# Genetic Modifiers Predisposing to Congenital Heart Disease in the Sensitized Down Syndrome Population

Huiqing Li, PhD*; Sheila Cherry, PhD*; Donna Klinedinst, PhD; Valerie DeLeon, PhD;
Jennifer Redig, PhD; Benjamin Reshey, BS; Michael T. Chin, MD, PhD; Stephanie L. Sherman, PhD;
Cheryl L. Maslen, PhD; Roger H. Reeves, PhD

***Background***—About half of people with Down syndrome (DS) exhibit some form of congenital heart disease (CHD); however, trisomy for human chromosome 21 (Hsa21) alone is insufficient to cause CHD, as half of all people with DS have a normal heart, suggesting that genetic modifiers interact with dosage-sensitive gene(s) on Hsa21 to result in CHD. We hypothesize that a threshold exists in both DS and euploid populations for the number of genetic perturbations that can be tolerated before CHD results.

***Methods and Results***—We ascertained a group of individuals with DS and complete atrioventricular septal defect and sequenced 2 candidate genes for CHD: *CRELD1*, which is associated with atrioventricular septal defect in people with or without DS, and *HEY2*, whose mouse ortholog (*Hey2*) produces septal defects when mutated. Several deleterious variants were identified, but the frequency of these potential modifiers was low. We crossed mice with mutant forms of these potential modifiers to the Ts65Dn mouse model of DS. Crossing loss-of-function alleles of either *Creld1* or *Hey2* onto the trisomic background caused a significant increase in the frequency of CHD, demonstrating an interaction between the modifiers and trisomic genes. We showed further that, although each of these mutant modifiers is benign by itself, they interact to affect heart development when inherited together.

***Conclusions***—Using mouse models of Down syndrome and of genes associated with congenital heart disease, we demonstrate a biological basis for an interaction that supports a threshold hypothesis for additive effects of genetic modifiers in the sensitized trisomic population. (***Circ Cardiovasc Genet***. 2012;5:301-308.)

**Key Words:** congenital heart disease ■ genetic modifier ■ Down syndrome

Congenital heart disease (CHD) is the most common congenital anomaly in humans, occurring in as many as 9 of every 1000 infants born each year (http://www.heart.org/HEARTORG/). The frequency is greatly elevated in people with Down syndrome (DS), as nearly half of all people with trisomy 21 exhibit some form of CHD.[1] One of the more severe structural anomalies, atrioventricular septal defect (AVSD), affects about 1/10 000 live births in the population at large but one fifth of individuals with DS; however, trisomy 21 alone is insufficient to cause CHD, as half of all people with DS have a normal heart, suggesting that genetic modifiers interact with dosage sensitive gene(s) on human chromosome 21 (Hsa21) to result in CHD.[2]

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Attempts to identify dosage-sensitive Hsa21 genes that contribute to CHD have generally focused on individuals with partial trisomy for Hsa21 to identify critical regions: the smallest regions of overlap between individuals who share a DS-associated phenotype[3–5]; however, the resolution of this approach is limited by the rarity of the condition, the complex karyotype of such individuals (usually including other chromosomal anomalies in addition to partial trisomy 21), and the heterogeneity of the phenotype. Several heart-critical regions have been defined, including a minimal region of just 1.7 Mb from *DSCAM* to *PKNOX1* that is based on individuals with DS and a variety of CHDs.[3] To date, this kind of analysis has

not considered additional (disomic) genetic modifiers that have been associated with CHD.

One such genetic modifier is cysteine-rich with EGF-like domains 1 (*CRELD1*), initially identified as a candidate for the AVSD2 locus.[6] Missense mutations in *CRELD1* cause protein misfolding[6] and are associated with AVSD in some individuals but are also present in unaffected family members.[7,8] This finding suggests that *CRELD1* is neither necessary nor sufficient to cause AVSD but may increase the risk of developing a defect, making it a reasonable candidate as a susceptibility locus (modifier) for AVSD. Indeed, in an earlier study, we detected mutations in *CRELD1* in a small population of people with DS and CHD.[2] Other genetic modifiers have been shown to affect heart development in either individuals with DS or model organisms. For example, somatic mutations in *HEY2* have been identified in CHD in people with DS but not in euploid populations with heart defects,[9,10] and *Hey2*$^{-/-}$ mice die in early postnatal stages from cardiac abnormalities that always include septal defects.[11,12]

Animal models are crucial to our understanding of the pathogenesis of CHD and the molecular mechanisms underlying these conditions. Orthologs of many genes on Hsa21 are found on mouse chromosome 16 (Mmu16), with smaller subsets on Mmu10 and Mmu17.[13] The most widely used DS mouse model, Ts65Dn, is trisomic for a segment of Mmu16 containing about half of the Hsa21 orthologs.[14] Ts65Dn mice display a number of the features of DS, including cardiac abnormalities, albeit at a lower frequency than in humans.[15] The information derived from mouse models suggests that, in mouse as in human, heart phenotypes likely result from a complex genetic insult, of which dosage imbalance represents only a part. Additional genetic and environmental factors must contribute to these phenotypes. In DS, polymorphic disomic loci on other chromosomes may act as genetic modifiers when combined with trisomic genes.

