Penetrance of Congenital Heart Disease in a Mouse Model of Down Syndrome Depends on a Trisomic Potentiator of a Disomic Modifier

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ABSTRACT Down syndrome (DS) is a significant risk factor for congenital heart disease (CHD), increasing the incidence 50 times over the general population. However, half of people with DS have a normal heart and thus trisomy 21 is not sufficient to cause CHD by itself. Ts65Dn mice are trisomic for orthologs of >100 Hsa21 genes, and their heart defect frequency is significantly higher than their euploid littermates. Introduction of a null allele of *Creld1* into Ts65Dn increases the penetrance of heart defects significantly. However, this increase was not seen when the *Creld1* null allele was introduced into Ts1Cje, a mouse that is trisomic for about two thirds of the Hsa21 orthologs that are triplicated in Ts65Dn. Among the 23 genes present in three copies in Ts65Dn but not Ts1Cje, we identified *Jam2* as necessary for the increased penetrance of *Creld1*-mediated septal defects in Ts65Dn. Thus, overexpression of the trisomic gene, *Jam2*, is a necessary potentiator of the disomic genetic modifier, *Creld1*. No direct physical interaction between Jam2 and Creld1 was identified by several methods. Regions of Hsa21 containing genes that are risk factors of CHD have been identified, but *Jam2* (and its environs) has not been linked to heart formation previously. The complexity of this interaction may be more representative of the clinical situation in people than consideration of simple single-gene models.

KEYWORDS trisomic potentiator; disomic modifier; congenital heart disease; Down syndrome

CONGENITAL heart disease (CHD) is the most frequent birth defect in human beings, affecting nearly 1% of all newborns (9/1000) (http://www.heart.org/HEARTORG). This frequency is far higher in Down syndrome (DS) where almost half of newborns have CHD (Freeman *et al.* 2008). Many genes have been implicated as potential modifiers of heart development (Locke *et al.* 2010; Sailani *et al.* 2013; Glessner *et al.* 2014); Online Mendelian Inheritance in Man (http://OMIM.org) lists 11,000 genes or syndromes of which CHD is a feature. We proposed a genetic model in which inheritance of multiple, individually benign genetic variants combine effects to reach a threshold beyond which heart development does not proceed normally (Li *et al.* 2012). On a euploid background, a large number of modifiers of small risk might be required. In this model, trisomy 21 (ts21) contributes a large fraction of risk. As ts21 is not sufficient to cause CHD by itself, it follows that additional risk factors must be necessary to reach the threshold for disease.

We provided biological support for this genetic model using mice with trisomy for regions orthologous to human chromosome 21 (Hsa21). In particular, the Ts65Dn mouse has been studied in this regard (Moore 2006; Williams *et al.* 2008; Li *et al.* 2012). We found a significant increase in septal defects in newborn trisomic mice that also carried a null allele of *Creld1*, a gene that has been associated with atrioventricular septal defect (AVSD) (Maslen 2004; Li *et al.* 2012). About 4% of newborn Ts65Dn mice have a septal defect and no defects were seen in *Creld1*$^{+/-}$ mice,

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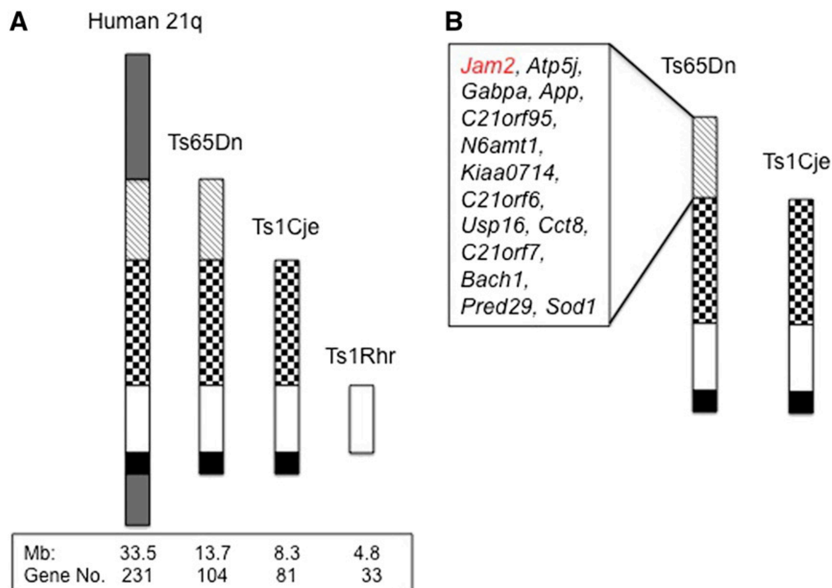

Figure 1 Down syndrome mouse models used in this study. (A) Sizes and gene numbers of the three trisomic mouse models used in this study. (B). Fourteen Hsa21-orthologous genes that are expressed in the developing heart which are localized on Ts65Dn but not on Ts1Cje.

however, a third of Ts65Dn;*Creld1*$^{+/-}$ mice were affected. A similar observation was made with a null allele of *Hey2* in place of *Creld1*. These individually benign mutations complemented each other in a euploid background: 9.7% of *Creld1*$^{+/-}$;*Hey2*$^{+/-}$ mice have septal defects. It has recently been recognized that the freely-segregating marker chromosome that carries these extra Hsa21 orthologous genes in Ts65Dn also contains a third copy of some genes not conserved with Hsa21 (Duchon *et al.* 2011; Reinholdt *et al.* 2011). However, the pattern of septal defects in Ts65Dn is similar to that reported for Dp(16)1Yey mice that carry a direct duplication of all Hsa21 orthologous genes on Mmu16 (Liu *et al.* 2014), albeit at a lower frequency. Thus this trisomic model is not only useful for uncovering individually benign modifier genes, but appears to be relevant to understanding the genetic basis for the high frequency of CHD in DS. We interrogated additional mouse models with segmental trisomy in an effort to localize genes that might contribute to the increased frequency of CHD.

