Chordin Is a Modifier of Tbx1 for the Craniofacial Malformations of 22q11 Deletion Syndrome Phenotypes in Mouse

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Abstract

Point mutations in TBX1 can recapitulate many of the structural defects of 22q11 deletion syndromes (22q11DS), usually associated with a chromosomal deletion at 22q1.2. 22q11DS often includes specific cardiac and pharyngeal organ anomalies, but the presence of characteristic craniofacial defects is highly variable. Even among family members with a single Tbx1 point mutation but no cytological deletion, cleft palate and low-set ears may or may not be present. In theory, such differences could depend on an unidentified, second-site lesion that modifies the craniofacial consequences of Tbx1 deficiency. We present evidence for such a locus in a mouse model. Null mutations of chordin have been reported to cause severe defects recapitulating 22q11DS, which we show are highly dependent on genetic background. In an inbred strain in which chordin−/− is fully penetrant, we found a closely linked, strong modifier—a mutation in a Tbx1 intron causing severe splicing defects. Without it, lack of chordin results in a low penetration of mandibular hypoplasia but no cardiac or thoracic organ malformations. This hypomorphic Tbx1 allele per se results in defects resembling 22q11DS but with a low penetrance of hallmark craniofacial malformations, unless chordin is mutant. Thus, chordin is a modifier for the craniofacial anomalies of Tbx1 mutations, demonstrating the existence of a second-site modifier for a specific subset of the phenotypes associated with 22q11DS.

Citation: Choi M, Klingensmith J (2009) Chordin Is a Modifier of Tbx1 for the Craniofacial Malformations of 22q11 Deletion Syndrome Phenotypes in Mouse. PLoS Genet 5(2): e1000395. doi:10.1371/journal.pgen.1000395

Editor: Bernice E. Morrow, Albert Einstein College of Medicine, United States of America

Received October 17, 2008; Accepted January 28, 2009; Published February 27, 2009

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Funding: This work was supported by NIH grant DE13674 and HL685308 to JK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

In approximately 1 in 4000 human births, syndromic congenital malformations are associated with deletions in chromosomal region 22q11.2. DiGeorge syndrome (OMIM 188400), velocardiofacial syndrome (OMIM 192430), and related syndromes are all associated with this deletion; these are collectively called the 22q11 deletion syndromes, 22q11DS [1]. At least 20 genes are contained within the region typically deleted, the DiGeorge Critical Region (DCR). To understand the roles of particular DCR genes in the etiology of 22q11DS, the functions of many of these genes have been assessed in the mouse. Tbx1 null homozygotes show severe defects in all the structures variably affected in these syndromes [2,3,4], while heterozygotes show aortic arch artery defects similar to some mildly affected patients [2]. Several different point mutations in Tbx1 have been identified in patients with 22q11DS but without cytological deletions at 22q11 [5,6]. Thus, defective Tbx1 function is a key factor in the pathogenesis of the 22q11DS malformations.

Despite the identification of the DCR, and the key role of Tbx1 in particular, the genetics of 22q11DS pathogenesis remains unclear. Significant numbers of 22q11DS patients don’t possess deletions at 22q11.2 or known Tbx1 point mutations, while deletions in other regions of the genome have been observed [7,8]. These and related considerations suggest that other loci, as yet unknown, play an important role in the etiology of 22q11DS [9]. In mouse models, mutations in Cdk1, which lies in 22q1.2, and in Fgfl, which is unlinked, have both been shown to modulate the developmental phenotype by enhancing the effects of a Tbx1 null mutation [10,11]. However, none of the genetic results to date suggest an explanation for the high variability of disease symptoms among 22q11DS patients, or why subsets of patients present with particular structural malformations but not others.