We have created a null allele of mouse *Creld1* to study the contributions of this gene to developmental processes (Redig and Maslen, submitted). Homozygous *Creld1*$^{-/-}$ embryos die by embryonic day 11.5 and exhibit several defects, including perturbations in heart development. In particular, the endocardial cushions form but are hypocellular, with few mesenchymal cells evident. Little more is known about *Creld1* function during development and how it affects heart formation. Further, there is no description of whether and how *Creld1* interacts with Hsa21 genes in normal heart development.

We hypothesize that a threshold exists, in both DS and euploid populations, for the number of genetic perturbations that can be tolerated before CHD results (Figure 1). In this model, CHD in euploid individuals is caused by multiple additive factors that could be genetic, environmental, or entirely stochastic. Given the significant increased risk for CHD in people with trisomy 21, the DS population can be said to be sensitized, creating a greater signal-to-noise ratio for smaller risk factors. Identification of modifiers should therefore be enhanced in DS using genome-wide association studies, candidate gene sequencing in affected and control populations, and modeling of candidate gene mutations in the mouse. Here, we provide evidence supporting this additive hypothesis and demonstrate the use of
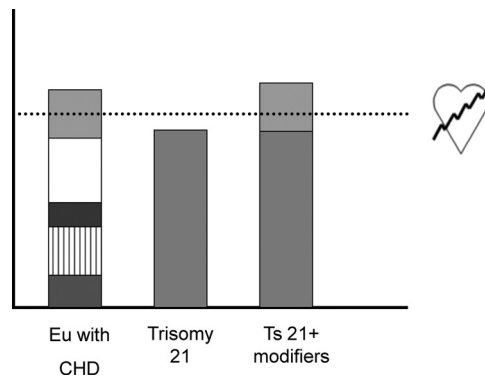


**Figure 1.** A threshold model for CHD. We hypothesize that the human population includes allelic variants in multiple genes that contribute to the risk of congenital heart disease, many of which have subtle or no effects by themselves. Additive effects of individual modifier genes can reach a threshold whereby heart septal development is disrupted (Euploidy with congenital heart disease [CHD]), but the likelihood of inheriting many predisposing modifiers is small. Trisomy 21 is a significant risk factor for CHD, but alone is not sufficient to produce heart defects; however, those people with an extra copy of Hsa21 may require fewer disomic or trisomic modifiers to reach the threshold (Ts21+modifier). The relative contribution of the modifier in the sensitized Down syndrome population is therefore more readily detectable.

this approach to confirm results from both human resequencing and mouse mutation analysis.

## Materials and Methods

### Patient Recruiting

Human subjects were recruited nationally through the Down Syndrome Heart Project (DSHP) (http://inertia.bs.jhmi.edu/ds/index.html). Individuals with DS due to an extra intact copy of Hsa21 verified by karyotype were included as cases if they had complete atrioventricular canal (DS+AVSD) and as controls if they had DS and a normal echocardiogram. All surgical records or echocardiograms were reviewed and confirmed by a pediatric cardiologist. All cases and controls were non-Hispanic whites. Consents and procedures were approved by Institutional Review Boards at the Johns Hopkins University School of Medicine, Emory University, and Oregon Health & Science University.

### Resequencing Summary

Human *CRELD1* and *HEY2* genes were resequenced, including all coding regions and at least 50 bp into each flanking intron and untranslated regions (UTR) to cover interstitial regulatory elements (Figure 2 and online-only Data Supplement Table I). Analyses were done by standard DNA sequencing of polymerase chain reaction (PCR)-amplified genomic DNA by the Oregon Clinical Translational Research Institute sequencing core facility. Electropherograms were transferred electronically for detailed analysis facilitated by the MutationSurvey or software suite (Soft Genetics). All sequences were compared with a reference sequence, and all variants were noted and further analyzed. Variants of interest were confirmed by resequencing a newly prepared amplicon. With 1 exception, all variants reported were heterozygous (Table 1).

Variants were categorized by the type of variant, and documented single-nucleotide polymorphism (SNPs) were annotated by query of the SNP database. We compared SNP frequency to identify any allele-specific disease associations. Variants were categorized as missense, insertion/deletion, noncoding, splice site, regulatory, and other and prioritized for disease-association potential (ie, the likelihood that the variant will alter gene expression or protein product structure or function). Missense variants were analyzed using the Polyphen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org/) algo-
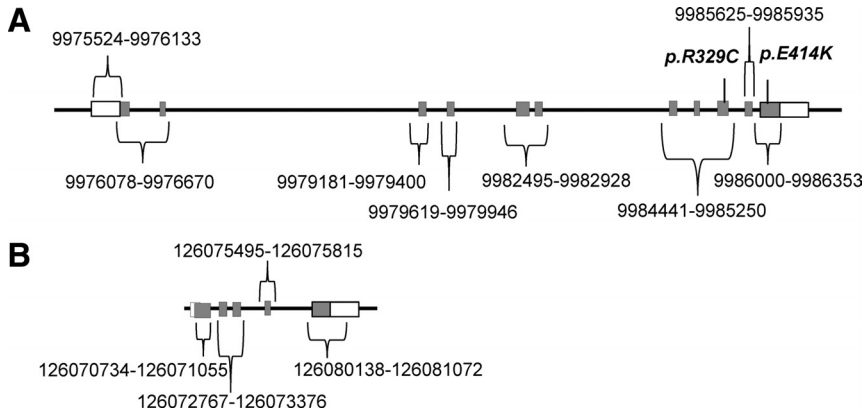
Figure 2. Resequencing strategy for *CRELD1* and *HEY2*. We assessed coding regions of these genes, including all exons with 50+ bp of sequence in the flanking introns, the 5′ untranslated region (UTR) and 50+ bp of the 3′ UTR. **A**, Resequencing strategy for *CRELD1*; **B**, Resequencing strategy for *HEY2*. For exact coordinates, see online-only Data Supplement Table I.

