]. Mutations in genes involved in the establishment and/or maintenance of epigenetic regulation in the genome have been identified in syndromes with heart abnormalities, e.g. CHARGE Syndrome [2]. Similarly, a recent study identified *de novo* mutations in histone modifying genes in patients with sporadic CHD [3]. Pre-natal deficiency of folate, a methyl donor important in the establishment of DNA methylation patterns during development, has also been associated with increased risk of CHD [4].

Although only a handful of epigenetic regulators have been implicated in human cases of CHD, mouse studies from our laboratory and others point to a broader role of epigenetic pathways in this set of diseases. Developmental heart defects are observed in mice null for a wide range of epigenetic modifiers, including the DNA methyltransferase *Dnmt3b* [5], the chromatin remodeller *Pbrm1* [6, 7] and the histone modifying gene *Dot1l* [8].

Re-arranged L-Myc Fusion (RLF) is a multi-Zinc finger protein that was first studied in the context of gene fusions with *L-myc*, found in small cell lung cancer [9]. Recent exome sequencing studies have also reported *Rlf-L-myc* gene fusions in lung cancer [10]. Such gene fusions lead to inactivation of L-MYC, a known tumour suppressor; however, the role of RLF itself remains poorly studied.

Epigenetic mechanisms, including DNA methylation, histone modifications, chromatin remodelling and non-coding RNAs, play a central role in the regulation of gene expression [11]. Mutations in genes responsible for establishing the epigenetic state of the genome, termed epigenetic modifiers, often result in developmental defects and disease. To uncover novel epigenetic modifiers, an ENU mutagenesis screen was established using mice carrying a multi-copy green fluorescent protein (GFP) transgene linked to an erythroid specific *α-globin* promoter and a *HS40* enhancer. Multi-copy transgenes have been shown to undergo silencing via epigenetic mechanisms [12]. Our screen was based on the variegated expression of GFP in red blood cells in the transgenic line, i.e. only 55% of red blood cells express GFP, as it is silenced in 45% of cells. Alleles that variegate in this way are known as metastable epialleles [13] and the mutant lines produced from the above ENU screen are known as *MommeD* (*Modifier of murine metastable epiallele Dominant*) lines [14].

Among the mutant lines produced in this screen, three independent lines carried mutations in *Rlf* - *MommeD8, MommeD28,* and *MommeD34* [6, 14]. Identification of *Rlf* as the gene carrying the causative mutations in these lines has been described previously [6]. Briefly, the lines were back-crossed for at least five generations before performing linkage analysis. For *MommeD8* and *MommeD34*, the linked intervals (<3.8 Mbp) were sequenced in full, using a custom capture array followed by deep sequencing. *Rlf* was the only gene for which mutations were identified in both lines. *MommeD8,* has a mutation changing a cysteine to a phenylalanine in the last predicted Zinc finger in the RLF region encoded by exon 8. *MommeD34* has a point mutation changing a cysteine to a stop codon. For the *MommeD28* line, linkage analysis produced a 3.5 Mbp interval that included *Rlf*. Sanger sequencing of *Rlf* revealed an adenine → guanine mutation at the splice acceptor site of intron 4 [6]. The

MommeD28 and *MommeD34* lines failed to produce any detectable RLF protein via Western blotting [6]. *MommeD8,* is a hypomorphic line and produces a mutant protein [6].

All three *Rlf* mutant lines showed 1) a decrease in the percentage of red blood cells expressing GFP and 2) increased methylation of the GFP transgene, compared to their wild-type (WT) littermates [6]. Mutation of *Rlf* was also demonstrated to have effects on A^{vy}, an independent epigenetically sensitive allele that contributes to coat colour in mice. $Rlf^{MommeD8/+}$ dams produced more pseudoagouti offspring than WT dams, a phenotype that correlates closely with increased methylation at the A^{vy} locus [6]. Whole genome bisulphite sequencing of $Rlf^{MommeD28/MommeD28}$ and WT fetal liver from E10.5 embryos showed that loss of *Rlf* results in an increase in methylation at hundreds of distinct loci across the genome. Many of these sites overlap putative regulatory elements [15]. We have also shown that RLF can bind to DNA in *in vitro* assays [15]. Taken together, these findings suggest that RLF acts as an epigenetic modifier that plays a role in transcriptional activation, though the biochemical function of RLF is still unclear. It likely forms part of a larger transcriptional complex that contains direct epigenetic modifiers.

Previous preliminary studies found that *Rlf* mutants weigh less than their WT littermates and are present in reduced numbers at weaning; however, no further analysis of their phenotype was undertaken [6, 16]. The data presented here demonstrates that RLF is required for normal heart development. Investigations of the placenta and lungs of $Rlf^{MommeD28/MommeD28}$ embryos found no gross morphological differences, consistent with loss of RLF causing cardiac defects directly. Heart defects in *Rlf* null embryos ($Rlf^{MommeD28/MommeD28}$ *and* $Rlf^{MommeD34/MommeD34}$) resembled Left Ventricular Noncompaction Cardiomyopathy (LVNC) in

humans. Isolated LVNC is now recognised as a distinct form of inherited cardiomyopathy [17]. Recently, defective NOTCH signalling has been linked to LVNC phenotypes in *Mib1* and *Numb* mouse mutants [18, 19], and in humans, Luxan et al reported causative mutations in *MIB1* in two LVNC families [18] Molecular analysis of *Rlf* homozygous mutant and wild-type hearts identified altered NOTCH pathway signalling, suggesting this as the likely causation.

MATERIALS AND METHODS

Mouse lines: The *MommeD28* and *MommeD34* mouse lines were produced in an ENU mutagenesis screen for epigenetic modifiers and have been described previously [6]. The ENU screen was carried out in *Line3* FVB/NJ mice carrying a multi-copy GFP transgene under the control of the human *α-globin* promoter and the *HS-40* enhancer. All mice used in this study were homozygous for the GFP transgene array. ENU mutant lines have been maintained as inbred colonies by backcrossing to unmutagenised *Line3* mice for at least ten generations [14]. All procedures were carried out with approval from the Animal Ethics Committee of the QIMR Berghofer Medical Research Institute.

DNA and RNA extraction: Embryonic tissue was obtained from natural timed matings of heterozygous individuals with the presence of a post-coital plug defined as E0.5. Pregnant dams were euthanized via cervical dislocation at the required time point. E13.5 fetal hearts were removed and homogenised in RLT Plus Buffer (Qiagen) using a syringe and needle. Genomic DNA and total RNA were prepared using an AllPrep DNA/RNA Micro Kit according to manufacturers' instructions (Qiagen). DNA from yolk sac tissue was extracted using lysis buffer and purified using the phenol-chloroform extraction method. Genotyping of

MommeD28 and *MommeD34* mice was performed using PCR followed by Sanger sequencing, as described previously [15].