Materials and Methods

Animal husbandry and genotyping

Mice used in the study were maintained in an American Association for Laboratory Animal Science (AAALAS)-certified clean facility with food and water *ad libitum*. Dp(16Cbr1-ORF9)1Rhr (Ts1Rhr) mice were maintained on the C57BL/6J background (B6J). Both B6EiC3Sn-Ts(16c-tel)1Cje/DnJ (Ts1Cje) and B6EiC3Sn *a/A*-Ts(17^{16})65Dn (Ts65Dn) were obtained from the Jackson Laboratory and maintained as a B6xC3H/HeJ advanced intercross. Dr. Akihiko Okuda of the Saitama Medical University in Japan kindly provided mice carrying a null allele of *Jam2* (Sakaguchi *et al.* 2006) on the C57Bl/B6N background through the Large Animal Resources and Genetic Engineering resource (http://www.cdb.riken.jp/arg/mutant%20mice%20list.html; Material Accession number CDB0413K). All procedures were approved by the Institutional Animal Care and Use Committee.

Genomic DNA was extracted from tail tips and used for genotyping by PCR. Ts1Cje mice were identified using the following primers:

CITE 19UP – CTCGCCAAAGGAATGCAAGGTCTGT,
CITE 324L – CCCTTGTTGAATACGCTTGAGGAGA,
GRIK1 F2 – CCCCTTAGCATAACGACCAG, and
GRIK1 R2 – GGAACGAGACAGACACTGAG.

Ts1Rhr and Ts65Dn PCR typing was performed as described (Duchon *et al.* 2011; Reinholdt *et al.* 2011). Genotyping of *Creld1* and *Jam2* knockout mice was performed by PCR as described (Li *et al.* 2012; Sakaguchi *et al.* 2006).

Histology

The progeny of various crosses were collected within hours of birth and processed, embedded, sectioned, and stained as described (Li *et al.* 2012). Heart morphology for each animal was analyzed with a dissecting stereomicroscope by at least two individuals blinded to genotypes. Photos were taken using a Nikon Digital Sight system (Japan).

Quantitative PCR analysis of Jam2 gene expression

Hearts of 4-week-old mice with different genotypes were dissected and homogenized. Total RNA was extracted using TRIzol (Life Technologies Corporation, Carlsbad, CA). Complementary DNA (cDNA) synthesis was carried out with the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Sciences, Cat.#LSK1200, Petersburg, FL) using 8 μg of

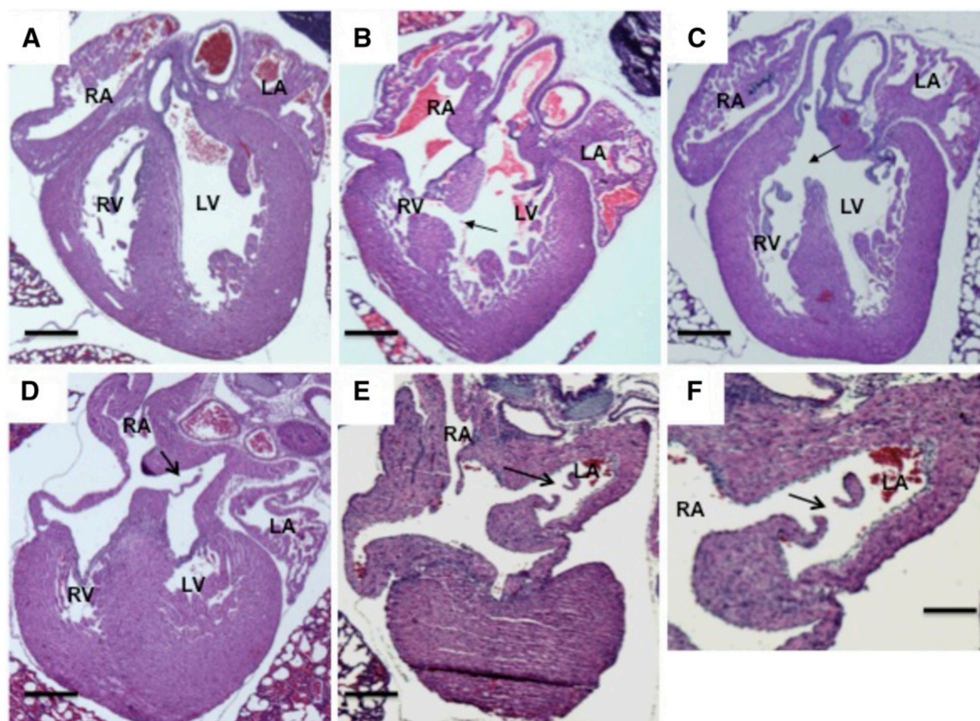

Figure 2 Different types of septal defects were observed in mutant and trisomic mice at P0. (A) Normal heart showing intact ventricular septum at P0; (B) muscular VSD; (C) membranous VSD; (D) normal heart showing atrial septum; (E) ostium secundum ASD; (F) ASD from E at higher magnification. For the incidence of defects in various models, see Table 1 and Table 2. Arrows indicate communication between the chambers. RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium; Bars: A–E, 400 μm; F, 150 μm.

total RNA as template. PCR was carried out using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA). Fluorescent (FAM)-labeled *Jam2* (Applied Biosystems) was normalized to a VIC-labeled internal control, *β-actin*. All comparisons refer to the wild type (WT).

In vitro transcription of messenger RNA

Plasmids were transcribed *in vitro* using the mMESSAGE mMACHINE SP6 kit (Ambion, Austin, TX). Plasmids were linearized, then purified by precipitation. Transcribed sequence reactions were treated with DNase I, and messenger RNA (mRNA) was purified with lithium chloride. mRNA quality and quantity were confirmed by formaldehyde agarose gel and the NanoDrop8000 (Thermo Fisher Scientific, Waltham, MA).

Zebrafish maintenance and injections

Tubingen Zebrafish were raised in the Zebrafish Core center at the Institute for Genetic Medicine (Johns Hopkins University) under protocol #FI12M263 as described (Westerfield 1993). Zebrafish were maintained at 28°. Males and females were placed together in the morning and embryos were collected 30 min later. One hundred embryos were then injected at the 1–4 cell blastula stage with JAM2 mRNA at 50 pg and 100 pg using a Zeiss Stemi 2000 microscope and PV820 Pneumatic picopump injector. Injected embryos were phenotyped at 24–96 hr postfertilization (hpf) using a Nikon SMZ1500 microscope and imaged with NIS Elements Imaging Software. After imaging, embryos were fixed in 4% paraformaldehyde and transferred to 100% methanol at −20°.

