LETTERS

Mutations in *NOTCH1* cause aortic valve disease

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Calcification of the aortic valve is the third leading cause of heart disease in adults[1]. The incidence increases with age, and it is often associated with a bicuspid aortic valve present in 1–2% of the population[2]. Despite the frequency, neither the mechanisms of valve calcification nor the developmental origin of a two, rather than three, leaflet aortic valve is known. Here, we show that mutations in the signalling and transcriptional regulator *NOTCH1* cause a spectrum of developmental aortic valve anomalies and severe valve calcification in non-syndromic autosomal-dominant human pedigrees. Consistent with the valve calcification phenotype, *Notch1* transcripts were most abundant in the developing aortic valve of mice, and Notch1 repressed the activity of Runx2, a central transcriptional regulator of osteoblast cell fate. The hairy-related family of transcriptional repressors (Hrt), which are activated by Notch1 signalling, physically interacted with Runx2 and repressed Runx2 transcriptional activity independent of histone deacetylase activity. These results suggest that *NOTCH1* mutations cause an early developmental defect in the aortic valve and a later de-repression of calcium deposition that causes progressive aortic valve disease.

Abundant evidence suggests a major inherited component to the aetiology of aortic valve disease in children and adults[3,4]. The most severe type of aortic valve obstruction in children results in failure of the fetal left ventricle to grow, a condition known as hypoplastic left heart syndrome. About 10% of relatives of hypoplastic left heart syndrome patients have bicuspid aortic valve, often undiagnosed, suggesting a common genetic aetiology with phenotypic heterogeneity[5]. The valve calcification often observed in bicuspid aortic valve is a result of inappropriate activation of osteoblast-specific gene expression[6], but the mechanism is unknown.