Western Blotting: Protein lysates from whole fetal tissue were prepared by homogenizing in ten volumes of urea lysis buffer, as described [6]. Samples were quantified using BCA Assay (Thermo Scientific), separated on polyacrylamide gels (Bio-Rad) and immunoblotted. Clarity Western ECL substrate (Bio-Rad) and the MF Chemi-Bis (DNR Bio-imaging systems) was used for visualization. A custom made anti-Rlf polyclonal (Ab1) was used for experiments presented in this paper. The antibody epitope is EAIQEIAKVDCKDV, and occurs after the *MommeD28* mutation. Initial testing of Ab1 was performed on fetal head protein lysates from both wild-type and *Rlf* $^{MommeD28/MommeD28}$ embryos. Blots using the same protein lysates were also performed using a commercially available anti-RLF antibody (Abcam). A 250 kda band was detected with both the Ab1 and Abcam antibodies in wild-type samples. This band was absent in *Rlf* $^{MommeD28/MommeD28}$ protein samples, indicating that the band detected is RLF. Both antibodies also detected lower molecular weight bands unique to the particular antibody. These bands were present in both the wild-type and *Rlf* $^{MommeD28/MommeD28}$ samples, suggesting that the antibodies may be cross-reacting with proteins other than RLF. Western blots are shown in **SUPPL.FIG 1A**. anti-γ-tubulin was supplied by Sigma-Aldrich (T5192).

Histology: Whole embryos or placentas were collected in cold PBS, fixed in 4% paraformaldehyde at 4°C overnight and processed for paraffin embedding. Sections were stained with haematoxylin and eosin using standard procedures.

Cardiac morphology quantification: Histology slides were scanned to create digital images using an Aperio AT Turbo slide scanner (Leica) at 40X magnification. Left and Right ventricle field images were acquired in ImageScope (v12.1.0.5029) and saved as individual files. Area quantification was performed using Amira® software. Morphological tissue

segmentation was applied on a minimum of 3 consecutive histological sections per embryo and a minimum of 4 embryos per developmental stage analysed. Once the different tissues were extracted from the image, area quantification was performed. The data collected was plotted and statistically analysed using GraphPad Prism 6.

ISH: cDNA probes for *in situ* hybridization (ISH) analysis were isolated by using either a Minelute gel extraction kit (Qiagen) or PCR purification (Qiagen) and sequence verified by the Australian Genome Research Facility (AGRF). Probe sequences were as follows: Gcm1 F: CAT CTA CAG CTC GGA CGA CA, R: CCT TCC TCT GTG GAG CAG TC; Syna F: ATG GAG AAA CCC CTT ACG CT, R: TAG GGG TCT TTG TGT CCC TG; Tpbpa F: AAG TTA GGC AAC GAG CGA AA, R: AGT GCA GGA TCC CAC TTG TC; Mest F: GAG AGA GTG GTG GGT CCA AG, R: CGA TCA CTC GAT GGA ACC TC; Ctsq F: TTC ATT GGC CCA ATA CCC TA, R: GAA AGC TCC CAG AAT TCA CA. *Rlf* probe sequence will be provided on request. Digoxigenin (DIG) labelled cRNA probes were synthesized in accordance with manufacturer's directions (Roche) and as previously described [20]. ISH on 10 μm paraffin-embedded sections were undertaken as previously described [21]. Negative controls included the use of sense probes for all mRNA analysed, and positive controls included the detection of antisense probes in positive control tissues **(**See **SUPPL.FIG 1)**.

Immunostaining: the detection of the active form of the NOTCH1 receptor (N1ICD) and JAGGED1 (Cell Signaling) was performed following the protocol described [22]. Myocardial counterstaining was performed as described [23]. Histological images were obtained by confocal microscopy (Zeiss Axio LSM Imager.Z1).

Proliferation and apoptosis: proliferation analysis was performed by immunostaining for Ki67 (Abcam), a marker for actively cycling cells, as described [22]. Analysis for apoptotic

cells was performed by immunostaining for cleaved Caspase 3 (Cell Signaling), as described [22]. Histological images were obtained by confocal microscopy. Proliferation and apoptosis quantifications were performed using Amira® software. Morphological tissue segmentation was applied on a minimum of 3 consecutive histological sections per embryo and a minimum of 3 embryos at E13.5. Once tissue was segmented into trabecular and compact myocardium, proliferative/apoptotic nuclei were counted within each tissue. The ratio between the proliferative/apoptotic cells and the total number of cells was plotted and statistically analysed using GraphPad Prism 6.

Stereology: Volume densities of placental compartments were calculated using the test-point counting method as previously described [24]. Briefly, 7 µm sections through half of each placenta were used to calculate length and distance between sections, stained with *Tpbpa* and four sections evenly spaced between midline and outer edge per placenta used to calculate total placenta, labyrinth and junctional zone volumes. Maternal and Fetal blood space volumes were calculated from H&E stained placentas. Randomly selected fields of view (three fields per section, three sections per placenta, 70 µm apart, and 40 X magnification) from across the labyrinth were used.

RNA-sequencing: mRNA-seq library preparation (Illumina TruSeq RNA Sample Preparation kit; Illumina, San Diego, CA) and Illumina HiSeq 2000 Sequencing with 50 bp single-end reads was performed by the Australian Genome Research Facility (AGRF, Melbourne, VIC, Australia). Reads were aligned to the mouse genome (NCBI37/mm9) using Tophat [25] with the parameters -I 100000 --no-coverage-search --read-mismatches 2 --library-type fr-unstranded. Read counts for mRNA transcripts were extracted from the mapped reads using htseq-count [26] with the options -s no -m intersection-strict and using gene annotations from Ensembl (release 67). Differential gene expression was assessed using the R-package EdgeR, using default parameters [27].

Ingenuity Pathway and Upstream Regulator Analysis: Genes identified as differentially expressed (> 1.5 fold change, $p \leq 0.05$) were uploaded to Qiagens' Ingenuity Pathway Analysis (IPA, Qiagen, www.qiagen.com/ingenuity) and a core biological pathway analysis performed to identify molecular networks and upstream regulators. GOplot was used in order to better visualise the connections between the genes and the canonical pathways highlighted in the Ingenuity pathway and upstream regulator analysis [28].

Bisulphite Sequencing at Basp1 locus: Bisulphite conversion of 1 µg of genomic DNA was carried out using the EpiTect Bisulphite Kit (Qiagen). Nested PCR was performed to analyse methylation at the Basp1 region with the following primers: Basp1 F1: GAG GTT AAA AAG ATT GAG GTT TT; Basp1 R1: TCC AAT TTA AAA CAA ATT AAT ATA AAA TAA; Basp1 F2: GTT AAG GTA GAG GAG AAG GAG GT; Basp1 R2: AAT AAA ACA CAT CCT CTT TAT TTT T. Cycling conditions were as follows: primary and secondary PCR, 94°C for 2 minutes for 1 cycle; 94°C for 30 secs, 47°C for 30 secs, 72°C for 45 secs for 35 cycles and 72°C for 6 mins for 1 cycle. The PCR product was ligated into the pGEM T Easy Vector (Promega) and transformed. DNA from individual colonies was sequenced using Sanger sequencing. The bisulphite conversion was >97% and sequences analysed using the BiQ Analyser software [29]. The primers used for allelic sequencing of *Jag1* promoter region were: GGG AGA GAG ATT TTT ATT TTG GTT (fwd) and ACT CTT AAA AAA ACT TTA AAA AAT AAT AT (rev). Putative upstream enhancer regions of *Jag1* and *Jag2* were also analysed (see **SUPPL.FIG 7 and 8**). These regions were characterised by enrichment for H3K4me1 by ChIP-seq based on Histone Mods from ENCODE/LICR (UCSC). The primers used were: *Jag1* putative enhancer: AAT TTG GTT TTT TTT GGG TGG (fwd), CCA TCA TAC CCT AAT ATC TAA AAA AAT TAA (rev); *Jag2* putative enhancer: TGT TTT GTT TTT TTA GGA AGG TTT G (fwd), CCC TCA CTC AAA TCT ACA ACC TAA C (rev). PCR products were purified using MinElute PCR Purification Kit (Qiagen) and were cloned

into pGEM-T (Promega) and transformed into DH5α competent *E.coli* cells (Life Technologies). Plasmid DNA from individual colonies was purified using QIAprep Miniprep Kit (Qiagen) and sequenced. Sequences were aligned using Needleman-Wunsch alignment method with the minimum sequence identification cut-off of 80% and analysed for cytosine methylation status with the minimum conversion rate of 90% (BiQ Analyser).