Mice lacking chordin (Chrd), a dedicated antagonist of Bone Morphogenetic Proteins (BMPs), have been reported to show a phenotype recapitulating many structural features of 22q11DS, and very similar to that of Tbx1 null embryos [12,13]. Especially given that both genes are expressed in or around the pharyngeal endoderm during early organogenesis, this implied a mechanistic link between BMP antagonism and Tbx1 function. Consistent with this, reduced levels of Tbx1 message were observed in the pharyngeal region of Chrd−/− embryos; moreover, Chrd transcript injected into early Xenopus embryos could increase the endogenous Tbx1 transcriptional level [12]. Taken together, these results suggested that chordin acts in the pharyngeal region to protect Tbx1 expression from inhibition by local BMPs. However, as detailed below, our breeding of Chrd in different wildtype genetic
Author Summary

A range of structural malformations is associated with 22q11 deletion syndrome (22q11DS), which is usually associated with microdeletions at chromosome 22q11.2. Variable defects in cardiovascular, pharyngeal, and craniofacial structures occur, but the basis for such variability is unknown. Mutations in Tbx1, a gene within the region typically deleted, can recapitulate the structural anomalies of 22q11.2 deletions. However, even among family members with a single Tbx1 point mutation, craniofacial defects are variable. In theory, such differences could depend on an unknown, second-site lesion that modifies the craniofacial consequences of Tbx1 deficiency. We identify such a locus in mice. In certain strains, lack of the chordin gene results in a phenotype resembling severe 22q11DS, also seen in mice lacking Tbx1. We find that the chordin phenotype depends on a closely linked, strong modifier—a cryptic, partial-function mutation in Tbx1. Without it, lack of chordin sometimes results in mandibular truncations but no cardiac or thoracic organ malformations. This novel Tbx1 allele per se results in defects resembling 22q11DS, but with a low frequency of hallmark craniofacial malformations, unless chordin is mutant. Thus chordin is a modifier for the craniofacial anomalies of Tbx1 mutations, demonstrating the existence of a second-site modifier for a specific subset of phenotypes associated with 22q11DS.

backgrounds has indicated that in most cases, embryos can lack Chrd entirely, yet exhibit no resemblance to 22q11DS or Tbx1 mutants. Such results reveal that an additional, unknown genetic mechanism was at play in the generation of these phenotypes. Here we report our elucidation of the relationship between Chrd, Tbx1, and the 22q11DS phenotypes.

Results

The previously reported Chrd+/− null phenotype is a completely penetrant constellation of defects— which we call hereafter the ‘full phenotype’—that includes dysmorphic ear (Figure 1A, B), absence of thymus (athyemia), persistent truncus arteriosus (PTA), abnormal aortic arch artery structure (Figure 1C–E), cleft palate (Figure 1F–G). This spectrum of defects is virtually identical to those of 22q11DS, also seen in mice lacking Tbx1. However, Chrd+−/− embryos, while F1 +/−/+ and B6−/− Chrd+/−/+ mice, no significant phenotype was observed in double heterozygotes (0/15, two cases of asymmetric thymus were detected). Thus the apparent interaction of the Tbx1 null locus with Chrd depends on a linked sensitizer in the 129S6 background. The simplest explanation is a cryptic mutation in Tbx1 itself.

Utilizing the Tbx1+/− mutation as a SNP marker, we addressed how strongly it segregates with the full phenotype. The high LOD score suggests that the Tbx1 mutation is indeed a strong modifier (Figure 2D). Since the mutation is in the boundary region of the second intron and is predicted to disrupt the splicing factor recognition sequence (ESEfinder 2.0; Figure 2E) [15,16], we examined splicing of Tbx1 in 129S6-Chrd+/− ES cell culture or in the establishment of the 129S6-Chrd colony. We detected exon skipping and intron retention of Tbx1 mRNA from such embryos, leaving little of the correct form (Figure 2F). This suggests that the Tbx1+/− mutation is at least partly responsible for the 22q11DS constellation of phenotypes observed in Chrd mutants in certain genetic backgrounds.