Morpholino rescue

A previously validated translation-inhibiting antisense morpholino (MO) was designed against zebrafish *Jam2a* (Powell and Wright 2011). One hundred embryos were injected with 2 ng MO, 100 embryos were injected with 100 pg of mRNA, and 100 embryos were injected with both 2 ng MO and 100 pg mRNA; 100 uninjected embryos were used as a control. Embryos were examined at 24 hpf.

Co-immunoprecipitation

Unless otherwise noted reagents were from Thermo Fisher Scientific. α-FLAG antibodies and affinity gel were from Sigma Chemical (St. Louis, MO). Protease inhibitor (PI) and Protein A agarose were from Roche.

Plasmids containing most of the human genes of interest were moved to pcDNA3.1/nV5-DEST with LR clonase. The stop codon was removed from *FAM126A*, *ARHGAP29*, and those genes encoding an N-terminal signal sequence, and the sequences moved to pEF-DEST51 by PCR cloning to add a C-terminal V5 tag. Human *CRELD1*- and *CRELD1*-R329C-FLAG C-terminal constructs were provided by Cheryl L. Maslen. The CRELD1-E414K construct was produced by site-directed mutagenesis of the WT CRELD1 construct using QuikChangeII XL site-directed mutagenesis kit (Agilent).

GripTite 293 MSR Cells were cotransfected with a V5-tagged gene of interest and FLAG-tagged *CRELD1* using Lipofectamine LTX and Plus reagent. After 48 hr, cells were washed with PBS, triturated from the plates in PBS, and pellets were frozen at −80° until use.

Table 1 Frequency of heart defects on mutant and trisomic genetic backgrounds

Phenotype	Genetic background	% of affected	Total no.	Type of septal defect
Creld1$^{+/-}$	B6J/C3H[a]	0	18	Not applicable
	B6J	0	27	
Ts1Cje	B6J/C3H[b]	17.2	29	4 membranous VSDs,[c] 1 secundum ASD[d]
Ts1Cje;*Creld1*$^{+/-}$	B6J/C3H[b]	13	31	3 membranous VSDs, 1 secundum ASD
Ts1Rhr	B6J	11.1	18	2 muscular VSDs
Ts1Rhr;*Creld1*$^{+/-}$	B6J	8	25	1 membranous VSD, 1 secundum ASD

[a] 50% B6, 50% C3H.
[b] 75% B6, 25% C3H.
[c] VSD, ventricular septal defect.
[d] ASD, atrial septal defect.

Cells were lysed using immunoprecipitation (IP) lysis buffer with PI, precleared with Protein A agarose and incubated with either α-FLAG- (30 μl) or α-V5-affinity gel (20 μl) for 2 hr at 4°. Eluted protein complexes were separated on denaturing NuPAGE gels and transferred to PVDF membranes. For Western blots of IPs using α-FLAG beads, coprecipitated V5-tagged proteins were detected with α-V5-HRP antibody or α-V5 and Clean Blot IP Detection Reagent (HRP). CRELD1-FLAG was detected with α-FLAG M2-AP and Lumi-Phos Western Blotting Reagent. For IPs using α-V5 beads, coprecipitating CRELD1 was detected with either rabbit α-FLAG and α-rabbit-HRP (Cell Signaling) or α-FLAG M2-AP. V5 proteins were detected with α-V5-HRP.

Protein microarray and data analysis

FLAG-tagged human CRELD1 cDNA with the two transmembrane domains removed (ΔCRELD1) (Rupp *et al.*, 2002) was expressed in GripTite 293 cells. The secreted ΔCRELD1 was purified by anti-FLAG M2 affinity gel (Sigma Chemical). The protein was incubated with 17,000 GST-tagged human proteins that were recovered from yeast, and arrayed in duplicate on microscope slides (Jeong *et al.* 2012). The microarrays were processed with α-FLAG or α-GST as described (Newman *et al.* 2013). The signal intensity (SI) of each spot is defined as the odds ratio of median values of the foreground and background signals, where a value of one indicates that the query protein did not bind to the substrate protein on the chip. Within-chip normalization was performed and the SI of all spots approximated a normal distribution. A spot was defined as positive if its SI was larger than mean ± 5 std. deviations.

Statistical analysis

Genotype ratios for the crosses produced in this study, the prevalence of heart defects in different mouse genotypes, and the penetrance of heart edema in zebrafish embryos after injection with JAM2 mRNA and/or MO were compared by Fisher's exact test using GraphPad Prism version 5. The relative quantification of gene expression from different genotypes was compared by Mann–Whitney test. All tests were two-tailed and *P*-values of $P < 0.05$ were considered significant.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article and Supplemental Material.

Results

Reduced Creld1 expression increases septal defect frequency in trisomic mice

We showed previously that reduced expression of *Creld1* acts in concert with trisomy in Ts65Dn to increase the occurrence of heart defects in Ts65Dn;*Creld1*$^{+/-}$ mice (Li *et al.* 2012). To further localize the trisomic genes contributing to CHD, *Creld1*$^{+/-}$ mice were crossed to Ts1Cje, a mouse model that is trisomic for about 80% of the Mmu16 genes triplicated in Ts65Dn (Figure 1A) (Das *et al.* 2013). Progeny were killed within hours of birth and evaluated histologically (Figure 2). The genotype ratio of the offspring from this cross was not significantly different from the expected frequency (Supplemental Material, Table S1). The baseline frequency of septal defects was higher in Ts1Cje (5 out of 29) than in Ts65Dn (2 out of 58) (Table 1) ($P = 0.04$). However, in contrast to the situation in Ts65Dn;*Creld1*$^{+/-}$ mice, there was no increase in septal defects in Ts1Cje;*Creld1*$^{+/-}$ mice. We observed defects in 17% of Ts1Cje mice and 13% in Ts1Cje carrying a null allele of *Creld1* ($P = 0.70$).