Whole genome DNA methylation: DNA methylation was quantified by LC-MS/MS as described previously [30]. On average 400 ng of genomic DNA was used as an input for DNA hydrolysis using DNA Degradase Plus (Zymo). The reaction mixture was incubated at 37 °C for two hours to ensure complete digestion. Typically, sample of hydrolyzed DNA (5 mL) were diluted with 35 mL of 0.1 % formic acid and then five microliters injected onto a reverse phase ultra-performance liquid chromatography (UPLC) column (Zorbax Eclipse C18, 2.1 x 50 mm, 1.8 mm particle size, Agilent) equilibrated and eluted (100 mL/ min) with water/methanol/formic acid (95:5:0.1, v/v/v). The effluent was directed to an electrospray ion source (Agilent Jet Stream) connected to a triple quadrupole mass spectrometer (Agilent 6490 QQQ) operating in the positive ion mode using previously optimized conditions. Quantification of 2-deoxy-cytidine (2dC) and 5-methyl-2-deoxycytidine (5mdC) was by multiple reaction monitoring (MRM) using m/z 228 to 112 and 242 to 126.1 as the fragment ion transitions for 2dC and 5mdC, respectively. The measured percentage of 5mdC in each sample was calculated from the MRM peak area divided by the combined peak areas for 5mdC plus 2dC (total cytosine pool).

Statistical analysis: Statistical significance of quantitative data was determined by two-tailed Student's T-test. The proportions of genotypes from timed matings were compared to expected Mendelian ratios using a $\chi 2$ test. All graphs and statistics were performed using GraphPad Prism6 software.

RESULTS AND DISCUSSION

RLF is expressed in a number of tissues across development

Human *RLF* mRNA has been found to be expressed in a number of tissues including adult and fetal heart, brain, spleen, liver, and muscle [31]. We analysed RLF protein expression in a panel of protein lysates prepared from whole E10.5 WT mouse embryos, as well as from a range of tissues from E14.5 and P0 WT mice (**FIG 1A**). Western blotting identified an ~ 280 kDa band in all tissues analysed, including brain, heart and liver (**FIG 1A**). Lower molecular weight bands were also observed in brain and whole embryo lysates (~100 kDa) and heart tissue lysates (~150 kDa) (**FIG 1A**). In the mouse, *Rlf* is predicted to encode three protein variants with molecular weights of 12, 206, and 217 kDa. To determine which bands were specific for RLF, P0 $Rlf^{+/+}$, $Rlf^{MommeD28/+}$, and $Rlf^{MommeD28/MommeD28}$ heart and brain tissue lysates were analysed. Western blotting with a custom designed anti-RLF antibody (Ab1) showed the ~280 kDa band to be reduced in $Rlf^{MommeD28/+}$ mice and absent in $Rlf^{MommeD28/MommeD28}$ mice in both heart and brain (**FIG 1B**). No change in expression was observed for the lower molecular weight ~150 kDa band detected in heart or the ~100 kDa band detected in brain (**FIG 1B**). These results suggest that these lower molecular weight bands are unrelated to RLF, and not alternative RLF isoforms. These data are also consistent with our previous published studies using two commercially available RLF antibodies (Abcam Ab115011 and Abnova, M05, clone2G2), which showed that the ~280 kDa RLF protein is detected specifically in fetal head protein lysates of E14.5 WT embryos, but very low or undetectable in $Rlf^{MommeD28/MommeD28}$ or $Rlf^{MommeD34/MommeD34}$ homozygotes [6]. Post-translational modifications may explain the discrepancy in size between the predicted and

observed molecular weights for RLF. Taken together these Western blots show that RLF is widely expressed in early development.

Loss of *Rlf* reduces viability of offspring in the mouse

Previously we have reported that the number of viable $Rlf^{MommeD28/MommeD28}$ mice is significantly reduced at three weeks of age [6]. Here we analysed additional *MommeD28* heterozygous intercrosses to determine when lethality of $Rlf^{MommeD28/MommeD28}$ mice occurs. Genotyping of offspring at E9.5, E13.5, E14.5 and E18.5 showed no significant reduction in the number of $Rlf^{MommeD28/MommeD28}$ mice (**FIG 1C**). However, observations of $Rlf^{MommeD28/+}$ intercrosses, in which pups were kept in the breeding cage until weaning at three weeks, revealed that pups found dead in the cage were always less than one week old. These data on the *MommeD28* line are consistent with previously published data from the *MommeD34 Rlf* null line, in which the number of homozygous offspring from intercrosses at both one and three weeks of age was significantly less than expected [6]. Taken together our data suggests that *Rlf* homozygous null mutants are viable up until birth and that death in the majority of mutants occurs within the first week after birth.

***Rlf* mutants weigh less than their WT littermates**

To determine whether there were any gross differences in development between genotypes, the weights of E18.5 and E14.5 embryos were measured. E18.5 $Rlf^{MommeD28/MommeD28}$ embryos were found to weigh significantly less (0.731 g, ± 0.028) than their WT (1.144 g, ± 0.019) and heterozygous littermates (1.049 g, ± 0.020) (**FIG 1D**). This striking difference in weight was also observed visually following removal of embryos from the uterus and yolk sac (**FIG 1F**). A significant difference in weight was also observed comparing E18.5 $Rlf^{MommeD28/+}$ embryos with their $Rlf^{+/+}$ littermates (**FIG 1D**). Investigation of E14.5 $Rlf^{MommeD28/MommeD28}$ embryos

also found a small, but statistically significant reduction in weight (0.206 g, ± 0.005) compared to their WT (0.217 g, ± 0.004) and heterozygous (0.224 g, ± 0.004) littermates (**FIG 1E**), although this was not overtly obvious following removal of embryos from the uterus (**FIG 1G**). No difference in weight was observed comparing $Rlf^{MommeD28/+}$ and $Rlf^{+/+}$ embryos at E14.5. The reduced weight of *Rlf* mutants may be indicative of growth restriction, common underlying causes of which include abnormal cardiovascular or placental development.

Rlf mutants display defects in cardiac development

Many causes of perinatal lethality exist. Cardiovascular and pulmonary defects often become evident after birth as a result of the transition from fetal to neonatal circulation [32]. A number of recent studies found epigenetic modifiers to be important in controlling cardiac maturation and differentiation [33, 34]. Indeed mice carrying mutations in other epigenetic modifiers identified in our ENU mutagenesis screen are known to have heart defects. For example, *Pbrm1* (Polybromo 1) mutant mice display severe hypoplasia of the ventricular walls as well as ventricular septal defects [7]. Prompted by these findings we investigated whether *Rlf* mutant mice displayed defects in cardiac development.