Having determined that a Tbx1 lesion is a linked modifier of Chrd, we wondered how we observed 44% penetrance of the 22q11DS phenotypes in F2 Chrd+/−− animals (Figure 1H), instead of the expected value of 25%. Breeding records and retrospective Tbx1 genotyping indicated that this resulted from using a subset of F1 hybrid males as studs that by chance had most often inherited their mutant Chrd allele from the 129S6 background, and thus were also carriers of the tightly linked Tbx1+/−− allele (the expected phenotypic penetrance would be 50% if this were the case for all chromosomal region with the Chrd locus to result in the full phenotype. Although there was evidence of more than one potential modifier, the data suggested that such a region resides on the same chromosome as Chrd (data not shown). In contrast to the situation in humans, Chrd is near the DCR in mice (Figure 2A), reflecting a relative chromosomal translocation between Chrd and the DCR in these species. Since lesions within the DCR are implicated in similar phenotypes in mouse models [10,14], we investigated whether a linked modifier might account for the strain-dependence of the Chrd phenotypes.

Using several SSLP and SNP markers on our F2 recombinant genomic DNAs, we located a region of 129S6-derived chromosome segregating with the full Chrd phenotype; this region lay close to the marker rs1465069, just proximal to the DCR (Figure 2A). These data imply a physically-linked, recessive lesion specific to the 129S6-derived DCR area that is associated with the full Chrd+− phenotype.

Because the null phenotype of Chrd in the 129S6 background is essentially identical to that of Tbx1+−−, the simplest explanation is that this region contains a recessive, loss-of-function mutation affecting Tbx1 activity. If so, the Chrd mutant chromosome and linked modifier(s) should be unable to complement a Tbx1 null mutation. Accordingly, we crossed 129S6-Chrd+−/− with B6−/− Tbx1+/− animals, generating double heterozygotes, with one Chrd allele and one Tbx1 allele being null created by gene targeting, in trans on the two cognate chromosomes. Among Chrd+−−, Tbx1+/−−/+ embryos, 5/12 showed the ‘full phenotype’ and 6/12 showed a partial DGS-like phenotype. This indicates the Tbx1 null allele interacts strongly with the Chrd mutant chromosome (Figure 2B). In contrast, when B6−/− Tbx1+/−−/+ mice, no significant phenotype was observed in double heterozygotes (0/15, two cases of asymmetric thymus were detected). Thus the apparent interaction of the Tbx1 null locus with Chrd depends on a linked sensitizer in the 129S6 background. The simplest explanation is a cryptic mutation in Tbx1 itself.
Figure 1. Phenotype of Chrd mutant embryos in 129S6 inbred and hybrid backgrounds. (A–G) Comparison of organ structures in wildtype and Chrd mutant embryos at embryonic day (E) 15.5. Ears of Chrd+/− embryos are abnormally located and fail to form auricle structures (A, B). Athymia, persistent truncus arteriosus (PTA; failure of outflow tract septation) and abnormal aortic arch structure are observed in the mutant (C–E). Cleft palate (CP) is also a feature of the mutant embryos (F, G). (H) Penetration of Chrd null mutation in various genetic backgrounds; it shows complete penetrance in the 129S6 strain and partial penetrance in the F2 hybrid (129SB6F2) strain. (I) Phenotypic pattern of 129SB6F2-Chrd+/− embryos. The majority of embryos showed either a ‘Full phenotype’ (as defined in the main text) or no phenotype. Several embryos (11/97) showed variable DGS-like phenotypes. Despite this variability, the embryos can be classified into two groups; those that retain DGS-like phenotypes (in red), and those devoid of DGS-like phenotypes (in blue). a, aorta; cp, cleft palate; HPE, holoprosencephaly; p, pulmonary trunk; pta, persistent truncus arteriosus; t, thymus.

doi:10.1371/journal.pgen.1000395.g001

studs used). This caused a non-random bias in transmitting the Tbx1G/G allele to F2 hybrid animals. Our F1 and F2 genotyping also revealed that Chrd−/−,Tbx1+/G mice (in which one Chrd null allele was inherited from the129S6 strain and one from B6) had very few 22q11DS phenotypes (1/53 for F2 animals; Figure 2D).