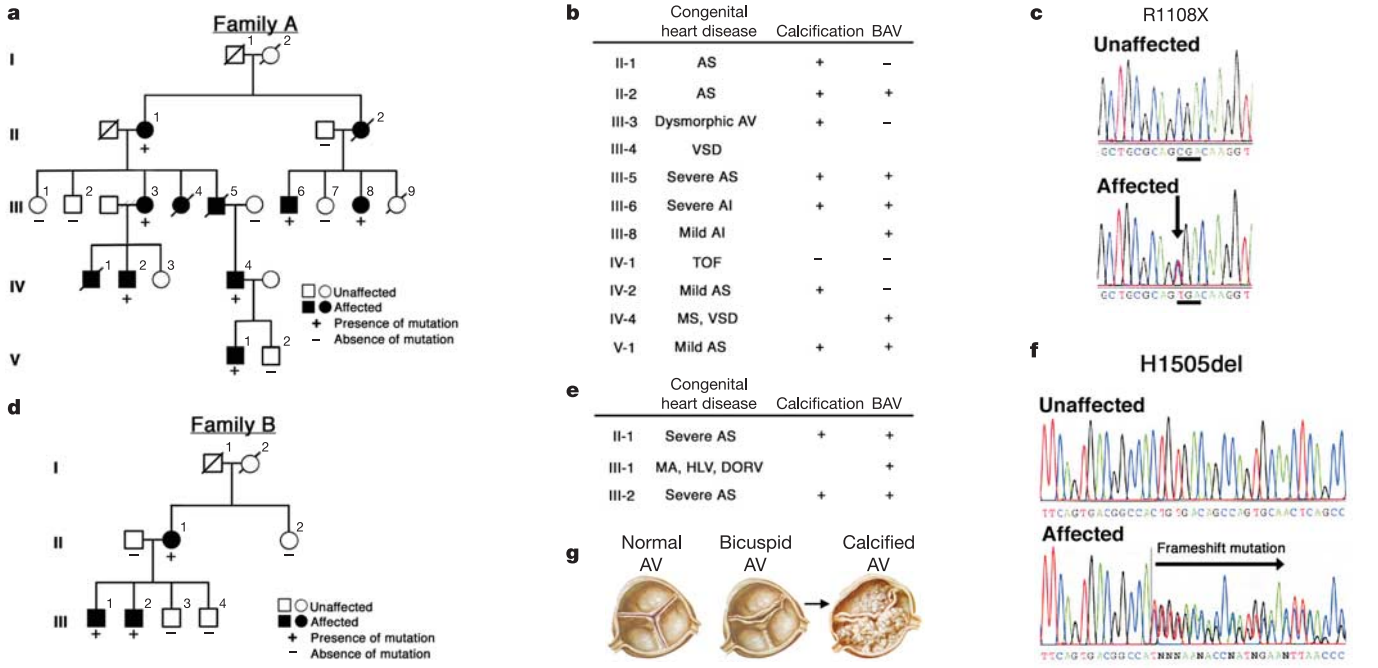

Figure 1 | *NOTCH1* mutations segregate with familial aortic valve disease. **a**, Kindred with five generations (indicated with Roman numerals) affected by congenital heart disease and valve calcification. Participating members of each generation are indicated numerically. Deceased family members (slash) were unavailable for mutation analysis. Squares, males; circles, females. **b**, Cardiac phenotype in affected family members. AI, aortic insufficiency; AS, aortic stenosis; AV, aortic valve; BAV, bicuspid aortic valve; TOF, tetralogy of Fallot; VSD, ventricular septal defect. **c**, Sequence chromatogram of affected family members. **d**, Kindred with three members affected by congenital heart disease. **e**, Cardiac phenotype of family B. DORV, double-outlet right ventricle; HLV, hypoplastic left ventricle; MA, mitral atresia; MS, mitral stenosis. **f**, Sequence chromatogram of affected members in family B. **g**, Schematic of normal trileaflet aortic valve, bicuspid aortic valve and calcified aortic valve.

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We identified a family of European–American descent spanning five generations with 11 cases of congenital heart disease (Fig. 1a). Clinical evaluations demonstrated autosomal-dominant inheritance of congenital heart disease. Nine affected family members had aortic valve disease (Fig. 1b,g). In eight, an abnormal aortic valve was the only cardiac malformation; six had bicuspid aortic valve, and seven developed calcific aortic stenosis, including three cases in the setting of a three leaflet valve. One family member (IV-4) had an associated abnormal mitral valve, resulting in mitral stenosis, and a ventricular septal defect. An isolated ventricular septal defect or tetralogy of Fallot with a bicuspid pulmonary valve was identified in two other affected family members (III-4 and IV-1, respectively). Four family members have required aortic valve replacement for severe calcification. No cardiac conduction abnormalities, neurological deficits, or other birth defects were identified. Detailed clinical phenotype information for this family is shown in Supplementary Fig. 1a.

A genome-wide scan of available family members revealed linkage of the congenital heart disease phenotype to a single locus on chromosome 9q34-35 between D9S1826 and D9qter (logarithm of odds (LOD) score, 3.5, $\theta = 0$), spanning approximately 3 megabases ($\sim$9 cM) (for haplotype data, see Supplementary Fig. 2). Review of 30 known (and 57 predicted) genes revealed *NOTCH1*, which encodes a transmembrane receptor (2,556 amino acids) that functions in a highly conserved intracellular signalling pathway involved in cellular differentiation, cell fate and lateral inhibition[7]. Direct sequencing of *NOTCH1* in an affected patient revealed a heterozygous C-to-T transition of nucleotide 3322 that predicted a premature stop codon instead of arginine at position 1108 in the extracellular domain (Fig. 1c). All affected subjects who were clinically evaluated had the R1108X mutation, suggesting autosomal-dominant inheritance of the disease phenotype with complete penetrance (Fig. 1a). The mutant allele was not detected in unaffected family members or in 1,136 unrelated subjects of diverse ethnicity (Supplementary Fig. 3), making it unlikely that R1108X is a rare polymorphism. In the proband (the index case), sequencing of 100 additional regulatory genes essential for, or expressed during, cardiac development identified no other linked mutations, consistent with a monogenic aetiology (V.G. and D.S., unpublished observations).

Direct sequencing of *NOTCH1* in a smaller, unrelated Hispanic family with aortic valve disease revealed a second mutation that segregated with three affected family members, all with bicuspid aortic valve (Fig. 1d, e). The proband (III-1) also had mitral valve atresia, hypoplastic left ventricle and double-outlet right ventricle; his sibling (III-2) and mother (II-1) had aortic valve calcification and stenosis. Family member II-2 had an ascending aortic aneurysm (Fig. 1e; see also Supplementary Fig. 1b) but no aortic valve disease. A single base pair deletion at position 4515 that segregated with aortic valve disease in this family was not found in 1,138 ethnically diverse controls (Fig. 1f; see also Supplementary Fig. 3). This deletion resulted in a frameshift mutation (H1505del) that predicted a severely altered protein containing 74 incorrect amino acids at the carboxy terminus of the extracellular domain followed by a premature stop codon (Fig. 1f). These *NOTCH1* mutations generate truncated transcripts that probably undergo nonsense-mediated decay[8] and provide compelling genetic evidence that *NOTCH1* haploinsufficiency results in human congenital heart disease, although dominant-negative effects cannot formally be ruled out.