Histological analysis of sections was performed on $Rlf^{MommeD28/MommeD28}$ mutant embryos at E9.5, E11.5, E13.5 and E14.5. Area quantifications of contributing heart tissues at these stages were then carried out. At E9.5, no morphological differences were observed in $Rlf^{MommeD28/MommeD28}$ compared to WT hearts (**SUPPL.FIG 2**). However, E11.5 mutant hearts showed a significant reduction in total heart tissue area compared to WT (**FIG 2A,E**). Segmentation of the organ into the different heart regions and quantification of each area separately showed that the defect was mainly localized to the ventricular chambers. However, only significant differences were observed in the trabecular myocardium area of the right

ventricle (**FIG 2F**). Tissue areas in other regions of the heart - inter-ventricular septum, auricles, and the atrio-ventricular canal were unchanged (**FIG 2F**). At E13.5, chamber defects were evident macroscopically as a thinner compact layer and a less mature or disorganised trabecular myocardium in mutants (**FIG 2B-D**). Quantifications showed no significant changes in total heart area at E13.5 (**FIG 2E**), but a clear increase in ventricular trabecular area in LV and RV, and a decrease in the area of the compact layer in both ventricles (**FIG 2B-D,G**). The significant increase in the trabecular myocardial area in both ventricles, in contrast to the reduction of trabecular area at E11.5, suggests hypertrabeculation (**FIG 2C,D,G**). Formation of the trabeculae-like pectinate muscles of the right and left atrial chambers, the inter-atrial septum primum and the inter-ventricular valves, appeared grossly normal at E13.5 (**SUPPL.FIG 3A,B**). Analysis of E14.5 $Rlf^{MommeD28/MommeD28}$ hearts confirmed the observations described at E13.5 – presence of a thin compact layer in left and right ventricles, and less mature, more disorganised trabeculae, including the presence of deep trabecular crypts (**FIG 2H,I,J;** see brackets in panel I). Morphological quantification confirmed the reduction in compact myocardium thickness and the significant increase in trabecular myocardial area (**FIG 2J**). In a recent publication, Captur et al. described trabecular compaction as involving a process of trabecular complexity simplification by trabecular fusion [35]. Trabecular complexity, in this setting, involves its 3D qualities including the degree and orientation of branching. Thus, the apparent qualitative differences in trabecular myocardium between $Rlf^{MommeD28/MommeD28}$ mutant and WT hearts might be explained by differences in trabecular complexity. A subset of mutants also displayed a fenestrated interventricular septum (n=7/12), although no peri-membranous ventricular septal defects, as found in another mouse model of LVNC [35], were evident in these hearts (**SUPPL.FIG 3C-F**). At all stages analysed, the RV was more severely affected than the left ventricle (LV).

These results are consistent with an underlying defect in expansion of the outer (compact) layer and in the process of simplification of trabeculation during maturation of the ventricular chamber walls. During development, the trabecular myocardium is critical for force generation, fast conduction of action potentials throughout the ventricles, and exchange of oxygen and nutrients in the absence of a coronary circulation. Development of the coronary vasculature occurs within the growing compact layer and marks a key transition allowing an increase in chamber wall thickness. During ventricular chamber maturation, the complex spongy-like trabecular myocardium network is simplified and drawn into the compact layer, a process called trabecular compaction [17]. In humans, dysregulation of chamber wall thickening and/or trabecular compaction leads to the thin walled, hypertrabeculated phenotype seen in LVNC. Patients with LVNC also often have ventricular septal defects [36, 37]. These human phenotypes are highly reminiscent of those observed in *Rlf* mutants and in other mutant mouse models [18, 38]. Our results provide the first evidence that RLF plays a role in heart development and more precisely in the processes of compact layer expansion and ventricular compaction during chamber maturation.

The *Rlf* mutant chamber phenotype is associated with proliferation defects in compact myocardium

In order to further investigate the causes of the myocardial defects in $Rlf^{MommeD28/MommeD28}$ mutants, we performed proliferation and apoptosis studies at E13.5. KI67 immunostaining detecting cycling cells, and subsequent determination of the proliferation rate by tissue segmentation and quantification, revealed a slight but non-significant increase in trabecular myocardium proliferation, together with a striking decrease in proliferation in compact myocardium (**SUPPL. FIG 4 A-B', E**). Similar analysis and quantification of the cleaved form of CASPASE-3, induced in apoptotic cells, showed a significant reduction in compact myocardium of the RV (**SUPPL. FIG 4 C-D',F**). The lack of a significant increase in

proliferation in the trabecular layer suggests that the hypertrabeculation phenotype described at E13.5-E14.5 above is due to defective compaction and/or simplification, and not to excessive proliferation of the trabecular myocardium. Defects in these and potentially other processes together with defective proliferation of the compact myocardium likely cause the thinner ventricular walls observed in the $Rlf^{MommeD28/MommeD28}$ mutants.

Defects in cardiac development are observed in an independent *Rlf* mutant line

To confirm whether cardiac defects observed in $Rlf^{MommeD28/MommeD28}$ mice are attributable to loss of RLF, we investigated the independent *Rlf* null mouse line, *MommeD34*. Histological analysis of E14.5 $Rlf^{MommeD34/MommeD34}$ embryos showed a similar cardiac phenotype to that observed in $Rlf^{MommeD28/MommeD28}$ embryos (**FIG 2K,L)**. Morphological quantification of the ventricular walls of $Rlf^{MommeD34/MommeD34}$ hearts revealed them to be significantly thinner than those of $Rlf^{+/+}$ hearts (**FIG 2M**). Observations of cardiac defects in both $Rlf^{MommeD28/MommeD28}$ and $Rlf^{MommeD34/MommeD34}$ embryos further supports the hypothesis that *Rlf* is critical for normal heart chamber development.

***Rlf* is expressed in endocardium and epicardium, and transiently in myocardium, during development.**

Western blotting analysis (**FIG 1A,B**) clearly showed expression of RLF in the heart at different developmental stages. However, its specific expression in the different heart lineages remains unknown. In order to investigate where *Rlf* is expressed in the heart, we performed a time course expression analysis using *in situ* hybridization (ISH) on sections. In general, *Rlf* was expressed widely during embryonic development (data not shown). At E8.5, *Rlf* expression appeared homogeneous in the endocardium (**FIG 3A**, arrow). In the myocardium, *Rlf* was expressed more strongly in working myocardium of the atrial and ventricular chamber regions (**FIG 3A**, white arrow), being low or absent in the non-chamber

myocardium of the atrioventricular canal and in the inter-ventricular region. By E9.5, *Rlf* was strongly expressed in the proepicardium (**FIG 3B**, arrowhead) and endocardium (**FIG 3B**, arrow). Myocardial expression was observed across both left and right ventricles and in both compact and trabecular myocardial layers, occurring in a somewhat patchy pattern (**FIG 3B**, white arrows). At E10.5, there was expression in endocardium (**FIG 3C-C'**, arrow), valve mesenchyme and epicardium (**FIG 3C-C'**, arrowhead). From this stage, the expression in the myocardium became stronger (**FIG 3C-C'**, white arrows), peaking at E11.5 (**FIG 3D,D'**) before fading progressively at E12.5 (**FIG 3E,E'**) whereas expression in endocardium and epicardium expression was maintained. At E14.5 also, expression in the endocardium (**FIG 3 F-F'''**, arrow) and epicardium (**FIG 3F-F'''**, arrowhead) was still evident. However, there was very low expression in myocardium across the whole heart at this stage. While expression in endocardium associated with the atrioventricular valve leaflets was evident, expression in valvular mesenchyme seen earlier at E10.5 (**FIG 3C**), E11.5 (**FIG 3D**) and E12.5 (**FIG 3E**), had diminished. As an apparent repressor of DNA methylation, the reduction in expression of *Rlf* in myocardium between E10.5 and E14.5 suggests an important transition in heart development after which gene silencing through increased DNA methylation plays an increasingly important role. In contrast, the relative lack of change in the expression of *Rlf* in endocardium and epicardium, suggests the maintenance of low DNA methylation and potentially more plastic states in these tissues. Further genome-wide studies in specific cardiac tissues are required to investigate evolving patterns of RLF expression and DNA methylation, and their relative biological importance during embryogenesis and cardiac development.