Consistent with the idea that a gene that is trisomic in Ts65Dn but not Ts1Cje is required to see the *Creld1*$^{+/-}$-influenced increase in heart defects, we detected no interaction between *Creld1*$^{+/-}$ and trisomy in another model, Ts1Rhr (Olson *et al.* 2004). These mice are trisomic for 33 of the genes that are triplicated in Ts1Cje and Ts65Dn. The genotype ratio of the offspring from this cross was not significantly different from the expected frequency (Table S2). Septal defects were seen in 8% of Ts1Rhr;*Creld1*$^{+/-}$ mice, which was not significantly different than the 11.1% frequency in Ts1Rhr itself ($P = 0.12$) (Table 1). The different outcomes in Ts65Dn compared to both Ts1Cje and Ts1Rhr suggest that a trisomic gene(s) that is necessary (but not necessarily sufficient) for the *Creld1* modifier effect on

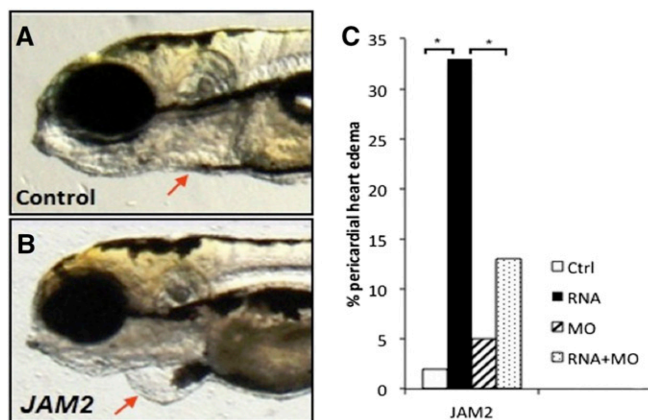

Figure 3 Effects of JAM2 expression in zebrafish embryos. (A) Control and (B) 100 pg JAM2-injected embryos at 48 hpf showing pericardial edema. (C) MO rescue, JAM2 mRNA alone, jam2 MO alone, or co-injection mRNA + MO each injected into 100 embryos and phenotyped at 24 hpf. * indicates $P < 0.01$.

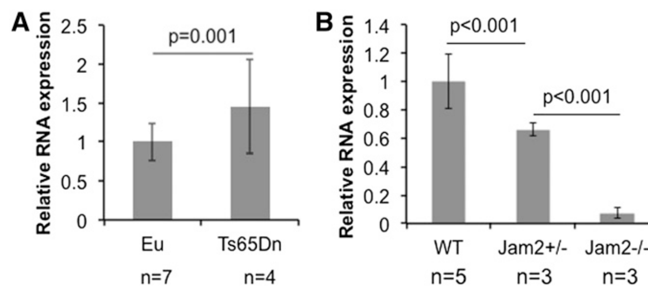

Figure 4 Real-time PCR showing the relative RNA expression level of *Jam2* in mice with different genotypes. (A) TaqMan assay showed about 1.5-fold increase of *Jam2* expression in Ts65Dn mice compared to WT. (B) TaqMan assay showed about 40% decrease of *Jam2* expression in *Jam2*$^{+/-}$ mice compared to WT, only background level of *Jam2* mRNA expression can be detected in *Jam2*$^{-/-}$ mice. *Jam2* mRNA was normalized to *β-actin* mRNA, *P*-value is indicated (Mann–Whitney U test).

penetrance is localized on the proximal portion of the segment that is triplicated in Ts65Dn (Figure 1B).

Trisomy for Jam2 acts in concert with Creld1

Twenty-three orthologs of Hsa21 genes plus a cluster of KRTAP-related genes that are trisomic in Ts65Dn are not triplicated in Ts1Cje (Starbuck *et al.* 2014). We identified 14 of these that are expressed in the developing heart (Figure 1B) (http://www.tigem.it/ch21exp/AtlasNewL.html; http://www.genecards.org/; http://www.ncbi.nlm.nih.gov/pubmed). We considered these to be candidates for increased CHD in the presence of decreased Creld1 expression on a trisomic background. Three of these are membrane proteins; Creld1 has been identified as a cell surface protein and more recently has been described in endoplasmic reticulum as well (Rupp *et al.* 2002; Maslen 2004; Mass *et al.* 2014). Among the 14 heart-expressed genes, JAM2 is a cell membrane protein with immunoglobulin-like domains that is concentrated at cell-to-cell junctions in heart endothelial cells of both large and small vessels, and it has been implicated in angiogenesis defects in Tc1 mice (Reynolds *et al.* 2010). Mouse *Jam2* was identified in a gene expression-based search for stemness genes in embryonic stem (ES) cells where it was highly expressed in ES cells but quickly down regulated as they began to differentiate (Cunningham *et al.* 2000). Surprisingly, no phenotype was detected in a thorough study of *Jam2*$^{-/-}$ mice (Sakaguchi *et al.* 2006). However, in a screen of Hsa21 gene effects on early embryonic zebrafish development (S. Edie, N. A. Zaghloul, D. K. Klinedinst, J. Lebron, N. Katsanis, R. H. Reeves, in preparation), we found that overexpression of *JAM2* causes maldevelopment of the heart.

We cloned a human *JAM2* ORF into the pCS2 vector, synthesized mRNA and injected zebrafish embryos with *JAM2* mRNA. Injected embryos showed a high frequency of pericardial edema and this phenotype was robustly replicated over multiple injections ($P < 0.0001$, Fisher's exact test) (Figure 3). The edema phenotype was partially rescued by co-injecting translation-blocking MOs targeted against the zebrafish ortholog, *jam2a* ($P = 0.001$), indicating that the effect is due to mRNA expression and not to nonspecific toxicity. Further, the pericardial edema phenotype was not observed when any of >100 other Hsa21 cDNAs was injected in the same paradigm.

Based on these observations, we tested the hypothesis that *Jam2* must be trisomic in mice to see the greatly increased penetrance of septal defects that occurs in Ts65Dn;*Creld1*$^{+/-}$, but not in Ts1Cje;*Creld1*$^{+/-}$ mice that are not trisomic for *Jam2*. Initial experiments showed that the frequency of heart defects in Ts65Dn seen previously on the trisomic B6J.C3H background (Moore 2006; Williams *et al.* 2008; Li *et al.* 2012) was attenuated or lost on the B6N.C3H background. Accordingly, B6N.*Jam2*$^{-/-}$ mice were backcrossed onto a C57BL/6J background for six or more generations. We used qPCR to compare *Jam2* mRNA level in hearts of euploid (WT), Ts65Dn, and *Jam2*$^{-/-}$ mice. We found that *Jam2* expression was increased by about 1.5-fold in Ts65Dn compared to the WT, there was a 40% decrease of *Jam2* expression in *Jam2*$^{+/-}$ compared to the WT, and only background signal was detected in *Jam2*$^{-/-}$ mice (Figure 4).