To determine whether *Notch1* expression during development correlates with the predominant phenotype in humans, we performed *in situ* hybridization at multiple stages during cardiogenesis, focusing on *Notch1* transcripts in cardiac valves and their precursors in mice. At mouse embryonic day (E) 11.5, *Notch1* messenger RNA transcripts were abundant in the outflow tract mesenchyme, which gives rise to the valves, and in the endocardium (Fig. 2a–d). By E13.5, when septation of the common arterial trunk occurs, *Notch1* was expressed at high levels in the endothelial layer (Fig. 2e–h) and mesenchyme of aortic valve leaflets (Fig. 2g, h), possibly explaining the increased dose sensitivity of the aortic valve. These findings suggest a role for Notch signalling in the morphological development of the aortic valve. Disruption of *Notch1* in mice results in death by E9.5 from vascular endothelial defects, precluding analysis of the aortic valve[9], but in fish and frogs Notch1 appears to be important for early valve development[10].

NOTCH1 encodes a large protein containing an extracellular domain with 36 tandem epidermal growth factor (EGF)-like repeats and three cysteine-rich Notch/LIN-12 repeats, an intracellular domain with six ankyrin repeats, and a transactivation domain. The Notch signalling pathway is highly conserved across species[7]. Notch receptors (1–4) interact with Delta(1–4) or jagged(1, 2)/serrate, resulting in two independent cleavages, first by a metalloprotease[11,12] and then by presenilin[13,14], that release the Notch

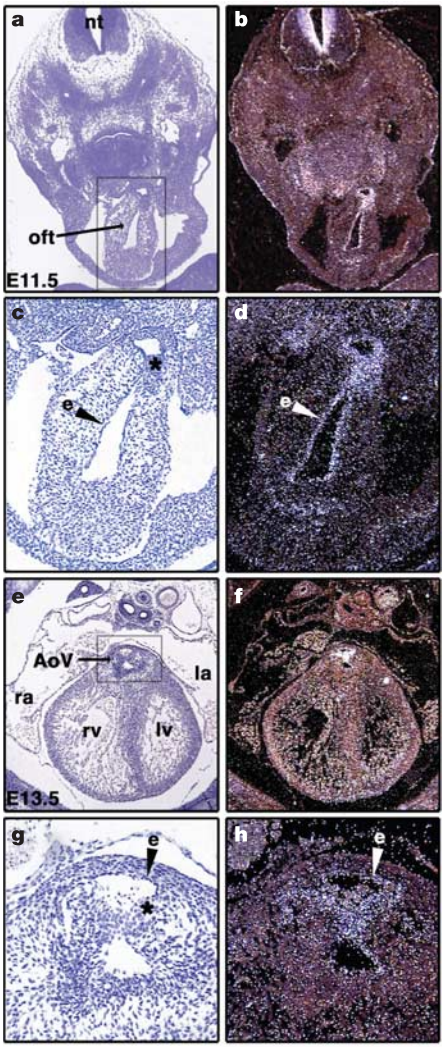

Figure 2 | Cardiac expression of mouse *Notch1* mRNA by radioactive-section *in situ* hybridization. **a**–**d**, Transverse sections of E11.5 mouse embryos through endocardium (arrowhead) and endocardial cushions (asterisk) of the outflow tract (oft). **c**, **d**, High-magnification images of **a**, **b**, as depicted by box in **a**. e, endocardium; nt, neural tube. **e**–**h**, Transverse sections through E13.5 embryonic heart. **g**, **h**, High-magnification of aortic valve (AoV) region outlined by box in **e**. The asterisk indicates AoV mesenchyme. Sections in **a**, **c**, **e** and **g** are bright-field images of **b**, **d**, **f** and **h**, respectively. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.

intracellular domain from the membrane, in a manner similar to that first described for sterol response element binding protein[15]. Notch intracellular domain translocates to the nucleus, where it interacts with the DNA-binding protein CSL (CBF-1, suppressor of hairless, and Lag-1) to activate downstream target genes, including members of the hairy/enhancer of split (Hes) family of transcriptional repressors. This pathway participates in cell fate determination and differentiation during organogenesis throughout the embryo and is regulated by glycosylation of the extracellular EGF-like repeats in Notch1 (ref. 16).