Loss of RLF does not affect placental or lung morphology

Structural changes in the placenta, leading to altered hemodynamics or surface area available for nutrient exchange, have been shown to result in reductions in growth, heart defects and

perinatal morbidity [39, 40]. Publicly available RNAseq datasets indicate that *Rlf* is expressed in the placenta [41]. Given this, we investigated whether loss of *Rlf* altered the morphology of the placenta in $Rlf^{MommeD28/MommeD28}$ conceptuses. The mouse placenta is made up of three zones – the maternal decidua, junctional zone and labyrinth zone, in which the maternal and fetal vasculature is closely intertwined [42, 43].

Stereological analysis of $Rlf^{MommeD28/MommeD28}$ placentas found no significant difference in total placenta, placental zone (junctional and labyrinth) volumes, or maternal and fetal blood space ratio compared to $Rlf^{+/+}$ placentas (**FIG 4A**). Investigation of markers specific to the junctional zone (*Tbpba*) and labyrinth cell types (*Ctsq, Gcm1, Syna¸ Mest*) found expression in the expected patterns and at similar levels in both genotypes (**FIG 4B**).

Currently, we cannot exclude a subtle placental defect; however, if present, a placental defect of the nature reported by others in the literature to underlie cardiac defects should have been detected by our analysis. For example, abnormal placental vascularisation in mice deficient in *Pparγ* (Peroxisome proliferator-activated receptor γ) has been shown to underlie the thinning of the ventricular walls observed in these animals [44]. When *Pparγ* null morula embryos were aggregated with tetraploid morulae (which can give rise to a normal placenta in such chimaeras), the cardiac defect was corrected [44]. Similar studies in *Rlf* mutants would be needed to definitively rule out a placental origin for the observed heart defect; however, the lack of gross morphological placental differences between $Rlf^{+/+}$ and $Rlf^{MommeD28/MommeD28}$ conceptuses and the dynamic expression of *Rlf* in the developing heart, supports a model in which loss of *Rlf* expression in the heart leads directly to cardiac phenotypes.

Developmental defects in lung morphogenesis and/or the function of the multiple epithelial and mesenchymal cell types that make up the lung [45], may also affect postnatal growth and lethality. We examined histological sections of the lung at E13.5 (not shown) and E14.5

(**SUPPL. FIG 3G,H**) and did not see any gross morphological defects in mutants. While we cannot exclude functional or more subtle anatomical defects, it seems likely that the cause of death in *Rlf* mutants is related to the cardiovascular defects described above and that loss of RLF is directly related to these cardiac malformations.

Loss of RLF affects methylation in the fetal heart

Our previous studies using whole genome bisulphite sequencing of E14.5 fetal liver DNA showed that loss of RLF results in increased methylation at hundreds of sites across the genome [6, 15]. Comparison of our data to publicly available ChIP-seq data sets showed that a sub-set of differentially methylated regions (RLF DMRs) occur at sites that are likely to be inactive enhancers in the normal fetal liver, but active enhancers in other tissues.

Here we investigate a candidate regulatory site to determine whether RLF influences methylation in heart tissue. We chose a region which we have previously shown to be increased in methylation in $Rlf^{MommeD28/MommeD28}$ fetal liver DNA, compared to WT liver DNA. This region is located on mouse chromosome 15, over the last exon of the gene for Brain abundant, membrane attached signal protein 1 (*Basp1*) which is enriched in H3K4me1 in E14.5 fetal hearts, suggesting that the site may act as a cardiac enhancer. Bisulphite sequencing of this region revealed increased methylation of CpGs in $Rlf^{MommeD28/MommeD28}$ fetal heart DNA (70 %) compared to WT DNA (45 %) (**FIG 5A**). Total genome levels of cytosine methylation were also assessed in DNA extracted from whole E13.5 fetal hearts after hydrolysis and analysis using liquid chromatography/mass spectrometry (LC/MS) relative to 2-deoxycytosine (unmethylated) and 5-methyl 2-deoxycytosine (methylated) nucleoside standards (n=4 per genotype). No differences in global DNA cytosine methylation were detected between WT, heterozygous or homozygous $Rlf^{MommeD28}$ samples (**FIG 5B,C**).

Dynamic changes in cytosine DNA methylation are known to occur during cardiomyocyte development and maturation and also in cardiac disease [46]. Our results show that loss of RLF is associated with increased cytosine methylation at a select cardiac gene locus; however, further studies are necessary to identify the genome-wide effects of *Rlf* mutation on DNA methylation at other cardiac loci and whether such changes underlie the cardiac phenotypes observed in the *Rlf* homozygotes.

Attenuation of the NOTCH signalling pathway underlying the *Rlf*$^{MommeD28/MommeD28}$ mutant phenotype

In order to more clearly define how loss of *Rlf* may alter cardiac development, we analysed the transcriptome of whole fetal hearts from E13.5 *Rlf*$^{MommeD28/MommeD28}$ and *Rlf*$^{+/+}$ embryos using RNA-seq (n = 4 per genotype). The R package EdgeR and Ensembl transcript annotation, NCBI37/mm9, were used to identify differentially expressed genes. Applying a fold change cut-off of 1.2 and an adjusted p value ≤ 0.05; 511 genes were found to be differentially expressed in *Rlf*$^{MommeD28/MommeD28}$ fetal hearts (285 genes downregulated, 226 genes upregulated, **FIG 6A**). Analysis of up- and downregulated genes found them to be associated with several pathways including NOTCH signalling and cardiac hypertrophy. NOTCH pathway genes found to be altered by *Rlf* mutants included those encoding JAGGED1, JAGGED2, HEY1, CNTN1 and DLL1 (**FIG 6B, Supplementary Table 1**). Ingenuity's Upstream Regulator Analysis identified several inhibited upstream regulators including, NOTCH1 (z score = -2.356), SRF (z score = -2.327), a cardiac transcription factor, and SMARCA4/BRG1 (z score = -2.132), a member of the SWI/SNF ATPase chromatin modifier. Activated upstream regulators included RNF2 (z = 2.236), NRL|2 (z = 2.219), HDAC2 (z score = 2.219) and RARA (z score = 2.449) (**FIG 6C and data not shown**). Genes for VEGFA (*Vegfa*) and CONEXIN40 (*Gja5*), important for heart chamber development, were also found to be differentially expressed (**Supplementary Table 1**).

qPCR analysis on whole heart samples at E13.5 confirmed downregulation of a selection of genes found to be strongly downregulated by RNA-seq (*Aldh1a7*, *Apoc1*, *Tex11*, *Cpa1*) (**SUPPL. FIG 6A-D**). However, only non-significant changes were seen for *Vegfa* and *Gja5*, and other cardiac genes tested (*Wt1*, *Bmp10*, *Hey1*, *Jag1*, *Jag2*) (**SUPPL FIG 6 E-K**). This may be due to the modest changes in expression levels of these genes between mutants and WT, and their spatiotemporally restricted expression patterns.