To determine the phenotypes resulting from the Chrd null or the Tbx1G/G allele individually, we bred 129S6-Chrd+/+,Tbx1+/G/Tbx1G/G with 129S6 wild-type mice to generate recombinant animals (Figure 3A). We genotyped 540 offspring and identified one recombinant carrying only the Chrd null allele (Chrd+/−,Tbx1+/G), and three carrying only the Tbx1+/G allele (Chrd+/+,Tbx1+/G/G). We assessed the phenotypic consequences of the isolated mutations. Animals heterozygous for the Tbx1+/G/G mutation are healthy and fully viable, displaying no visible phenotype. We crossed Tbx1+/G/G animals to themselves and also to Tbx1−/−/−. Analysis of the three classes of Tbx1 mutant embryos for the DGS phenotype demonstrates genetic rescue: Tbx1−/−/− homozygotes show rescue of the major craniofacial defects of the Tbx1 null phenotype (n = 18), while the compound heterozygote (Tbx1+/G/G) is intermediate (n = 13), relative to the fully penetrant, strong DGS-like phenotype of the null homozygote. Thus Tbx1+/G/G is a hypomorphic allele (Figure 3B). That craniofacial development is much less affected in the hypomorphic homozygote relative to the null demonstrates that craniofacial structures are more sensitive to Tbx1 dose than cardiovascular structures, consistent with a previous report [17].

We also prepared homozygotes for the Chrd allele alone, recombined away from Tbx1+/G/G. These Chrd null homozygotes did not exhibit phenotypes similar to 22q11DS, but showed a low penetrance of variable mandibular truncations, comparable in extent and frequency to what we have observed for B6.129-Chrd−/− embryos (Figure 3C–H and Table S1). A previous study revealed redundant but essential roles of chordin and noggin, another BMP antagonist, in mandibular outgrowth, during which these BMP antagonists promote cell survival in the developing 1st pharyngeal arch [18]. That study used the outbred Chrd strain, which does not carry the Tbx1 hypomorphic allele. Our study confirms a role for chordin in promoting mandibular development. Interestingly, we also observed a severe mandible truncation in one of the double mutant embryos (Chrd−/−,Tbx1+/G/G, Table S1). This result indicates that the craniofacial phenotype caused by loss of Chrd is not suppressed by the Tbx1 mutation, suggesting that Tbx1 is not in turn a modifier of Chrd.

Our data indicating that Chrd deficiency is a modifier of Tbx1 action raises the issue of the molecular basis of this modifier effect. Previous work has suggested that Chrd activity promotes Tbx1 expression in the pharyngeal region of the mouse embryo. Tbx1 expression is reduced in this area of Chrd null embryos [12]. However, the pharyngeal tissues themselves are deficient from early stages in the Chrd mutant (if the cryptic Tbx1 allele is also present). Nonetheless, ectopic Chrd activity in early Xenopus embryos can cause increased Tbx1 transcript levels [12]. In a
Figure 2. Characterization of a linked modifier of Chrd in the 129S6 strain. (A) Schematic diagram of mouse chromosome 16, showing three genetic markers used and genotyping results of F2 hybrid DNAs using these markers. Information on the markers is shown in the table at the bottom of the panel. The second marker rs4165069 shows the strongest co-segregation with the trait. The novel SSLP marker 'ChTb03' produces a 271 bp long PCR fragment from 129S6, and 262 bp from B6 (Figure S4). (B) Cross between 129S6-Chrd++/+ and B6-Tbx1++/+ mice produced Chrd++/+, Tbx1++/+ embryos with highly penetrant 22q11DS phenotypes, supporting the existence of modifier linked to Chrd and suggesting the possibility of a mutation in Tbx1 itself. (C) Sequencing of the Tbx1 locus of 129S6-Chrd++/+ and 129S6-Chrd++/2 strains, revealing a specific point mutation (Tbx1G>T) located in the second intron of the 129S6-Chrd++/2 allele (asterisk). (D) Genotyping results of F2 hybrid DNAs using the Tbx1G>T mutation as a SNP marker, showing that it is more strongly linked to the phenotype than rs4165069. (E) Tbx1G>T is located at an exon-intron boundary. Consensus, wild-type, and mutant sequences encompassing the mutation are displayed. (F) RT-PCR analysis demonstrates that Tbx1G>T disrupts normal splicing of Tbx1 in 129S6 mice carrying the Chrd null allele. As a result of the point mutation, both exon skipping (left) and intron retention (right) occur in the generation of Tbx1 mRNA, but very little normal message is produced. Diagrams of mRNA with asterisks (*) denote mutant splicing variants that would invariably produce truncated Tbx1 protein.