Although haemodynamic alterations induced by bicuspid aortic valve may contribute to calcification, several family members with tricuspid aortic valves also developed calcification. This observation, along with the severity of calcification, led us to test whether NOTCH1 may directly affect calcium deposition. A proposed cellular mechanism by which valvular calcification develops is via differentiation of valvular cells into osteoblast-like cells[6], including upregulation of genes, such as osteopontin[17]. Expression of osteopontin, osteocalcin and other osteoblast-specific genes is directly regulated by upstream *cis* elements that bind to the transcription factor Runx2 (ref. 18). Because Runx2 is upregulated in mouse and rabbit models of valvular calcification[19,20], we investigated whether Notch1 normally represses Runx2 activation. In the fibroblast cell line COS7, constitutively active Notch1 intracellular domain repressed Runx2-induced activation of luciferase through a multimerized Runx2-binding *cis* element normally present upstream of osteocalcin (Fig. 3a). Although Runx2 can be inhibited by histone deacetylase (HDAC) activity[21], Notch1-mediated repression of Runx2 was unaffected by the potent HDAC inhibitor trichostatin A, suggesting that it was HDAC-independent.

Notch directly activates the hairy family of transcriptional repressors, the central mediators of Notch's effects on gene expression. The heart and vasculature are enriched in the hairy-related transcriptional repressors Hrt1 and Hrt2, which mediate the Notch signal[22,23]. Hrt1 and Hrt2, also known as Hey1 and Hey2, were co-expressed in the endothelial lining of the murine aortic valve leaflet at E17.5, as well as in the endocardium and vascular endothelium (Fig. 3b–d). As with Notch1, Hrt1 and Hrt2 inhibited Runx2 activation of the osteocalcin enhancer. Using Hrt2 truncation mutants, we determined that the basic helix–loop–helix (bHLH) domain of Hrt2 is necessary for full Hrt2-mediated repression of Runx2 (Fig. 3e, f). Moreover, semi-quantitative RT–PCR demonstrated upregulation of Hrt1 and Hrt2 transcripts in COS7 cells transfected with Notch1 intracellular domain (Fig. 3g). In glutathione *S*-transferase (GST) pull-down studies performed to investigate the mechanism of Hrt2-mediated repression, Hrt2 and Runx2 specifically interacted (Fig. 3h), consistent with the repression we observed and the reported *in vitro* interaction between Hes1 and Runx2 (ref. 24). The bHLH domain of Hrt2 can recruit HDACs[25]; however, as with Notch1, Hrt repression of Runx2 activation was not dependent on HDAC activity (Fig. 3). These data suggest that Hrt proteins repress Runx2 through a physical interaction, and may mediate Notch1 repression of Runx2.

Somatic *NOTCH1* mutations have been identified in human blood cancers[26], but the discovery of *NOTCH1* mutations as a cause of aortic valve calcification and aortic valve anomalies represents the first demonstration of *NOTCH1* germline mutations in human disease. The families reported here provided insights into the cause of a common human developmental malformation (bicuspid aortic valve) and revealed a potential mechanism mediated by NOTCH1 mutations that may predispose to endothelial dysfunction and inflammation underlying abnormal cardiovascular calcification events. Further studies of the NOTCH1 signalling pathway in the adult calcific process may identify preventative and pharmacological approaches to slow this age-related disease.

Whereas the role of NOTCH1 in preventing aortic valve calcification is relevant for adult-onset disease, its essential function in normal development of the valve is equally intriguing. Bicuspid