However, the identification of the NOTCH pathway as a potential upstream regulator of genes differentially expressed in *Rlf* mutant hearts is of particular note. Work from others has shown that NOTCH1 membrane signalling receptor is required for normal ventricular development [47] and, in mice, inactivation of *NOTCH1* or the gene for the NOTCH pathway regulator Mindbomb homolog 1 (*Mib1*), results in LVNC [18]. *MIB1* mutations have also been shown to cause LVNC in humans [18]. A recent publication described the NOTCH ligand DELTA4 as being important during early stages of trabecular development, whereas related ligands JAGGED1 and JAGGED2 play important roles during the subsequent compaction and maturation phases of chamber development [38]. In the same study, the genetic deletion of *Jag1* and *Jag2* in mice induced phenotypes typical of human LVNC. As noted above, qPCR analysis of whole heart RNA samples did not show significant differences in the expression of the NOTCH pathway genes *Hey1*, *Jag1* and *Jag2* (**SUPPL. FIG 6I-K**). However, the central role of NOTCH signalling in heart chamber development and the dysregulation of NOTCH1 pathway genes in *Rlf* mutants, and in LVNC in humans and animal models, prompted us to investigate the activity of the NOTCH pathway further by examining expression of the NOTCH ligand JAGGED1 and the cleaved, active form of the NOTCH1 receptor (N1ICD; NOTCH1 intracellular domain), by immunofluorescence. The level of N1ICD in the nucleus provides a surrogate readout of NOTCH1 signalling activity.

At E11.5, prior to the appearance of the hypertrabeculation phenotype, the overall spatial expression of JAGGED1 in trabecular myocardium and N1ICD in endocardium in *Rlf* mutant hearts appeared normal, although there was a clear reduction in the intensity of staining for both N1ICD and JAGGED1 compared to WT hearts (**SUPPL FIG 5A-B'**). At E13.5 also, only low levels of JAGGED1 and N1ICD were detected in the mutant left and right ventricles (**FIG 7A-D'**). These results support the dysregulation of NOTCH1 pathway genes seen in the RNAseq analysis. Specifically, they suggest that the downregulation of NOTCH pathway signalling (observed as reduced N1ICD levels by immunostaining) in *Rlf* mutants may be due at least in part to downregulation of the expression of *Jag1* and *Jag2* (encoding NOTCH ligands JAGGED1 and JAGGED2) observed by the RNA-seq analysis. As noted above, D'amato et al. proposed that the NOTCH1 receptor was induced by the DELTA4 ligand, expressed in endocardium during the early phases of ventricular development (until ~E11.5), after which JAGGED1 and JAGGED2, expressed from myocardium, became the dominant ligands for chamber maturation and compaction [38]. In our study, the reduction of N1ICD was evident at E11.5 and more severe at E13.5, suggesting that the downregulation of the transcripts for *Jag1* and *Jag2* in *Rlf* mutants contributes to the reduced levels of JAGGED1 and JAGGED2 ligands, perturbing the normal switch from DELTA to JAGGED ligand activation of NOTCH1 signalling, and leading to ventricular maturation and LVNC-like defects.

Our previous analysis of changes in the methylation status of DNA in WT and *Rlf* mutant livers demonstrated that loss of RLF leads to an increase in cytosine methylation at select CpG islands and regulatory regions across the genome [6]. While methylation status at *Jag1* and *Jag2* was not dependent on RLF in liver, we investigated whether increased methylation could be involved in the downregulation of *Jag1* and *Jag2* in *Rlf* mutant hearts. We analysed DNA methylation at putative enhancer regions upstream of *Jag1* (**SUPPL.FIG 7**) and *Jag2*

(**SUPPL. FIG 8**) in whole WT and *Rlf*$^{MommeD28/MommeD28}$ hearts at E13.5 using bisulphite sequencing. Regions for analysis were identified as the coincident peaks of H3K4me1 in E14.5 and 8 week-old hearts as seen in publically available ChIPseq data. Results showed no striking differences in the methylation pattern across these potential *Jag1* and *Jag2* enhancers in *Rlf* mutants. We also analysed a region spanning the *Jag1* promoter, which is embedded within a CpG island (**SUPPL.FIG 7**), but again saw no difference. These results suggest that RLF may have an indirect role in the downregulation of *Jag1* and *Jag2* in *Rlf* mutants, although we cannot rule out hypermethylation over other regulatory elements. Further investigation is needed to determine the specific mechanisms through which loss of RLF impacts the NOTCH pathway.

CONCLUSION

Congenital heart disease (CHD) is the most common birth defect from non-infectious causes, affecting ~1% of live births; however, the underlying genetic cause is not known in the majority of cases [1]. Recently, the contribution of the epigenetic state of the genome to CHD has begun to emerge [48, 49]. Mutations in genes involved in the establishment and/or maintenance of epigenetic states have been identified in syndromes with heart abnormalities [2] and an increased burden of *de novo* mutations in histone modifying genes has been identified in patients with sporadic CHD [3]. Developmental heart defects are also observed in mice null for a wide range of epigenetic modifiers [5, 7, 8].

Here we show, in two independent mouse lines, that loss of the epigenetic modifier RLF leads to ventricular chamber growth and compaction defects resembling LVNC. The lack of any significant changes in placental and lung morphology suggests that the cardiac defects described may be a direct effect of loss of *Rlf* expression in the heart. Our transcriptome and

immunofluorescence data also point to the LVNC phenotype in mutants being caused at least in part by inactivation of the NOTCH1 pathway through the downregulation of JAGGED1 (and potentially JAGGED2) ligand expression. Our previous studies carried out in non-cardiac tissues have shown that RLF is necessary for maintaining cytosine hypomethylation at hundreds of regulatory regions across the genome, in particular at enhancers and CpG island shores [15]. Further studies are needed to define the molecular mechanism via which this occurs, and to uncover how RLF influences the epigenome during cardiac development and how this drives the hypertrabeculation and compaction defects observed in *Rlf* mutant mice. The ENU mutants described here involve global knock-down of RLF. Conditional *Rlf* knock-outs are needed to identify the cell types involved in the cardiac phenotypes observed in the *Rlf* mutant lines and to determine whether the heart defect underlies the postnatal lethality in these lines. This information may provide new insights into the aetiology of LVNC in humans and provide new tools to analyse the many layers of epigenetic regulation in development and organogenesis.

FIGURE LEGENDS

Figure 1: Rlf is expressed throughout development and loss of *Rlf* alters viability and weight of mice

A. Western blotting shows RLF protein expression (▶) in a number of fetal and postnatal (P0) tissues. Non-specific bands indicated by open triangle (▷). **B.** Western blotting of P0 $Rlf^{+/+}$(+/+), $Rlf^{MommeD28/+}$ (*D28*/+) and $Rlf^{MommeD28/MommeD28}$ (*D28/D28*) heart tissue. γ-tubulin was used as a loading control for all Western blots. **C**. $Rlf^{MommeD28}$ mice; data shows the number of mice observed (percentage in brackets) at E9.5, E13.5, E14.5 and E18.5 and at postnatal week three [6]. **D, E**. Embryonic weights of E18.5 (**D**) and E14.5 (**E**) offspring from $Rlf^{MommeD28}$ heterozygous intercrosses. Homozygous embryos show a significant decrease in weight versus WT littermates at both time-points. n = 7 litters (E18.5), 12 litters (E14.5). **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$. Weights for each litter were normalised to the average weight of WT littermates, and only litters with more than one WT embryo were included. **F, G**. Representative $Rlf^{+/+}$ and $Rlf^{MommeD28/MommeD28}$ littermates at E18.5 (**F**) and E14.5 (**G**) found an observable difference in size between genotypes at E18.5 only. Scale bar = 1 cm (E18.5), 5mm (E14.5).