We performed a two generation, three-way cross to subtract one copy of *Jam2* from Ts65Dn;*Creld1*$^{+/-}$ mice by crossing male *Jam2*$^{+/-}$;*Creld1*$^{+/-}$ to Ts65Dn females. The genotype ratio of the offspring from this cross was not significantly different from the expected frequency (Table S3). In contrast to the 18.3% septal defects in Ts65Dn;*Creld1*$^{+/-}$ mice, only 4.5% (2 out of 44) of Ts65Dn;*Creld1*$^{+/-}$;*Jam2*$^{+/-}$ (triple) mice had septal defects ($P = 0.015$) (Table 2). The septal defect penetrance in the triple mice was not different from that in Ts65Dn and Ts65Dn;*Jam2*$^{+/-}$ (3.4% and 3.8%, respectively). The defect seen in the two affected triple mice was membranous ventricular septal defect (VSD), the most frequent septal defect in Ts65Dn, while half of the affected Ts65Dn;*Creld1*$^{+/-}$ mice had a secundum atrial septal defect (ASD) (seven membranous VSD and eight secundum ASD).

Table 2 Type and frequency of heart defects in Ts65Dn×*Creld1*$^{+/-}$*;Jam2*$^{+/-}$

	Type of defect			
	Membranous VSD	Secundum ASD	% of total	Total
Ts65Dn	2	0	3.4	58[a]
Ts65Dn;*Creld1*$^{+/-}$	8	9	18.3	93
Ts65Dn;*Jam2*$^{+/-}$	1	0	3.8	26
Ts65Dn;*Creld1*$^{+/-}$,*Jam2*$^{+/-}$	2	0	4.5	44[b]
Creld1$^{+/-}$	0	0	0	45
Creld1$^{+/-}$;*Jam2*$^{+/-}$	0	0	0	25

[a] Ts65Dn *vs.* Ts65Dn;*Creld1*$^{+/-}$: $P = 0.01$.
[b] Ts65Dn;*Creld1*$^{+/-}$ *vs.* Ts65Dn;*Creld1*$^{+/-}$,*Jam2*$^{+/-}$: $P = 0.03$.

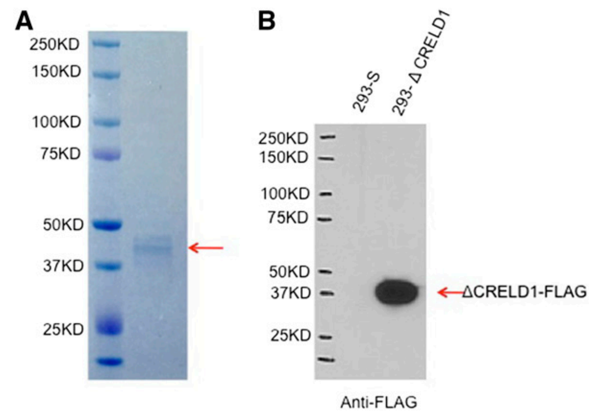

Figure 5 Expression and purification of ΔCRELD1-FLAG protein. (A) SDS-PAGE and Coomassie blue staining of purified ΔCRELD1-FLAG protein; (B) Western blot to detect the purified protein by anti-FLAG antibody, the first lane is the supernatant of untransfected GripTite 293 cells, the second lane is the supernatant of GripTite 293 cells transfected with pCS2/ΔCRELD1-FLAG.

No septal defects were detected in *Creld1*$^{+/-}$*;Jam2*$^{+/-}$ mice (0 out of 25) nor in *Creld1*$^{+/-}$ mice (0 out of 45). Since subtraction of the third copy of *Jam2* from Ts65Dn; *Creld1*$^{+/-}$ mice eliminated the interaction that elevates the penetrance of CHD in these mice; our results indicate that *Jam2* plays a necessary role in the cross-talk between trisomy and *Creld1* in Ts65Dn. However, carrying two *vs.* three copies of *Jam2* by itself did not affect the frequency of septal defects in Ts65Dn.

No evidence of direct interaction between Jam2 and Creld1 proteins by co-immunoprecipitation

Both *Creld1* and *Jam2* have been shown to encode membrane proteins (Rupp *et al.* 2002; Maslen 2004; Sakaguchi *et al.* 2006). We assessed the possibility that these proteins may interact directly to produce the effects observed in genetic models. Two investigators independently attempted co-immunoprecipitation (co-IP) using both Creld1 and Jam2 as drivers. We could find no evidence for interaction (Figure S2). We then searched for Creld1-interacting proteins that might be possible intermediates for communication between Creld1 and Jam2 using a human protein array.

Proteome microarray using purified human CRELD1 recombinant protein

Purified FLAG-tagged Human CRELD1 without the two transmembrane domains (rhΔCRELD1-FLAG) (Figure 5 and Figure S1) was incubated with protein microarray slides on which about 17,000 yeast-expressed human GST fusion proteins were printed. A negative control (without rhΔCRELD1-FLAG) was included. Signal intensities were determined with a GenePix 4000 scanner. GENEPIX PRO 5.0 software analysis identified about 2000 out of 17,000 proteins that were considered positive using a cut-off of 2 for the signal-to-noise ratio, and the remaining ~15,000 proteins were considered as negative.

We prioritized the list of 2000 putative CRELD1-interacting proteins using multiple criteria. First, the proteins were ordered based on intensity of hybridization to the CRELD1 probe peptide. We then assessed groups of 100 proteins using the START program developed by Vanderbilt University (http://bioinfo.vanderbilt.edu/webgestalt/option.php) which is based on Gene Ontology (GO Slim). GO Slim classification includes cellular component, molecular function and biological process. We focused on the cellular component classification because CRELD1 is a membrane protein and we reasoned that true hits would include a large percentage in this category. We found a large number of hits in this category among the top 100 proteins, fewer hits among the 101–200 strongest signal targets, fewer still among the next 100 and so on (Table S4). Based on this we assessed the top 300 proteins using GeneALaCart (A GeneCards Batch Queries Engine; http://gene4.weizmann.ac.il/cgi-bin/BatchQueries/Batch.pl) and made a new target protein list for CRELD1 using the membrane-related and heart expression criteria. We identified 38 such proteins among the top 300 strongest signals (Table S5). JAM2 was not among the top 2000 proteins identified in the protein array.