rithms for predictions of likelihood of damage to the protein product. We also monitored lack of expected SNP occurrence as an indicator of gene deletion. All variants were analyzed in control individuals (DS without heart defects) using DNA resequencing of the amplicon, encompassing the alteration, allele-specific PCR analysis or restriction enzyme digestion, as previously described.[7]

### Animal Husbandry

Mice were maintained in an AAALAS-certified clean facility with food and water ad libitum. Ts65Dn mice (B6EiC3H-a/A-Ts65Dn, Jackson Laboratory) were maintained on the B6;C3H background. Mice used in this study were the progeny of female Ts65Dn mice. *Creld1* null mice were generated by gene targeting (J. Redig, PhD, and C.L. Maslen, PhD, unpublished data; see online-only Data Supplement Figure I) and backcrossed a minimum of 8 generations onto a C57BL/6J background. Mice bearing a null allele of *Hey2* were generated by gene targeting.[16] All procedures were approved by the Institutional Animal Care and Use Committee.

### Genotyping

Genomic DNA was extracted from tail tips of mice and was used for genotyping by PCR. Ts65Dn mice were identified by both PCR and fluorescence in situ hybridization as described.[17,18]

Genotypes of *Creld1* and *Hey2* knockout mice were determined by PCR. For *Creld1* genotyping, 2 sets of primers that amplify the deleted region of *Creld1* and a portion of the neomycin resistance gene were added together in each reaction. The sequences of the primers for *Creld1* genotyping are as follows: E1-2F: 5′-CATCCTTCTC-CCCGAGCTGAG-3′; G2-F: 5′-CCAGTCAAAAACCACAGA-GAGGG-3′; E1-2R: 5′-GTGTTTCCACCCCCGAAGT-3′. PCR was done under the following cycling conditions: 95°C, 1 minute, (94°C, 30 s; 68°C, 30 s; 72°C, 1 minute) for 3 cycles; (94°C, 30 s; 67°C [−1°C/cycle], 30 s; 72°C, 1 minute) for 10 cycles, (94°C, 30 s; 58°C, 30 s; 72°C, 1 minute) for 25 cycles; 72°C, 5 minutes.

For *Hey2* genotyping, 2 sets of primers that amplify the deleted region of *Hey2* and a portion of the neomycin resistance gene were added together in each reaction. The sequences of the primers for *Hey2* genotyping are as follows: pK065A: 5′-CACTAAGAACTAG-CGATCTGG-3′; pGK: 5′-GCACGAGACTAGTGAGACGTG-3′; CHF-1WT3P: 5′-CTCAGGGGATTTTGAAAGC-3′. The PCR was done under the following cycling conditions: 95°C, 1 minute, (94°C, 30 s; 68°C, 30 s; 68°C, 1 minute) for 3 cycles, (94°C, 30 s, 67°C [−1°C/cycle], 30 s; 68°C, 1 minute) for 16 cycles; (94°C, 30 s; 52°C, 30 s; 68°C, 1 minute) for 25 cycles; 68°C, 5 minutes.

**Table 1.    *CRELD1* and *Hey2* Sequencing Results**

| Gene | Variants Detected (dbSNP rs Nos.) | Amino Acid Change | SIFT/Polyphen Predictions | MAF in DS Cases | MAF in Controls (in dbSNP) |
|---|---|---|---|---|---|
| *CRELD1* | NM_001031717.2:c.1−770G>A (rs279551) | NA (5′ UTR) | | *60/270 | 0/110 (0.009) |
| | NM_001031717.2:c.1-756C>G | NA (5′ UTR) | | 1/270 | 0/110 |
| | NM_001031717.2:c.1-550C>T | NA (5′ UTR) | | 1/270 | 0/110 |
| | NM_001031717.2:c.261+41C>T | NA (intron 2) | | 1/270 | 0/110 |
| | NM_001031717.2:c.111+96C>A | NA (intron 3) | | 2/270 | 0/110 |
| | NM_001031717.2:c.985C>T | R329C | Intolerant/possibly damaging | 2/270 | †0/400 |
| | NM_001031717.2:c.37G>A (rs279552) | V13M | Tolerant/benign | 1/270 (0.004) | 1/132 (0.006) |
| | NM_001031717.2:c.945G>A (rs76764016) | P315P | | 4/270 | 0/110 (0.009) |
| | NM_001031717.2:c.1104G>A | Q368Q | | 1/270 | 1/110 |
| | NM_001031717.2:c.1240G>A | E414K | Intolerant/possibly damaging | 1/270 | 0/200 |
| *HEY2* | NM_012259.2:c.588G>C (rs61737181) | L196L | | 20/180 (0.111) | 8/100 (0.056) |
| | NM_012259.2:c.84-94C>G (rs2875881) | NA (intron 1) | | 63/180 (0.350) | 37/100 (0.403) |
| | NM_012259.2:c.163-13T>C | NA (intron 2) | | 1/180 (0.006) | 0/100 |
| | NM_012259.2:c.246+39C>G | NA (intron 3) | | 6/180 (0.033) | 1/100 |
| | NM_012259.2:c.328+6T>C (rs1935978) | NA (intron 4) | | 12/180 (0.067) | 1/110 (0.054) |

MAF indicates minor allele frequency; DS, Down syndrome.