doi:10.1371/journal.pgen.1000395.g002
Figure 3. Phenotypes of $Chrd^{-/-}, Tbx1^{G>T}$ and $Chrd^{-/-}, Tbx1^{+/-}$ mutant embryos. (A) Crossing scheme to generate recombinant animals. 129S6-Chrd$^{-/-}$ females were mated with 129S6 wild-type studs and offspring were screened for recombination of markers. (B) Penetrance of five phenotypes associated with DGS from each different $Tbx1$ mutant class, displaying dosage-dependent rescue of craniofacial phenotypes. (C–H)
Variable mandible defects in Chrd<sup>+/−</sup> mutant at late gestation stages. (C, D) Mild mandible outgrowth defect in B6-Chrd<sup>+/−</sup> mutant embryo (arrowhead). (E–H) Total absence of mandibular elements accompanying incomplete midline structure (arrowhead) is displayed in lateral (E, F) and ventral view (G, H) of 129S6-Chrd<sup>+/−</sup> mutant embryo. (I–L) Developmental defects of Chrd null, Tbx1 hypomorphic, and compound mutations. (I) Organs that are defective in various classes of mutant embryos. (J) Tbx1 hypomorphic embryos develop very mild craniofacial defects (note partially dysmorphic ear) compared to Tbx1 null embryos.

doi:10.1371/journal.pgen.1000395.g004

Chordin Is a Tbx1 Modifier

Discussion

Our search for the modifier of Chrd identified an unexpected, linked mutation in the Tbx1 locus in the 129S6-Chrd<sup>+/−</sup> strain, which causes mis-splicing. Comparison of phenotypes between the separated, isolated Tbx1<sup>G>T</sup> and Chrd<sup>+/−</sup> mutations and those associated with homozygosity for the original chromosome, i.e. Chrd<sup>−/−</sup>-Tbx1<sup>G>T</sup> (outer ear) was approximately 20%, while cleft palate was about 50%. However, when the two mutations are together, all mutant embryos developed fully penetrant, highly consistent craniofacial phenotypes; these defects appear to be identical to those of Tbx1<sup>−/−</sup> null animals, with cleft palate, reduced, low-set ears, athymia, and a number of craniofacial defects. Second, embryos mutant for the hypomorphic Tbx1<sup>G>T</sup> mis-splicing allele show several cardiac and pharyngeal defects nearly as severe as those of the Tbx1 null mutants, but without the major craniofacial phenotypes of the null (reduced pinna and cleft palate). Finally, our results show that whereas homozygosity for the hypomorphic Tbx1<sup>G>T</sup> allele rarely results in major craniofacial defects alone, it does so when Chrd is absent. In contrast, the cardiovascular defects are unaffected. Thus, Chrd is a modifier specifically for the craniofacial phenotypes of Tbx1 lesions.

The craniofacial defects associated with homozygosity for the cryptic double mutant reflect a synergistic relationship between the Chrd and Tbx1 lesions. Although there was a low penetrance of striking mandibular outgrowth defects in Chrd<sup>−/−</sup> mutants, most homozygotes (73%) looked normal. The Tbx1<sup>G>T</sup> animals in turn showed a limited penetrance of craniofacial defects that are reminiscent of 22q11DS. Penetration of low-set, reduced pinna (outer ear) was approximately 20%, while cleft palate was about 50%. However, when the two mutations are together, all mutant embryos develop fully penetrant, highly consistent craniofacial phenotypes; these defects appear to be identical to those of Tbx1<sup>−/−</sup> null animals, with cleft palate, reduced, low-set ears, athymia,
persistent truncus arteriosus, etc (Figure 5). These phenotypes occur against a background of the low-penetrance of severe mandibular truncation defects caused by absence of Chrd per se, much more pronounced than the subtle mandibular hypoplasia reported for the Tbx1 null [3]. Therefore, the absence of Chrd leads to a synergistic (as opposed to additive) worsening of the defects caused by the hypomorphic Tbx1 allele, to generate the complete constellation of craniofacial defects seen in the Tbx1<sup>−/−</sup> null and reminiscent of 22q11DS.