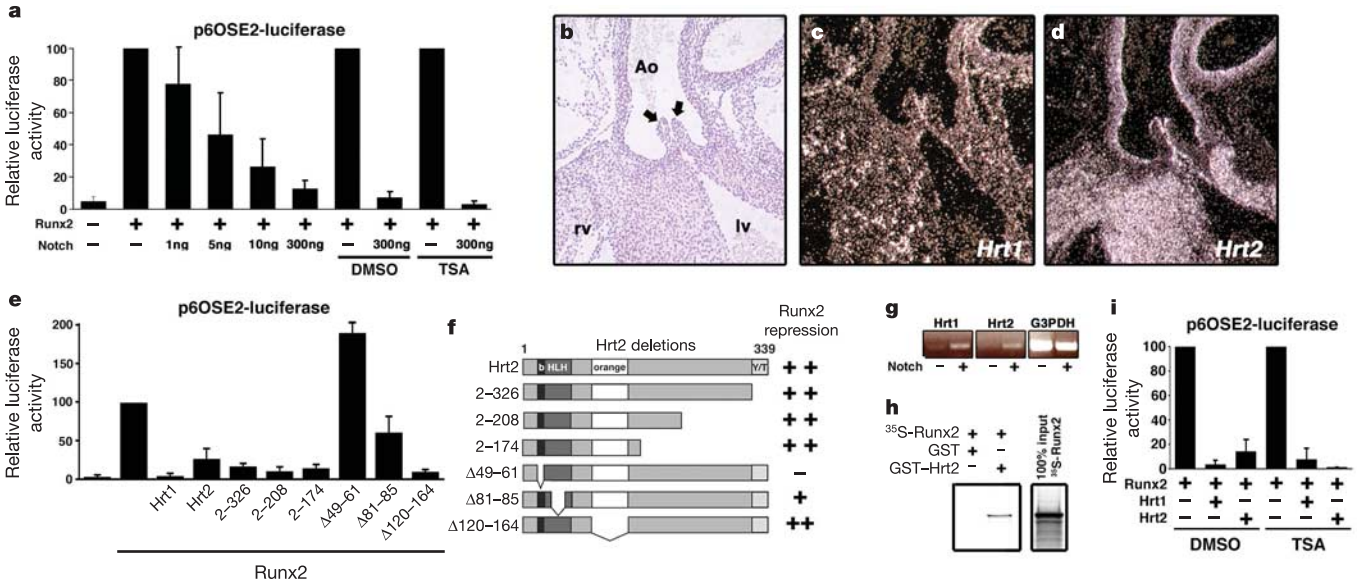

Figure 3 | Notch1, Hrt1 and Hrt2 repress Runx2 transcriptional activity. **a**, Relative luciferase activity in COS7 cells transfected with Flag-Runx2 and Runx2-dependent osteocalcin enhancer (p6OSE2) luciferase reporter with or without co-transfection of the indicated concentrations of Myc-Notch1 intracellular domain, in the presence of trichostatin A (TSA) or vehicle (DMSO). **b**–**d**, Coronal section *in situ* hybridization through aortic valve (arrows) of E17.5 mouse embryos (**c**, **d**). Panel **b** is a bright-field image. Ao, aorta; lv, left ventricle; rv, right ventricle. **e**, Relative luciferase activity directed by Runx2 with co-transfection of Hrt1, Hrt2 or Hrt2 mutant proteins. Numbers indicate amino acid positions of mutant proteins. **f**, Schematic and summary of Hrt2 mutant protein effects on Runx2. b, basic domain; HLH, helix–loop–helix domain; orange, orange domain; Y/T, YXPW-TEIGAF motif. **g**, Semi-quantitative RT–PCR of Hrt1, Hrt2 and G3PDH in COS7 cells with or without transfection of Notch intracellular domain. **h**, Pull-down assays with GST–Hrt2 fusion protein and ^{35}S-labelled Runx2. **i**, Relative luciferase activity of Runx2 with Hrt1 or Hrt2 in the presence of trichostatin A or DMSO control. Luciferase data are shown as percentage of Runx2 activation (normalized to 100%); mean ± s.d. are shown.

and even unicuspid aortic valves typically contain a ridge where the valve leaflets did not separate *in utero* (Fig. 1g). In extreme cases, blood flow may be so restricted that the left ventricle fails to grow, resulting in hypoplastic left heart syndrome, the most frequent cause of death in children with congenital heart disease. As bicuspid aortic valve and hypoplastic left heart syndrome may represent extremes of the aortic valve disease spectrum, the discovery of *NOTCH1* as a cause of bicuspid aortic valve and a hypoplastic left ventricle in the same family suggests that *NOTCH1* mutations may be the genetic basis for hypoplastic left heart syndrome in some patients. Future studies of *NOTCH1* mutations in this population may reveal those at risk for a subset of severe congenital heart lesions.