To verify these interactions, we subcloned these 38 target genes into a mammalian expression vector to produce a V5-tagged protein and performed co-IP experiments with FLAG-tagged full-length human CRELD1. Of the 38 proteins, 10 gave a positive result for association of CRELD1 and the target with α-FLAG antibody on the affinity column. In the inverse experiment, CRELD1-FLAG was pulled down with 9 of the 10 V5-tagged proteins (Table S6). Thus at least 9 of 38 proteins (24%) identified on the large protein array were correctly identified as CRELD1 interactors by this independent measure.

We repeated the co-IPs of the 10 positive proteins with CRELD1 clones that carry the R329C or E414K mutations that have been described in AVSD patients (Robinson *et al.* 2003; Maslen *et al.* 2006) with essentially identical results, indicating that mutations in CRELD1 did not affect the interaction with these proteins. We also carried out triple transfections of the V5-tagged target proteins, CRELD1-FLAG and JAM2-myc to determine if JAM2 interacts with CRELD1 indirectly through one of these binding partners. However, Jam2 did not coprecipitate with any of these protein pairs.

Discussion

Our previous demonstration that candidate genetic modifiers predisposing to CHD can be identified in human studies of the genetically-sensitized DS population and validated biologically in the laboratory mouse is expanded here to show a type of genetic relationship not previously described for trisomic gene effects. Variants of *Creld1* that are completely benign by themselves are risk factors for CHD that can act additively with other benign modifiers (*e.g.*, *Creld1*$^{+/-}$ and *Hey2*$^{+/-}$) or with trisomy for mouse orthologs of about half of the genes conserved with Hsa21 (Li *et al.* 2012). *Jam2* has no effect on heart development when present at 0, 1, 2, or 3 copies and shows no additive effect with trisomy. However, it must be trisomic and overexpressed to see the increased penetrance of septal defects in mice with only one copy of *Creld1*.

Our genetic data shows that *Jam2* is a potentiator of *Creld1* in an epistatic interaction leading to maldevelopment of the heart. This does not appear to be based on a direct protein interaction, nor did we identify potential intermediates that connect the two functionally. Indeed, the degree of the effect, while significant, is modest. Cohorts of Ts65Dn trisomic mice that also inherited a null allele of *Creld1* saw the incidence of septal defects rise from 4 to 18%, *i.e.*, $<$20% of offspring were affected.

We have shown that loss of function of *Creld1* can act in concert with the trisomic genes in Ts65Dn to create septal heart defects. The most frequent septal defect types we observed in our study are membranous VSD and secundum ASD. The atrioventricular cushions contribute to the formation and perhaps to closure of both the ventricular and atrial septa. CRELD1 is expressed in many human tissues by Northern blot, and it has high expression levels in heart. *In situ* hybridization using chick embryos showed high expression of CRELD1 in the cardiac atrial muscle and cushion tissue (Rupp *et al.* 2002), indicating a role for CRELD1 in endocardial cushion formation.

The endocardial cushions arise from a subset of endothelial cells that undergo epithelial-mesenchymal transition (EMT), a process whereby these cells break cell-to-cell adhesions and migrate into the inner heart wall to form endocardial cushions (Brade *et al.* 2006). In the Creld1 null mouse the endocardial cushions are smaller and hypocellular compared to developmentally matched WT littermates (Redig *et al.* 2014). Breakage of the cell-cell adhesion between the endothelial cells is an important process during endocardial cushion formation. Jam2 is a cell adhesion molecule that is specifically expressed in endothelial cells (Weber *et al.* 2007), suggesting that overexpression of the *Jam2* gene due to trisomy might slow or inhibit the EMT process by strengthening those cell-cell interactions. As Creld1 is also a membrane protein, it is reasonable to speculate that it may interact with Jam2. However, we did not detect either direct or indirect interaction between Jam2 and Creld1 by co-IP of candidate CRELD1 interacting proteins, nor was Jam2 bound by ΔCRELD1 on a large protein array. If *Jam2* is one of the genes responsible for the cross-talk between Ts65Dn and *Creld1*, it must do so by an indirect mechanism (not direct physical interaction) possibly by affecting endocardial cushion formation through the signaling pathways related to *Creld1*.

The genetics of CHD are complex, such that only a few highly-penetrant candidate genes have been implicated in human genetic syndromes. The vast majority of CHD is unexplained. If genetic contributions to this anomaly are due to small additive effects of a large pool of individually benign variants, identification of candidate targets for intervention will remain a challenge. The greatly increased frequency of CHD on the sensitized trisomic background will provide an important tool for finding and ameliorating the genetic variation contributing to the most frequent birth defect in human beings.

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Penetrance of Congenital Heart Disease in a Mouse Model of Down Syndrome Depends on a Trisomic Potentiator of a Disomic Modifier

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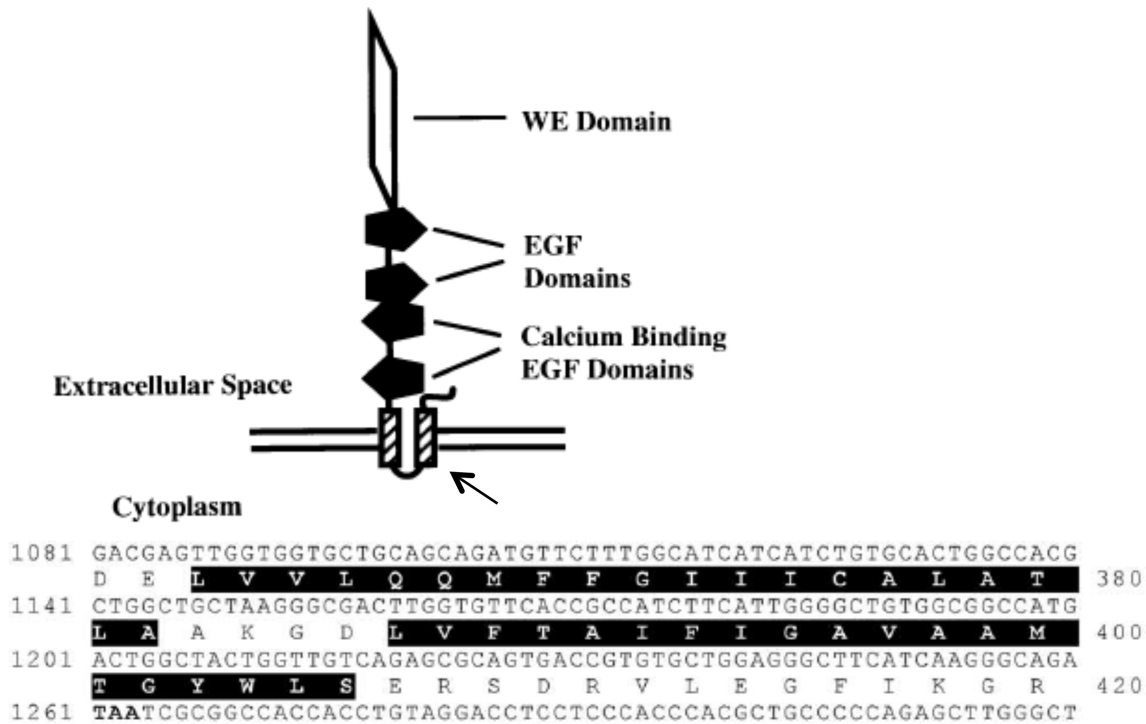