NA indicates not in coding region; coding variants shown as single letter amino acid code.

*All 30 individuals are GG genotype.

†200 race-matched control chromosomes without DS (Robinson et al, 2003) +200 control chromosomes with DS (Maslen et al, 2006).

MAF in controls; calculated from frequency in study controls; MAF from dbSNP when available.

## Histology

The progeny of various crosses were collected at postnatal day (P) 0 within hours of birth. Pups were euthanized, and thoraxes were removed and fixed in 10% formalin. Tissues were embedded in paraffin and sectioned at 7 μm by standard methods, followed by staining with hematoxylin and eosin using standard methods. Heart morphology for each animal was analyzed under a dissecting stereomicroscope by at least 2 individuals blinded to genotypes to evaluate the frequency and severity of phenotypes. Photos were taken using a Nikon Digital Sight system.

## Real-Time Analysis of Gene Expression

Total RNA was extracted from the L-cells or the hearts of 4-week-old mice with different genotypes by using TRIzol reagent (Invitrogen). cDNA synthesis was carried out with the First-Strand cDNA synthesis kit (Life Sciences) using 8 μg of total RNA as template. PCR was carried out using TaqMan Gene Expression Assays (Applied Biosystems) on a 7500 Real-Time PCR System (Applied Biosystems). For the *Creld1* and *Hey2* coregulation study, fluorescent (FAM)-labeled *Creld1*, *Hey2*, *Notch1*, and *GAPDH* were normalized to a VIC-labeled internal control, β-actin. *GAPDH* was used as a negative control. All comparisons refer to the wild type (WT).

## Cell Culture and Western Blotting

L-cells were a gift of Dr Gerry Weinmaster and were grown in Dulbecco's Modified Eagle Medium with high glucose and 10% heat-inactivated fetal bovine serum (FBS), supplemental nonessential amino acids and penicillin/streptomycin (Invitrogen) at 37°C and 5% $CO_2$. The $pCS2^+/Hey2$ construct was kindly provided by Dr Manfred Gessler. The $pCS2^+$ or $pCS2^+/Hey2$ construct was transfected into the L-cells by Lipofectamine Ltx (Invitrogen). Forty-eight hours later, half of the transfected cells were harvested and total RNA was extracted; the other half was lysed in RIPA buffer (Invitrogen) for Western Blot. The anti-*Hey2* antibody was purchased from PROTEINTECH (Cat. No. 10597-1-AP) and the anti-actin antibody from DSHB (JLA20).

## Statistical Analysis

Mendelian inheritance of alleles and trisomies were assessed by $\chi^2$ tests. The prevalence of heart defects for different mouse genotypes was compared by Fisher exact test using GraphPad Prism version 5 (GraphPad Software). The relative quantification of gene expression from different genotypes was compared by Mann-Whitney test. All tests were 2-tailed, and $P<0.05$ were considered significant.

# Results

## Candidate Gene Mutations in a Sensitized Population

We identified genes that could be involved in congenital heart disease based on data from either human populations or mouse experimental data and chose 2, *CRELD1* and *HEY2*, for resequencing in cases (individuals with DS and complete AVSD). The heart condition was defined by strict criteria, including 2 independent assessments of surgical notes for AVSD or a normal echocardiography report. *CRELD1* was resequenced in 135 cases (DS+AVSD), including 39 cases from a previous study[2] and 96 new cases. Four individuals each contained 1 of 3 different missense variants (online-only Data Supplement Table 1 and online-only Data Supplement Figure 2). One variant (p.V13M) is a known SNP (rs279552) that has been seen in equal numbers of nonsyndromic cases and controls and is predicted to be benign with regard to protein structure/function. The previously described p.R329C mutation was found in 2 cases, 1 reported before, and the

other is a new occurrence in the greatly expanded population in this study. This variant was identified originally in individuals with nonsyndromic AVSD[7] and has not been identified in >400 control chromosomes.[2,7] The p.R329C variant has been shown to affect *CRELD1* protein structure.[7] In each case, the p.R329C mutation was inherited from an unaffected parent. The third missense variant, p.E414K, predicted to be damaging to the protein, was identified in 1 case among the 39 individuals examined previously and was not detected in 200 control chromosomes.

As part of our candidate gene study, we also resequenced *HEY2* in 90 cases (Table 1). A single coding region variant was identified (p.L196L), which appears to be a relatively common SNP. Four noncoding variants of unknown significance were identified. All are predicted to be benign. For both *HEY2* and *CRELD1*, all variants were heterozygous.