Figure 2: *Rlf* mutant mice display ventricular defects from E11.5

A-D). Time-course morphological analysis by Haematoxylin and Eosin (H&E) staining of $Rlf^{+/+}$(WT) and $Rlf^{MommeD28/MommeD28}$ (*D28/D28*) embryo sections at E11.5 (**A**, n=36) and E13.5 (**B-D**, n=10). **A.** At E11.5, loss of *Rlf* results in reduced heart size. **B.** At E13.5, heart size is normal but there is defective trabecular and compact myocardium development. **C.** Higher magnification from B showing the trabecular and compact myocardium defects in the right ventricle (RV). **D.** Higher magnification from B showing the trabecular and compact myocardium defects in the LV. **E.** Morphological quantification of the total heart area at

E11.5 and E13.5. **F.** Morphological quantification of the area from different heart regions normalized to the total heart area at E11.5 localising the heart defects mainly to the ventricular myocardium of the *Rlf* $^{MommeD28/MommeD28}$ *(D28/D28)* mutants compared to WT. **G.** Morphological quantification of the different ventricular regions normalized to the total heart area at E13.5 showing the reduction of the compact myocardium area and the increase of the trabecular myocardium area in the *D28/D28* mutant hearts compared to WT. n=4 per genotype for E11.5 and n=5 per genotype for E13.5. **H-L.** Comparative morphological analysis by Haematoxilin and Eosin staining between *Rlf*$^{MommeD28/MommeD28}$ (n=12) and *Rlf*$^{MommeD34/MommeD34}$ (*D34/D34*) (n=6) embryos and their WT littermates (n=11; n=5) at E14.5. Note the similarities between the *Rlf*$^{MommeD28/MommeD28}$ and *Rlf*$^{MommeD34/MommeD34}$ mutant hearts in the reduced compact myocardium thickness. Boxed sections in **H** and **K** are shown at higher magnification in **I** and **L**. **J,M.** Morphological quantification of *Rlf*$^{MommeD28/MommeD28}$ and *Rlf*$^{+/+}$ hearts (**J**), and *Rlf*$^{MommeD34/MommeD34}$ and *Rlf*$^{+/+}$ hearts (**M**), showing the reduction of the compact myocardium area and the increased trabecular myocardium area in both right and left ventricles compared to WT. Both mutants showed a significant reduction in the IVS area. n = 12 per genotype (J) and n = 6 per genotype (M). *$p < 0.05$. Scale bar = 300µm (A), 390µm (B), 400 µm (H and I), and 100 µm (C, D, J and K).

Figure 3: *In situ* hybridisation analysis of *Rlf* mRNA expression during heart development

A-F’’’. *Rlf in situ* hybridization on paraffin sections at different developmental stages. **A.** At E8.5, *Rlf* is expressed in the endocardium (arrow) and in the chamber myocardium (white arrow). a=atrium, lv=left ventricle. **B.** At E9.5, *Rlf* is strongly expressed in the proepicardium (pe, arrowhead) and the endocardium (arrow), and presents patchy expression in cardiomyocytes of both trabecular and compact myocardial layers (white arrow). **C.** *Rlf* expression at E10.5 is evident in the myocardium (white arrow), endocardium (arrow) and

epicardium (arrowhead). rv=right ventricle. **C'.** High magnification from C showing *Rlf* expression in the endocardium (arrow) and the myocardium (white arrows) of the left ventricle. **D-D'.** *Rlf* expression in the myocardium (white arrow), endocardium (arrow) and epicardium (arrowhead) at E11.5. **D'.** High magnification from D showing *Rlf* expression in the left ventricle. **E-E'.** *Rlf* expression in the myocardium (white arrow), endocardium (arrow) and epicardium (arrowhead) at E12.5. **E'.** High magnification from E showing *Rlf* expression in the left ventricle. **F-F'''**. At E14.5, *Rlf* myocardial expression is no longer detectable but remains expressed in the endocardium (arrow) and epicardium (arrowhead), as shown in the general view (**F**), and in the higher magnification images of the right atrium (ra, **F'**), atrioventricular valves (avv, **F''**) and right ventricle (**F'''**). Scale bar = 95μm (A), 130μm (B), 200μm (C), 120μm (C'), 300μm (D), 60μm (D') 400μm (E), 110μm (E'), 500 μm (F), 80μm (F'-F''').

Figure 4: No gross morphological differences in placental morphology are observed in *Rlf*$^{MommeD28/MommeD28}$ placentas

A. Volume of total placenta, junctional zone and labyrinth zone in *Rlf*$^{+/+}$ and *Rlf*$^{MommeD28/MommeD28}$ showing no difference in total placenta or placental zone volumes in *Rlf*$^{MommeD28/MommeD28}$ placentas. *n* = 4 per genotype. **B.** *In situ* hybridizations for the labyrinth markers *Gcm1, Syna, Ctsq* and *Mest,* and the junctional zone marker *Tpbpa* found all markers to be present in both genotypes. *n* = 4 per genotype. Scale bar = 900 μm (blue) and 200 μm (red).

Figure 5: Bisulphite sequencing of *Basp1* region on Chromosome 15 reveals an increase in DNA methylation in *Rlf*$^{MommeD28/MommeD28}$ compared to WT E13.5 heart.

A. Bisulphite sequencing of *Basp1* region on Chromosome 15 reveals an increase in DNA methylation in *Rlf*$^{MommeD28/MommeD28}$ compared to WT fetal heart. Each column represents

DNA from a single embryo (n = 2 for each genotype), each row is the sequence from a single cell and each circle represents one CpG site. Black circles represent methylated CpGs, white circles represent unmethylated CpGs, and lines (-) represents an ambiguously sequenced position where a CpG exists in the genomic sequence. **B.** Representative LC-MS/MS chromatograms of 2-deoxycytidine (2dC) and 5-methyl-2-deoxycytidine (5mdC) derived from genomic DNA of $Rlf^{+/+}$, $Rlf^{+/-}$ and $Rlf^{-/-}$ embryos. Bottom chromatogram represents a standard mixture of 25 nM 2dC and 5mdC. **C.** Whole-genome 5-methylcytosine (m5C) content in 13.5 dpc embryonic heart DNA from control, RLF homozygous and RLF heterozygous mutant mice as assessed by LC/MS (n = 4 per group). Error bars indicate SEM

Figure 6: Transcriptome analysis shows dysregulation of the NOTCH signalling pathway in the *Rlf*$^{MommeD28/MommeD28}$ mutant hearts

A. Schematic showing the number of differentially expressed genes following RNA-seq analysis of whole E13.5 fetal hearts from the *MommeD28* line. **B.** GOplot representation of the analysis of canonical pathway enriched in the 511 differentially expressed genes in $Rlf^{MommeD28/MommeD28}$ fetal hearts. *P* value, Benjamini-Hochberg-adjusted *P* value. **C.** Putative upstream regulator analysis showing activated upstream regulators (red) and inhibited upstream regulators (blue), predicted utilising differentially expressed genes identified following RNA-seq analysis (511 genes).

Figure 7: Molecular analysis confirmed the downregulation of the Notch pathway.