Rupp et al., 2002

Figure S1. **The structure of human CRELD1.** The sequences highlighted in black correspond to the two transmembrane domains. These and the 44 nucleotide residues encoding the C-terminal portion of the protein were deleted. The N-terminal portion of the Creld1 gene was fused with a FLAG tag in the pCS2 vector and transfected to GripTite 293 to make ΔCRELD1-FLAG protein.

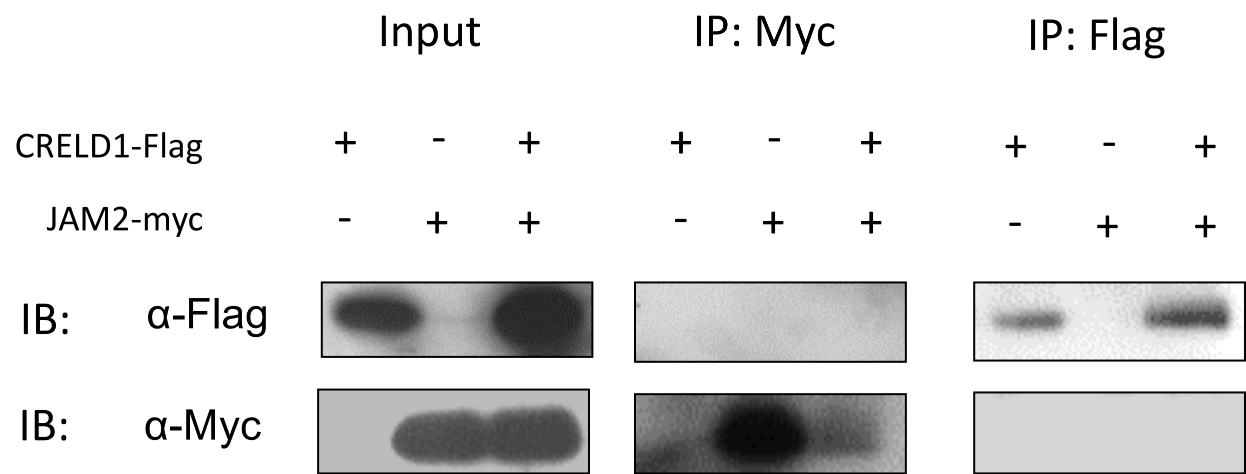

Figure S2: **Co-immunoprecipitation experiments with CRELD1-Flag and JAM2-myc**.

Table S1. Genotypic ratios of offspring generated by the Ts1Cje x *Creld1*$^{+/-}$ crosses

Genotype	**Number**	**Ratio to Total**	**Mendelian Ratio**	***p* Value**
Eu, Creld1$^{+/+}$	57	33.9%	25%	0.09
Eu, Creld1$^{+/-}$	43	25.6%	25%	1.00
Ts1Cje, Creld1$^{+/+}$	37	22%	25%	0.61
Ts1Cje, Creld1$^{+/-}$	31	18.5%	25%	0.19
Total	168			

Table S3. Genotypic ratios of offspring generated by the Ts65Dn x *Creld1*$^{+/-}$;*Jam2*$^{+/-}$ crosses

Genotype	Number	Ratio to Total	Mendelian Ratio	*p* Value
Eu, Creld1$^{+/+}$,JamB$^{+/+}$	49	15.5%	12.5%	0.36
Eu, Creld1$^{+/-}$,JamB$^{+/+}$	50	15.8%	12.5%	0.31
Eu, Creld1$^{+/+}$,JamB$^{+/-}$	34	10.8%	12.5%	0.54
Eu, Creld1$^{+/-}$,JamB$^{+/-}$	34	10.8%	12.5%	0.54
Ts, Creld1$^{+/+}$,JamB$^{+/+}$	35	11.1%	12.5%	0.62
Ts, Creld1$^{+/-}$,JamB$^{+/+}$	37	11.7%	12.5%	0.81
Ts, Creld1$^{+/+}$,JamB$^{+/-}$	33	10.4%	12.5%	0.46
Ts, Creld1$^{+/-}$,JamB$^{+/-}$	44	13.9%	12.5%	0.73
Total	316			

Table S4. Informatics analysis of putative CRELD1-interacting proteins, the final 38 candidate proteins were determined by both GO analysis and the protein's expression pattern.