## Reduced Expression of *Creld1* Increases Septal Defects in Ts65Dn Mice

Human association studies[7,8] and our resequencing analysis in a sensitized DS population indicate that inactivating mutations in *CRELD1* may increase the likelihood of developing a septal defect in individuals with DS. We hypothesized that reduced expression of *Creld1* would interact with trisomic genes in a mouse DS model to increase the occurrence of heart defects. Based on this hypothesis, we created mice carrying a null allele of *Creld1* that reduces gene expression to ~50% of normal in heterozygotes. No expression is detected in $Creld1^{-/-}$ embryos (online-only Data Supplement Figure I). $Creld1^{+/-}$ mice were crossed to Ts65Dn, and progeny were collected within hours of birth, prepared for histology, and assessed for the presence of septal defects. All classes of progeny were recovered at expected frequencies (online-only Data Supplement Table III).

A recent study detected septal defects in 1 out of 18 similarly analyzed newborn (P0) Ts65Dn mice.[15] We expanded this observation and saw a single septal defect among 25 Ts65Dn mice at P0 (combined frequency of septal defects=2/43 or 4.7%) (Table 2). $Creld1^{+/-}$ itself had no obvious effect on phenotype and none of the $Creld1^{+/-}$ pups that we analyzed at P0 was found to have a septal defect (n=45; 18 were on the B6; C3H background of Ts65Dn mice; and 27 were inbred on B6); however, the frequency of septal defects increased dramatically to 33% (6/18) in $Ts65Dn, Creld1^{+/-}$ mice (Table 2). Among those pups with heart defects, 3 had ostium secundum atrial septal defects (ASDs), and 3 had membranous ventricular septal defects (VSDs) (Table 2 and Figure 3). Thus, there is a significant difference in the frequency of heart defects between either Ts65Dn ($P=0.006$) or $Creld1^{+/-}$ ($P=0.0003$) mice and those with both genetic conditions (Ts65Dn, $Creld1^{+/-}$). This observation demonstrates a biological basis for a phenotype-altering interaction between the effects of trisomic genes and reduced *Creld1* expression and delimits a region orthologous to part of Hsa21q22.1 (the trisomic segment in Ts65Dn) trisomy, which is sufficient to predispose to CHD.

## Reduced Expression of *Hey2* Increases Septal Defects in Trisomic Mice

Somatic variants of *HEY2* have been identified in hearts from humans with septal defects.[9] In $Hey2^{-/-}$ mice, the reported

**Table 2.    Frequency of Heart Defects on Mutant and Trisomic Genetic Backgrounds**

| Phenotype | Genetic Background | % of Affected | With Septal Defect | Without Septal Defect | Type of Septal Defect |
|---|---|---|---|---|---|
| Ts65Dn | B6/C3H* | 4.7% | 2 | 41 | 2 membranous VSD |
| *Creld1*$^{+/-}$ | B6/C3H† | 0 | 0 | 18 | N/A |
|  | B6 | 0 | 0 | 27 | N/A |
| *Hey2*$^{+/-}$ | B6/C3H† | 0 | 0 | 20 | N/A |
|  | B6 | 0 | 0 | 25 | N/A |
| Ts65Dn, *Creld1*$^{+/-}$ | B6/C3H* | 33.3%‡ | 6 | 12 | 3 membranous VSDs, 3 secundum ASDs |
| Ts65Dn, *Hey2*$^{+/-}$ | B6/C3H* | 24%§ | 6 | 19 | 3 membranous VSDs, 1 muscular VSD, 2 secundum ASDs |
| *Creld1*$^{+/-}$, *Hey2*$^{+/-}$ | B6 | 9.7%‖ | 3 | 28 | 3 secundum ASDs |
| *Hey2*$^{-/-}$ | B6 | 78.6% | 11 | 3 | 11 membranous VSDs |
| *Creld1*$^{+/-}$, *Hey2*$^{-/-}$ | B6 | 90% | 9 | 1 | 9 membranous VSDs; among those 9, 1 has extra secundum ASD, and 1 has extra muscular VSD |

VSD indicates ventricular septal defect; ASD, atrial septal defect.

*75% B6, 25% C3H.

†50% B6, 50% C3H.

‡Indicates a significant difference between Ts65Dn, *Creld1*$^{+/-}$, and Ts65Dn ($P=0.006$) or *Creld1*$^{+/-}$ ($P=0.0003$).

§Significant difference between Ts65Dn, *Hey2*$^{+/-}$, and Ts65Dn ($P=0.04$) or *Hey2*$^{+/-}$ ($P=0.0014$).

‖Significant difference between *Creld1*$^{+/-}$, *Hey2*$^{+/-}$, and the single mutants ($P=0.016$).

frequency (penetrance) of septal defects varies with genetic background,[11,12,16,19,20] reaching >80% on an inbred C57Bl/6J (B6) background. We detected membranous VSDs in 11 out of 14 *Hey2* null neonates (79%).

We crossed a null allele of *Hey2* onto the Ts65Dn trisomic background. Ts65Dn, *Hey2*$^{+/-}$ pups were underrepresented among the progeny, suggesting a deleterious additive effect of reduced *Hey2* expression with the trisomic genes from Ts65Dn (online-only Data Supplement Table III). A single null allele of *Hey2* was not sufficient to cause septal defects (0 out of 45); however, the frequency of septal defects was significantly increased in Ts65Dn, *Hey2*$^{+/-}$ mice, with 6 out of 25 (24%) displaying a septal defect ($P=0.04$ or $P=0.0014$ compared with Ts65Dn or Hey2$^{+/-}$, respectively) (Table 2).