A-B'. Immuno-staining showing the reduction of JAGGED1 expression in the $Rlf^{MommeD28/MommeD28}$ mutants (**A', B'**, arrowhead) compared to WT (**A, B** arrow) at E13.5 in both the right (**A-A'**) and left (**B-B'**) ventricles. **C-D'.** Immunostaining showing the absence of the active form of the NOTCH1 receptor (N1ICD) in the $Rlf^{MommeD28/MommeD28}$ mutants (**C',D'**, arrowhead) compared to WT (**C,D**, arrow) at E13.5 in both the right (**C-C'**) and left

(**D-D'**) ventricles. In all immunostainings: marker of interest (red), nuclei (blue), myocardium (green). Scale bar = 70µm (**A-D'**)

Supplementary Figure 1: Western blot and ISH controls

A. Western blots showing initial testing of Ab1 on fetal head protein lysates from both wild-type and *Rlf* $^{MommeD28/MommeD28}$ embryos. Western blots using a commercially available anti-Rlf antibody (Abcam) were also performed for comparison. ▶ indicates the Rlf specific band; Non-specific bands detected with Ab1 (▷) or the Abcam antibody (▷▷) are also indicated **B.** Coomassie stained membranes for Western blots present in Figure 1A (upper membrane) and Figure 1B (lower membrane) demonstrating protein loading **C-F'.** ISH controls by comparing the expression pattern obtained with antisense and sense probes for *Rlf* at E10.5 (**C,C'**), E11.5 (**D,D'**), E12.5 (**E,E'**) and E13.5 (**F, F'**). a=atrium, rv=right ventricle, lv=left ventricle. Scale bar = 200µm (C,C'), 300µm (D-E'), 500 µm (F,F').

Supplementary Figure 2: E9.5 Morphological analysis

A-D. Haematoxylin and eosin staining of *Rlf*$^{MommeD28/MommeD28}$ embryos at E9.5 showing no morphological differences compared to WT. **A-B.** Lower magnification of a histological section of a WT (**A**) and a *Rlf*$^{MommeD28/MommeD28}$(**B**) hearts. **C-D**. Higher magnification of a histological section of a WT (**C**) and a *Rlf*$^{MommeD28/MommeD28}$(**D**) left ventricle (lv). a=atrium, rv=right ventricle. Scale bar = 120µm (A, B) and 60µm (C, D).

Supplementary Figure 3: E14.5 Morphological Analysis

A,B. Haematoxylin and eosin staining of representative WT (**A**) and *Rlf*$^{MommeD28/MommeD28}$ (**B**) embryos at E13.5 showing the absence of defects in the atrioventricular valves (arrows), atrial septum (arrowheads) and atrial appendages (white arrows). **C-F.** Haematoxylin and eosin staining of representative WT and *Rlf*$^{MommeD28/MommeD28}$ embryos at E14.5 indicating

fenestrations (F) in the inter-ventricular septum of *Rlf*$^{MommeD28/MommeD28}$ embryos. **C-D.** Lower magnifications of a histological section of a WT (**C**) and *Rlf*$^{MommeD28/MommeD28}$ (**D**) hearts. **E-F.** Higher magnification of a histological section of a WT (**E**) and a *Rlf*$^{MommeD28/MommeD28}$ (**F**) inter-ventricular septum (ivs). **G-H.** Haematoxylin and eosin staining of representative WT and *Rlf*$^{MommeD28/MommeD28}$ embryos at E14.5 showing the absence of macroscopic defects in the lungs. Scale bar= 450µm (A,B), 500µm (C,D), 200µm (E,F) and 700µm (G,H). rv=right ventricle, lv=left ventricle, ivs=inter-ventricular septum, l=lungs.

Supplementary Figure 4: Proliferation and Apoptosis Analysis.

A-B'. Proliferation analysis by KI67 immunostaining on the trabecular (arrow) and compact myocardium (arrowhead) of the ventricular chambers of *Rlf* mutants at E13.5 compared with WT. **(A, A')** Right ventricle (RV) and **(B,B')** Left ventricle (LV). **C-D'**. Apoptosis analysis by cleaved CASPASE-3 immunostaining on the trabecular (arrow) and compact myocardium (arrowhead) of the ventricular chambers of *Rlf* mutants at E13.5 compared with WT. (**C, C'**) Right ventricle (RV) and (**D,D'**) Left ventricle(LV). **E**. Proliferation quantification shown as the percentage of proliferating cells calculated as the ratio between the KI67 positive cells and the total number of cells in the trabecular myocardium and the compact myocardium. **F**. Apoptosis quantification shown as the percentage of apoptotic cells calculated as the ratio between the c-Caspase-3 (cCas3) positive cells and the total number of cells in the trabecular myocardium and the compact myocardium. In all immune-stainings: marker of interest (red), nuclei (blue), myocardium (green). Scale bar = 70µm (**A-D'**)

Supplementary Figure 5: Molecular analysis of *Rlf* mutants at E11.5

A-A'. Immunostaining showing the reduction in intensity of JAGGED1 in the *Rlf*$^{MommeD28/MommeD28}$ mutants (**A'**, arrowhead) compared to WT (**A**, arrow) at E11.5. **B-B'.** Immunostaining showing the reduction in intensity of the active form of the NOTCH1

receptor (N1ICD) in the *Rlf*$^{MommeD28/MommeD28}$ mutants (**B'**, arrowhead) compared to WT (**B**, arrow) at E11.5. In all immunostainings: marker of interest (red), nuclei (blue), myocardium (green). Scale bar = 60µm (A-B')

Supplementary Figure 6: qPCR analysis

A-K. qPCR analysis of the differentially expressed genes by RNAseq in whole heart samples at E13.5. **A.** *Aldh1a7*. **B.** *Apoc1*. **C.** *Tex11*. **D.** *Cpa1*. **E.** *Vegfa*. **F**. *Gja5*. **G.** *Wt1*. **H.** *Bmp10*. **I.** *Hey1*. **J.** *Jagged1*. **K.** *Jagged2*.

Supplementary Figure 7: DNA methylation analysis of Jagged1 regulatory regions

A. Schematics of the *Jagged1* loci showing the regions interrogated by allelic bisulphite sequencing, including transcription start site, CpG island and heart 8w and E14.5 H3K4m1 signals from ENCODE/LICR. Modified from images derived from UCSC genome browser. **B-C.** Bisulphite allelic sequencing profiles of promoter (**B**) and enhancer (**C**) regions from 13.5 dpc wild-type and *Rlf*$^{MommeD28/MommeD28}$ mouse heart DNA (n=3 per group).

Supplementary Figure 8: DNA methylation analysis of Jagged2 regulatory region.

A. Schematics of the *Jagged2* loci showing the regions interrogated by allelic bisulphite sequencing, including transcription start site, CpG island and heart 8w and E14.5 H3K4m1 signals from ENCODE/LICR. Modified from images derived from UCSC genome browser. **B.** Bisulphite allelic sequencing profiles of enhancer regions from 13.5 dpc wild-type and *Rlf* $^{MommeD28/MommeD28}$ mouse heart DNA (n=3 per group).

Supplementary Table 1: Differential expression RNA-seq analysis

Differential expression analysis comparing RNA-seq datasets from *Rlf*$^{+/+}$ mice to *Rlf*$^{MommeD28/MommeD28}$ mice.

CONTRIBUTIONS:

SKH conceived the study and project design and secured funding for the project. LMB, GDM, RPH, and SKH contributed to study design, carried out experiments, interpreted results and helped to write and edit the manuscript. JEO, VB, AA, SSJH and GJM carried out experiments and interpreted results. EW, DGS, PMP, RS, CMS provided intellectual input with regard to both experimental design and data interpretation and edited the manuscript. All authors read and approved the final manuscript.

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