	Signal Intensity (F/B)	Gene Category	Nuclear Protein	Chromosome Protein	No. of membrane-related, heart expression (% of 38 total)
Top 100	>7.28	18	26.7%	0	18 (47%)
Top 200	5.70-7.28	20	26.6%	0.65%	18+12 (79%)
Top 300	4.89-5.70	21	26.8%	1.57%	18+12+4 (90%)
Top 500	4.09-4.89	21	28.6%	2.11%	18+12+4+4 (100%)

Table S5. Membrane related CRELD1 interacting proteins expressed in the heart

	Clone id	Accession	Symbol	ORF size	mRNA size	Gene description
1	IOH11246	NM_015423.2	AASHDPPT	930	2880	aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase (AASDHPPT),
2	IOH10813	BC022530.1	ACVR1c	1482	3267	activin receptor
3	IOH11130	BC022483.1	ARHGAP29	1182	2096	unknown
4	IOH11645	NM_001721.6	BMX	2028	2530	BMX non-receptor tyrosine kinase (BMX), transcript variant 2
5	IOH13563	BC021578	CLCN2	1164	1866	chloride channel 2
6	IOH3437	NM_001312.2	CRIP2	627	1236	cysteine-rich protein 2
7	IOH6848	BC007567.1	DBN1	1950	2328	drebrin 1 (DBN1), transcript variant 2,
8	IOH5481	NM_018110.3	DOK4	981	2750	DOCKING PROTEIN
9	IOH22214	BC030828.1	EDIL3	1443	2728	EGF-like repeats and discoidin I-like domains 3
10	IOH10679	BC022511.1	EDNRA	1284	2705	endothelin receptor type A (EDNRA), transcript variant 1,
11	IOH14139	NM_018948.3	ERRFI1	1389	3144	ERBB receptor feedback inhibitor 1
12	IOH21728	NM_016337.2	EVL	1257	1853	Enah/Vasp-like
13	IOH13296	NM_032581.3	FAM126A	1566	6165	family with sequence similarity 126, member A
14	IOH21425	BC018128.1	FGFR1	2463	4086	
15	IOH13516	BC011634.1	GPER	1128	2218	G protein-coupled estrogen receptor 1
16	IOH53821	NM_000598.4	IGFBP3	876	2620	insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2
17	IOH6284	BC006231	IKBKB	771	1167	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
18	IOH29625	NM_003636.3	KCNAB2	1104	4224	potassium voltage-gated channel, shaker-related subfamily, beta member 2 (KCNAB2), transcript variant 1,
19	IOH13864	NM_005558.3	LAD1	1554	2869	ladinin 1
20	IOH3460	NM_002436.3	MPP1	1401	2067	membrane protein, palmitoylated 1, 55kDa (MPP1), transcript variant 1
21	IOH4468	NM_015922.2	NSDHL	1122	1581	NAD(P) dependent steroid dehydrogenase-like (NSDHL), transcript variant 1,
22	IOH3934	NM_007217.3	PDCD10	639	1454	programmed cell death 10 (PDCD10), transcript variant 1
23	IOH4237	ENST00000284767	PDLIM3	564	1888	PDZ and LIM domain 3
24	IOH10635	NM_014172.4	PHPT1	378	1218	phosphohistidine phosphatase 1 (PHPT1), transcript variant 3
25	IOH11174	NM_001130848.1	PITPNM1	3732	4245	phosphatidylinositol transfer protein, membrane-associated 1 (PITPNM1), transcript variant 2
26	IOH12943	BC009196.2	PPAP2B	936	1442	phosphatidic acid phosphatase type 2B
27	IOH29864	NM_002706.4	PPM1B	1440	2636	protein phosphatase, Mg2+/Mn2+ dependent, 1B (PPM1B), transcript variant 1
28	IOH12805	BC010353.1	PTPLA	867	1029	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A
29	IOH3388	BC008692.1	PTPN11	1383	1892	protein tyrosine phosphatase, non-receptor type 11
30	IOH10004	NM_020673.2	RAB22A	585	8702	member RAS oncogene family
31	IOH22947	NM_020387.2	RAB25	642	1101	member RAS oncogene family
32	IOH14673	NM_004251.4	RAB9A	606	1377	member RAS oncogene family (RAB9A), transcript variant 1, mRNA RAB9A
33	IOH12305	NM_001018003.2	SORBS3	990	2057	sorbin and SH3 domain containing 3 (SORBS3), transcript variant 2,
34	IOH5103	NM_003900.4	SQSTM1	1323	2923	sequestosome 1 (SQSTM1), transcript variant 1,
35	IOH23086	NM_012456.2	TIMM10	273	684	translocase of inner mitochondrial membrane 10 homolog
36	IOH5297	NM_001001431	TNNT2	858	1123	troponin T type 2 (cardiac) (TNNT2), transcript variant 3
37	IOH5913	NM_172016.2	TRIM39	1467	3578	tripartite motif containing 39 (TRIM39), transcript variant 2
38	IOH6304	NM_032353.2	VPS25	531	1122	vacuolar protein sorting 25 homolog

Table S6. Available gene constructs and their interaction with CRELD1-FLAG by Co-IP.

		Location of V5-tag	Antibody for Co-IP
1	AASHDPPT	N	
2	ACVR1	C	**F,V
3	ACVR1c	C	**F,V
4	ARHGAP29	C*	
5	BMX	N	
6	CRIP2	N	
7	DBN1	N	
8	EDIL3	C	**F,V
9	EDNRA	C	**F,V
10	ERRFI1	N	
11	EVL	N	
12	FAM126A	C*	
13	FGFR1	C	**F,V
14	GPER	N	**F,V
15	IGFBP3	C	
16	IKBKB	N	
17	KCNAB2	N	+
18	MPP1	N	
19	NSDHL	N	F
20	PDCD10	N	
21	PDLIM3	N	
22	PHPT1	N	
23	PITPNM1	N	
24	PPAP2B	N	**F,V
25	PPM1B	N	
26	PTPLA	N	**F,V
27	PTPN11	N	
28	RAB25	N	
29	RAB9A	N	
30	SORBS3	N	
31	SQSTM1	N	**F,V
32	TIMM10	N	

* expressed poorly with N terminal tag

+ precipitated in the absence of CRELD1

** positive forCRELD1 Co-IP

Table S6 methods: Thirty-five candidate genes were expressed as V5 fusion proteins along with FLAG-tagged CRELD1 and tested for co-immunoprecipitation with CRELD1. The 10 proteins that co-precipitated with CRELD1 using FLAG antibody beads are indicated (F). Nine of the 10 (all except NSDHL) successfully co-precipitated FLAG-tagged CRELD1 with V5-antibody beads in the reciprocal experiment (V). The 10 V5-tagged proteins that co-precipitated with wild type FLAG-tagged CRELD1 were also tested with 2 mutants of CRELD1 that had been identified in screens of Down syndrome individuals with complete AVSD. The candidate genes all co-precipitated with both of the mutated FLAG-tagged CRELD1 proteins using FLAG antibody beads.