Like *Creld1*, expression variants of *Hey2* act as a modifier, contributing to CHD in a manner that is detectable on a

sensitized trisomic background but not in euploid mice. Of note, membranous VSDs are the only septal defect in *Hey2*$^{-/-}$ mice, but we detected additional structural defects in Ts65Dn, *Hey2*$^{+/-}$. Among the 6 pups with heart defects, 3 had membranous VSDs, 1 had a muscular VSD, and 2 had ostium secundum ASDs (Table 2 and Figure 3). Thus, the pattern, as well as the number of defects, is altered by the *Hey2* trisomy interaction.

## Combining Benign *Creld1* and *Hey2* Modifier Variants Results in Septal Defects

To test our initial hypothesis that additive effects of modifier genes contribute to CHD on a euploid background, *Creld1*$^{+/-}$ mice were crossed with *Hey2*$^{+/-}$. Genotypes of progeny were recovered at the expected frequencies (online-only Data Supplement Table III). No septal defects were detected at P0 in 90 pups
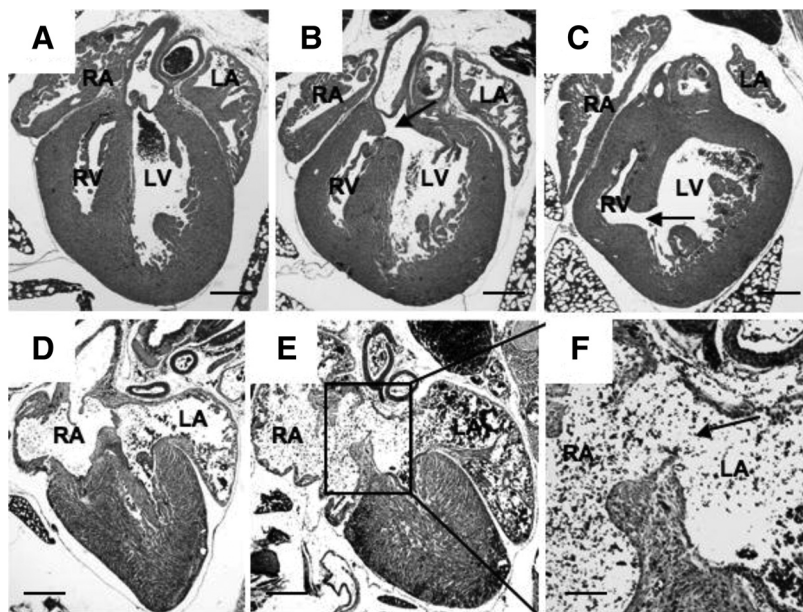


**Figure 3.** A variety of septal defects were observed in mutant and trisomic mice at the day of birth (P0). **A**, Normal heart showing intact ventricular septum at P0; **B**, membranous ventricular septal defect (VSD); **C**, Muscular VSD; **D**, Normal heart showing atrial septum; **E**, Ostium secundum atrial septal defect (ASD); **F**, ASD at higher magnification. For the incidence of defects in various models, see Table 2. Arrows indicate communication between the chambers. RA indicates right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; Scale bars: **A–E**, 400 $\mu$m, **F**, 150 $\mu$m.
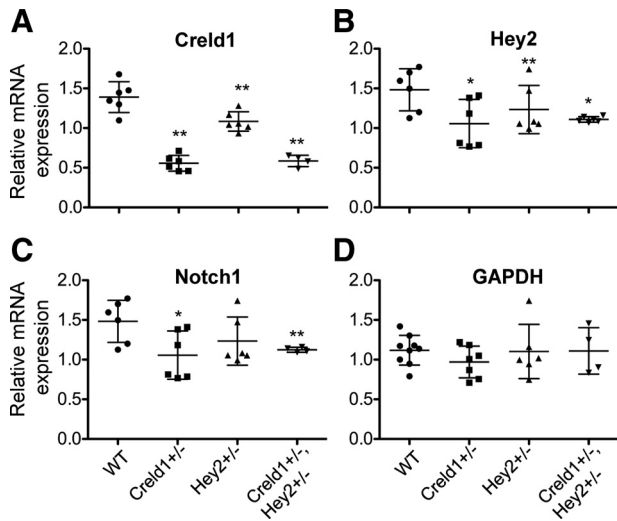
Figure 4. *Notch1*, *Creld1*, and *Hey2* interaction. **A-D**, TaqMan assay was used to show coordinate regulation of *Creld1* and *Hey2* transcript levels in hearts of heterozygous null mice of either genotype. *Notch1* was significantly downregulated in *Creld1*^{+/−} hearts, as well. FAM-labeled *Creld1*, *Hey2*, *Notch1*, and *GAPDH* were normalized to a VIC-labeled internal control, β-actin. *GAPDH* was used as a negative control. The number of hearts tested was 4 to 6 per analysis as indicated on the dot plot. All statistical comparisons are relative to WT. *$P<0.05$; **$P<0.01$ (Mann-Whitney test).

that were either *Creld1*^{+/−} (n=45) or *Hey2*^{+/−} (n=45); however, 3 out of 31 double heterozygous *Creld1*^{+/−}, *Hey2*^{+/−} pups had a septal defect (Table 2). This result indicates that *Creld1*^{+/−} and *Hey2*^{+/−} interact to increase the risk of CHD ($P=0.016$, Fisher exact test, 2-tailed).