METHODS

Clinical phenotype evaluation and DNA collection. The congenital heart disease families and individuals were ascertained for genetic linkage analyses at Children's Medical Center, Dallas (University of Texas Southwestern Medical Center) and the University of California, San Diego. Clinical evaluations and genetic studies were performed in accordance with human subject guidelines after informed consent according to the protocol approved by the individual Institutional Review Boards. Family members were studied by history, physical examination, 12-lead electrocardiogram, and echocardiography. Medical records were reviewed for individuals who had died. All phenotypic information was reviewed by cardiologists. Genomic DNA for genetic analyses was extracted from peripheral lymphocytes.

Genetic linkage analysis. Autosomal genome linkage analysis was performed with 372 polymorphic DNA markers at ~10-cM intervals (ABI Mapping Set v2.5). Markers were genotyped in all family members, and linkage analysis was performed with GENEHUNTER as described[27,28]. In brief, initial linkage analysis of family A, assuming 90% penetrance and a disease allele frequency of 1.5%, demonstrated the highest LOD score on chromosome 9q34-35. Phenotypic analysis assuming 100% penetrance yielded a single peak at 9q34-35 and a maximum LOD score of 3.5.

Identification of *NOTCH1* mutations. All *NOTCH1* exons were sequenced bidirectionally to search for sequence variations in the probands of families A and B. Exons containing R1108X and H1505del mutations were amplified by PCR for each additional family member and sequenced bidirectionally. Sequences of the 42 primer pairs for the 34 *NOTCH1* exons are available on request. PCR amplification was performed with the BD Biosciences Advantage GC Genomic PCR kit following the manufacturer's instructions, with annealing at 60 °C. Screening of identified human *NOTCH1* mutations was performed with allelic discrimination assays and the ABI Prism 7900 HT Sequence Detection System using TaqMan probes on DNA from participants in the Dallas Heart Study, as described[29].

Radioactive-section *in situ* hybridization. ^{35}S-labelled antisense riboprobes were synthesized with T7 RNA polymerase (MAXIScript, Ambion) from 400-bp partial mouse Notch1 cDNA or plasmids encoding Hrt1 and Hrt2. With these riboprobes, radioactive-section *in situ* hybridization was performed on paraffin-embedded sections of E11.5, E13.5 and E17.5 mouse embryos, as described[22].

Luciferase assays. COS7 cells were transfected using Fugene 6 (Roche) according to the manufacturer's instructions. The reporter plasmid (250 ng), p6OSE2 luciferase[18], and CMV β-galactosidase expression plasmid (50 ng) to control for transfection efficiency were transfected along with Runx2 expression plasmid (100 ng) and Hrt1, Hrt2, Notch1 intracellular domain and Hrt2 deletion expression plasmids (300–1,000 ng). Hrt2 deletion constructs were generated as described[30] and protein levels of mutants kept constant. To inhibit HDAC activity, trichostatin A diluted to 0.1 μM in dimethyl sulphoxide (DMSO) was added 24 h before collecting cell lysates. Simultaneous duplicate experiments with an identical amount of DMSO served as a control. Immunoblots verified appropriate protein expression. Luciferase activity was measured 40 h after transient transfection as described[30] and was normalized to LacZ expression to generate relative luciferase activity (Fig. 3a) or expression of Hrt and Runx2 proteins (Fig. 3e). At least three independent experiments were performed in duplicate.

Quantitative RT–PCR. Total RNA from COS7 cells collected 40 h after transfection with empty vector and Notch1 intracellular domain expression plasmid was purified with the Trizol method (Invitrogen). Total RNA (1 μg) was reverse transcribed using the Superscript First Strand Synthesis System for RT–PCR (Invitrogen). PCR analysis was performed using primers specific for Hrt1 and Hrt2. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) RNA was amplified as a loading control. An annealing temperature of 56 °C was used for PCR analysis. Negative controls for each sample used non-reverse-transcribed RNA.

GST pull-down assay. Mouse GST–Hrt2 fusion proteins were purified with glutathione Sepharose 4 Fast Flow beads (Roche) for 12 h and washed twice in binding buffer (300 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 1% NP-40, 0.1% SDS, 0.5 mM dithiothreitol). ^{35}S-labelled Runx2 protein was synthesized using the T7 TNT coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. Labelled protein was incubated with GST fusion protein (2 μg) for 8–10 h at 4 °C in binding buffer with 1 mg of nonfat dried milk to compete for nonspecific interactions. Bound proteins were analysed by SDS–PAGE and autoradiography.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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