The Tbx1<sup>G/G</sup> mutation we identified behaves according to the genetic definition of a hypomorphic allele: Tbx1<sup>G/T/G</sup> heterozygotes are less severely affected than homozygotes for a null allele, while the compound heterozygote is intermediate in severity. Molecular evidence to account for such reduced activity was apparent in the transcripts produced by the mutant allele. The mutation is predicted to disrupt normal splicing, and in fact we observed a truncated product lacking 25% of the T-box domain, essential for the proper function of the protein – if any protein is stabilly produced. In addition, these abnormal transcripts account for most of the spliceforms produced by the allele; we amplified little transcript corresponding to the correctly spliced wildtype version from Tbx1<sup>G/T/G</sup> homozygotes.

The consequences of the cryptic Tbx1<sup>G/T/G</sup> allele linked to Chrd in the initial 129S6 background account for much of the phenotype of Chrd<sup>−/−</sup> mutants as previously reported [12]. Nevertheless, our genomic scan to assess the possibility of additional specific modifiers at play, they have a very minor role.

Previous work and our unpublished observations suggest that Chrd functions to promote Tbx1 expression in the pharyngeal region. When we assayed Tbx1 expression in Chrd mutants with or without the cryptic Tbx1 hypomorphic mutation, consistent results were observed. In both 129S6- and B6-Chrd<sup>−/−</sup> embryos, there was mild but significant reduction of Tbx1 expression when compared to the wildtype embryos (Figure 4). Nevertheless, in all three backgrounds tested, Chrd null animals free of Tbx1<sup>G/G</sup> are viable and show no pharyngeal defects. Thus the decrease of Tbx1 expression in pure Chrd mutants is insufficient to cause a phenotype; however, loss of this activity could be a contributing factor in the functional synergy between the Chrd null and Tbx1<sup>G/G</sup> mutations in causing a more severe DGS-like phenotype than the hypomorphic Tbx1<sup>G/G</sup> mutation alone.

We note that Chrd<sup>−/−</sup>,Tbx1<sup>+/G</sup> mice very rarely show Tbx1 mutant phenotypes (1/53 in the F2 hybrids). Thus it is possible that the reduction in functional Tbx1 protein caused by a single allele of Tbx1<sup>G/G</sup>, compounded by the moderately decreased Tbx1 expression caused by loss of Chrd, is rare instances sufficient to generate phenotypes similar to 22q11DS.

Tbx1 also shows genetic interactions with other mutations both within and outside the DCR [10,11]. However, no second-site mutations has been found previously that can account for why DCR or Tbx1 mutations are sometimes associated with a particular defect but sometimes not. The result reported here show that Chrd is a modifier for the craniofacial anomalies of Tbx1 mutations, demonstrating the existence of a second-site modifier for a specific subset of the phenotypes associated with 22q11DS.

**Materials and Methods**

**Mouse Strains and Genotyping**

The Chrd null allele (Chrd<sup>tm1Emdr</sup>) was generated previously using R1 ES cells [12]. To generate outbred Chrd stock [18], germline chimeras were mated to random outbred ICR females, with backcrossing of F1 founders to ICR. To generate 129S6 inbred stock, germline chimeras were mated to 129S6/SvEvTac (Taconic) wild type mice, and Chrd heterozygotes backcrossed for