Further support for an interaction comes from the observation that although homozygosity for a null allele in *Hey2*^{−/−} mice results exclusively in membranous VSD, all 3 defects in the double heterozygotes were ostium secundum ASDs. We performed a second cross of a double heterozygous *Creld1*^{+/−}, *Hey2*^{+/−} mouse to *Hey2*^{+/−}. Ten pups were recovered that were homozygous for the *Hey2* null allele (which is expected to produce membranous VSD) and heterozygous at *Creld1* (Table 2). Of the 10, 1 had a normal heart and 9 had membranous VSDs; for those 9 mice with membranous VSDs, 1 also had an ostium secundum ASD, and 1 had a muscular VSD in addition to membranous involvement. This result suggests that reducing *Creld1* expression affected the pattern of defects in homozygous *Hey2* null mice, providing further support for an interaction between *Creld1* and *Hey2*.

### *Creld1* Interaction With *Hey2*

*Creld1* encodes a cell-surface protein, and *Hey2* is a nuclear transcription factor. Both are normally expressed in the heart during development and throughout life. We assessed transcript levels of both genes in hearts of mutant mice. As expected, *Creld1* RNA expression was reduced in *Creld1*^{+/−} mice, but, surprisingly, so was the expression of *Hey2* (Figure 4). The reverse was true, as well; *Hey2* and *Creld1* both showed reduced expression in hearts of *Hey2*^{+/−} mice. We saw no difference in expression of *Creld1* or *Hey2* in Ts65Dn compared with euploid hearts from mice that did not have the *Creld1* or *Hey2* null alleles.
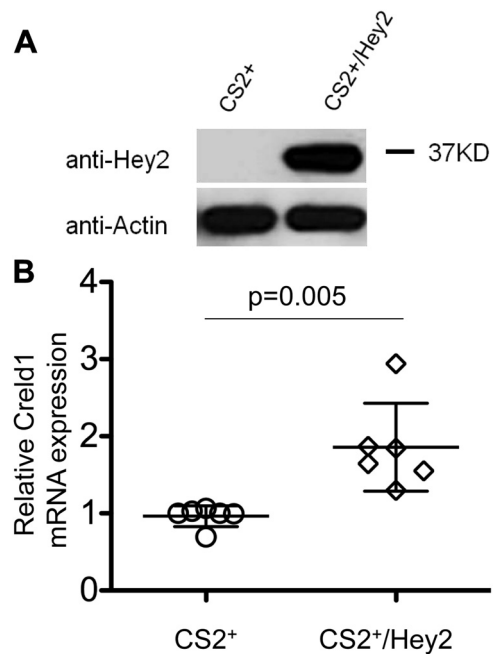


Figure 5. *Hey2* can activate *Creld1* expression in L-cells. **A**, Western blot showing *Hey2* protein expression in the L-cells transfected with *pCS2*[+]/*Hey2* but not in control cells transfected with *pCS2*[+] alone. An antibody to β-actin was used as a loading control. **B**, TaqMan assay showed a 1.9-fold increase of *Creld1* mRNA expression in *pCS2*[+]/*Hey2* transfected L-cells compared with controls. Plotted values are the average ratios of *Creld1* mRNA expression in 7 experiments. Standard deviation is indicated. *P* is indicated (Mann-Whitney test).

To explain the coregulation of *Creld1* and *Hey2*, we considered the Notch signaling pathway. Binding of a ligand to the Notch receptor causes cleavage and release of a transcriptional activator domain that, with further processing, is transported to the nucleus to activate transcription of target genes, including *Hey2*.[21] Since *Hey2* expression was downregulated in *Creld1*^{+/−} mice, we assessed the transcript level of *Notch1* and found it was also downregulated in these mice (Figure 4). This raises the possibility that *Creld1* might affect *Hey2* expression through the Notch pathway.

Since *Creld1* was downregulated in *Hey2*^{+/−} mice, we also tested whether *Hey2* might be a transcription factor for *Creld1*.[22] *Creld1* is expressed in L-cells. Transfection of a *pCS2*[+]/*Hey2* construct into L-cells significantly increased the *Creld1* mRNA expression by 1.9-fold compared with cells transfected with *pCS2*[+] alone (Figure 5).

### Discussion

Heart development is complex, and a complete genetic explanation for isolated CHD, which is generally believed to be a multifactorial condition, has proven to be a challenge; however, it is clear from extensive work with mutant mice that genetic background (modifiers) has a significant influence on both the type and the frequency of heart defects for a given mutation. For example, mice carrying mutant alleles of *Tbx5*, *Nkx2.5*, or *Hey2* show different outcomes both in terms of prevalence and also in changes in the timing or location of the maldevelopment when the same mutant allele is bred onto different genetic backgrounds.[20,23,24] The high

incidence but incomplete penetrance of CHD in the DS population suggests that variations at disomic loci (ie, on chromosomes other than Hsa21) may act as genetic modifiers in combination with trisomic genes. In other words, DS, the leading risk factor for CHD, in general, and septal defects in particular, represents a sensitizing genetic condition.