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**Figure 5. Developmental defects of Chrd, Tbx1, and compound mutants.** Organs that are defective in the various classes of Chrd and/or Tbx1 mutant embryos studied here. Degree of penetrance of phenotypes reminiscent of 22q11DS is shown by variable intensity of red shading, as defined to the upper right of the figure. (A) Normal morphology (indicated by green shading) of organs sensitive to loss of Chrd and/or Tbx1 and their location in wildtype embryos: mx, maxilla; mn, mandible; e, ear; t, thymus; h, heart; CP, cleft palate; ME, malformed ear; AT, athymia; PTA, persistent truncus arteriosus. (B) Chrd null mice display a markedly truncated mandible phenotype at low penetrance (blue shading), while other structures are unaffected. (C) Tbx1<sup>G−/−</sup> embryos sometimes develop mild craniofacial defects (note partially dysmorphic ear) compared to Tbx1<sup>−/−</sup> null embryos, while AT and PTA are fully penetrant. We did not quantitatively score for the presence of previously reported possible subtle mandibular craniofacial defects seen in the Chrd<sup>−/−</sup> background (Mouse Genome Informatics). To prove that coding regions of these genes are approximately 2 million bases could be outside the Chrd,Tbx1 locus, or even within it – the Chrd<sup>−/−</sup> phenotypic expression in the pharyngeal region (Figure 5). These phenotypes occur against a background of the low-penetrance of severe mandibular truncation defects caused by absence of Chrd per se, much more pronounced than the subtle mandibular hypoplasia reported for the Tbx1 null [3]. Therefore, the absence of Chrd leads to a synergistic (as opposed to additive) worsening of the defects caused by the hypomorphic Tbx1 allele, to generate the complete constellation of craniofacial defects seen in the Tbx1<sup>−/−</sup> null and reminiscent of 22q11DS.

The Tbx1<sup>G−/−</sup> mutation we identified behaves according to the genetic definition of a hypomorphic allele: Tbx1<sup>G−/−</sup> homozygotes are less severely affected than homozygotes for a null allele, while the compound heterozygote is intermediate in severity. Molecular evidence to account for such reduced activity was apparent in the transcripts produced by the mutant allele. The mutation is predicted to disrupt normal splicing, and in fact we observed a truncated product lacking 25% of the T-box domain, essential for the proper function of the protein – if any protein is stably produced. In addition, these abnormal transcripts account for most of the spliceforms produced by the allele; we amplified little transcript corresponding to the correctly spliced wildtype version from Tbx1<sup>G−/−</sup> homozygotes.

The consequences of the cryptic Tbx1<sup>G−/−</sup> allele linked to Chrd in the initial 129S6 background account for much of the phenotype of Chrd<sup>−/−</sup> mutants as previously reported [12]. Nevertheless, our genomic scan to assess the possibility of modifiers suggested more than one modifier was present. Thus there may be additional modifier(s) in the 129S6 background that influence the Chrd<sup>−/−</sup>,Tbx1<sup>G−/−</sup> phenotype. Such lesions could be outside the Chrd,Tbx1 region, or even within it – the coding regions of these genes are approximately 2 million bases apart (Mouse Genome Informatics). To prove that only the Chrd targeted mutation and the cryptic Tbx1 splice site mutation (but no other 129S6 allele) are sufficient for the full phenotype would require reconstituting only these mutations in a different inbred strain, a daunting task. We note that the behaviors of Chrd and Tbx1 alleles independently do not appear to be 129S6-specific. For Chrd<sup>−/−</sup> embryos, the penetrance and expressivity of the mandible phenotype in the 129S6 and B6 strains are quite comparable (Table S1). In the case of Tbx1, alleles have been made and studied in multiple strains and produced very consistent phenotypes (our observations, [2,3]). Altogether, it seems likely that if there are additional specific modifiers at play, they have a very minor role.

Previous work and our unpublished observations suggest that Chrd functions to promote Tbx1 expression in the pharyngeal region. When we assayed Tbx1 expression in Chrd mutants with or without the cryptic Tbx1 hypomorphic mutation, consistent results were observed. In both 129S6- and B6-Chrd<sup>−/−</sup> embryos, there was mild but significant reduction of Tbx1 expression when compared to the wildtype embryos (Figure 4). Nevertheless, in all three backgrounds tested, Chrd null animals free of Tbx1<sup>G−/−</sup> are viable and show no pharyngeal defects. Thus the decrease of Tbx1 expression in pure Chrd mutants is insufficient to cause a phenotype; however, loss of this activity could be a contributing factor in the functional synergy between the Chrd null and Tbx1<sup>G−/−</sup> mutations in causing a more severe DGS-like phenotype than the hypomorphic Tbx1<sup>G−/−</sup> mutation alone.

We note that Chrd<sup>−/−</sup>,Tbx1<sup>+/G</sup> mice very rarely show Tbx1 mutant phenotypes (1/53 in the F2 hybrids). Thus it is possible that the reduction in functional Tbx1 protein caused by a single allele of Tbx1<sup>G−/−</sup>, compounded by the moderately decreased Tbx1 expression caused by loss of Chrd, is in rare instances sufficient to generate phenotypes similar to 22q11DS.

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**DOI:** 10.1371/journal.pgen.1000395.g005
Isolation of RNA and Reverse Transcriptase-PCR (RT-PCR)

To assess Tbx1 splicing, total RNA was prepared from E9.5 embryos and subjected to RT-PCR. Pharyngeal tissues were homogenized and treated with TRIZOL Reagent (Invitrogen). Remaining tissues were used for genotyping. After further purification, the DNA pellet was dried and resuspended in DEPC-water for subsequent reverse transcription (RT) with random hexamer and mouse mammary tumour virus (MMTV) RT (Invitrogen). Resulting cDNA was used as a template for PCR. The following primers were used for testing Tbx1 splicing: TbxR1F 5’-TTTGTGCGCCCTGATGAGCAA-3’ (forward); TbxR2R 5’-TCATCCAGCAGGCTAIATG-3’ (reverse); TbxR3R 5’-AATGGGGCTGATACTGTG-3’ (reverse); TbxR4F 5’-TGTCGAGAGTCCAATCAG-3’ (forward).

Skeletal Preparation

Skeletal tissues were prepared and visualized as previously described [18].

Supporting Information

Figure S1 Absence of Tbx1 results in phenotypes identical to Chrd null homozygotes in a 129S6 inbred genetic background. Phenotype of Tbx1−/− embryo at E16.5 includes abnormal inner and outer ears (A, B); absence of thymus, PTA (C–E) and cleft palate (F, G); aorta; cp, cleft palate; p, pulmonary trunk; pta, persistent truncus arteriosus; t, thymus. Found at: doi:10.1371/journal.pgen.1000395.s001 (0.3 MB TIF)

Figure S2 Outer ear morphologies in embryos of different Chrd genotypes. (A, B) Normal ears of Chrd+/− and Chrd−/− embryos at E18.5, respectively. (C–F) Ears of F2 Chrd hybrid mutants (129S6F2-Chrd+/−) displaying partially-defective outer ear morphologies. (G) Representative ear of inbred Chrd mutant embryo, showing the total failure of auricle formation. Found at: doi:10.1371/journal.pgen.1000395.s002 (0.9 MB TIF)

Figure S3 Status of Tbx1 locus in various relevant strains. A number of substrains of 129, the C57BL/6 strain and the parental R1 ES cell line used for targeting are devoid of Tbx1+/− (represented by +/+/genotype). The diagram shows the lineage relationship of many of these strains. Found at: doi:10.1371/journal.pgen.1000395.s003 (0.2 MB TIF)

Figure S4 Sequences of novel SSLP locus ChrdB03 from B6 and 129S6 strains. Number of ‘CTT’ repetitions differs between the two strains, causing different lengths of amplified PCR product. Bold sequences are primers for PCR amplification. Found at: doi:10.1371/journal.pgen.1000395.s004 (0.02 MB DOC)

Table S1 Penetration of mandible defect phenotype in Chrd mutant embryos. Found at: doi:10.1371/journal.pgen.1000395.s005 (0.02 MB DOC)

Acknowledgments

We thank Dr. A. Baldini for the Tbx1 null allele and comments on the manuscript, Dr. R. Anderson for establishing the inbred Chrd strains, Drs. D. Threadgill and D. Marchuk for technical advice, K. Carmody for mouse husbandry, and Duke colleagues for helpful discussion.

Author Contributions

Conceived and designed the experiments: MC JK. Performed the experiments: MC. Analyzed the data: MC JK. Wrote the paper: MC JK.

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