Numerous studies in mouse models have shown that genetic background can affect the severity of heart defects.[24] For example, the phenotype and survival rate of $Hey2^{-/-}$ mice is strongly influenced by genetic background, with the highest mortality rates observed on C57BL/6 or 129Sv/J inbred backgrounds.[11,12,16,19,20] The correlation of variable phenotypes with different inbred backgrounds supports the idea that (disomic) allelic variants modify $Hey2$ function. Similarly, heterozygous $Nkx2.5$ knockout mice frequently have septal defects when the mutation is bred onto the B6 background, but the prevalence is substantially reduced in a first-generation (F1) outcross to FVB/N or A/J.[24]

In people, $CRELD1$ mutations have been specifically associated with AVSD[2,8]; however, this may be an ascertainment bias since most studies have focused on AVSD, and more partial AVSDs have been analyzed than complete AVSDs. Most $CRELD1$ missense mutations have been found in individuals with a partial AVSD (ostium primum ASD), occurring in ~2% (5/253) of cases from multiple studies.[7,25–27] $CRELD1$ mutations are also found in ~2.5% (4/159) of complete AVSD cases studied, including Heterotaxy syndrome-associated AVSD and AVSD in Down syndrome.[7,25,28] There were no $CRELD1$ mutations identified in a study of 110 individuals with isolated ostium secundum ASD.[29]

Although only membranous VSD is reported in $Hey2^{-/-}$ mice,[16,19,20] introduction of a null allele of $Hey2$ into Ts65Dn mice caused muscular VSD and ASD in addition to membranous VSD. Further, the structural defect noted in $Hey2^{+/-}$, $Creld1^{+/-}$ mice was secundum ASD. It will be instructive to determine whether timing or levels of expression, or the small structural differences in Creld1 or Hey2 proteins between mouse and human might contribute to these differences. Either way, a requirement for regulated $Creld1$ and $Hey2$ expression to achieve normal septation is clearly demonstrated here.

It should be possible to further localize the dosage-sensitive (trisomic) genetic modifiers present on the T65Dn marker chromosome by introducing $Creld1$ or $Hey2$ mutations onto other trisomic models of Down syndrome with smaller segmental trisomies, such as Ts65Dn/Ms1Rhr, Ts1Cje, and Ts1Rhr.[30,31] These mice overexpress subsets of the genes upregulated in Ts65Dn mice. The absence of AVSD in Ts65Dn mice may reflect the fact that they are trisomic for orthologs of only about half of Hsa21 genes. Mouse models trisomic for Hsa21-orthologous segments of Mmu17 and Mmu10 and for all of the Hsa21 orthologs on Mmu16 will prove useful to further characterize the contributions of dosage-sensitive genes to CHD.[13,32,33] These then become candidates for resequencing in the DS case and control populations described here.

The Ts65Dn populations generated in these studies will also be useful in the genome-wide search for disomic modifiers. As in our DS case and control human populations, we have generated trisomic Ts65Dn, $Creld1^{+/-}$ mice with and without septal defects that can be used for a genome-wide study of quantitative trait loci (QTLs) that explain the presence or not of CHD on this background.

The 2 disomic modifiers, $Creld1$ and $Hey2$, showed coregulation at the transcript level. These alterations might involve the Notch signaling pathway since Notch1 is a major transcription factor for $Hey2$. In that regard, it is interesting that the structure of Creld1, a predicted transmembrane protein with 4 EGF-like repeats in its extracellular domain,[6] is reminiscent of the noncanonical Notch ligands, DNER[34] and DLK-1.[35] Like CRELD1, both of these lack the DSL motif of canonical Notch ligands but contain tandem EGF repeats. We tried to determine whether CRELD1 is a noncanonical ligand of $Notch1$; however, we could not demonstrate a physical interaction of CRELD1 and Notch1 via co-IP nor a functional one using a Notch transcription reporter assay[36] *(data not shown)*.

Our results support a threshold hypothesis in which multiple genetic variants that are themselves benign can interact in an additive manner to produce a structural defect in the heart (Figure 1). We identify 2 such candidates, $Creld1$ and $Hey2$. The deleterious effects of mutations in $Creld1$ that were themselves insufficient to produce CHD were uncovered in human beings while $Hey2$ effects were first demonstrated in mice. Biological proof for interactions of both genes with trisomy and for direct interactions on a euploid background was obtained using mouse models. In combination, the benign single variants exhibit additive effects resulting in CHD in euploid individuals. This is 1 of several examples of how the genetic legacy of people with DS contributes to the health of the general population.

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## CLINICAL PERSPECTIVE

Our investigation supports a "threshold model for congenital heart disease (CHD), " in which CHD occurs owing to small genetic contributions by multiple genes that each increase the risk to some degree. The presence of trisomy 21 dramatically increases the frequency of CHD, thus Down syndrome (DS) is a sensitizing condition and a major risk factor. We confirmed that a disomic modifier, *Creld1*, can greatly increase the frequency of CHD in a mouse model of DS when its expression is reduced. Further, disomic modifiers *Creld1* and *Hey2*, which have no effect on heart development when either is heterozygous alone, result in a significantly increased frequency of CHD in doubly affected mice. Our study identifies candidate modifiers using the sensitized DS population that can be used to build a catalogue of modifier genes that may potentially help understand the pathogenesis of CHD. Such knowledge is essential for future work aimed at the prevention of CHD.