A phenotypic rescue approach identifies lineage regionalization defects in a mouse model of DiGeorge syndrome

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A phenotypic rescue approach identifies lineage regionalization defects in a mouse model of DiGeorge syndrome

Gabriella Lania1,4, Monica Franzese2, Noritaka Adachi4,*+, Marchesa Bilio1, Gemma Flore1, Annalaura Russo1, Erika D’Agostino1, Claudia Angelini3, Robert G. Kelly4 and Antonio Baldini1,5,6

ABSTRACT

TBX1 is a key regulator of pharyngeal apparatus (PhAp) development. Vitamin B12 (vB12) treatment partially rescues aortic arch patterning defects of Tbx1−/− embryos. Here, we show that it also improves cardiac outflow tract septation and branchiometric muscle anomalies of Tbx1 hypomorph mutants. At the molecular level, in vivo vB12 treatment enabled us to identify genes that were dysregulated by Tbx1 haploinsufficiency and rescued by treatment. We found that SNAI2, also known as SLUG, encoded by the rescued gene Snai2, identified a population of mesodermal cells that was partially overlapping with, but distinct from, ISL1+ and TBX1+ populations. In addition, SNAI2+ cells were mislocalized and had a greater tendency to aggregate in Tbx1+/− and Tbx1−/− embryos, and vB12 treatment restored cellular distribution. Adjacent neural crest-derived mesenchymal cells, which do not express TBX1, were also affected, showing enhanced segregation from cardiopharyngeal mesodermal cells. We propose that TBX1 regulates cell distribution in the core mesoderm and the arrangement of multiple lineages within the PhAp.

KEY WORDS: TBX1, DiGeorge syndrome, Pharyngeal apparatus, Cardiopharyngeal mesoderm, Phenotypic rescue

INTRODUCTION

The embryonic pharyngeal apparatus (PhAp) is a developmental system that provides progenitors and instructions to multiple organs and tissues, including, but not limited to, the craniofacial and mediastinic muscles and bones, most of the heart, and glands such as the thymus, parathyroids and thyroid. Developmental anomalies of the PhAp underlie numerous birth defects, highlighting its developmental and genetic complexity. A textbook example of PhAp maldevelopment is DiGeorge syndrome, the most common genetic cause of which is a heterozygous deletion of a chromosomal region within 22q11.2 (in which case the clinical presentation is more complex and is designated as 22q11.2 deletion syndrome), and it can also be caused by point mutations of the TBXI gene (Haddad et al., 2019; Paylor et al., 2006; Xu et al., 2014; Yagi et al., 2003; Zweier et al., 2007).

The development of the PhAp depends upon the contribution of tissues derived from all three germ layers: surface ectoderm, pharyngeal endoderm, neural crest-derived cells (NCCs) and the cardiopharyngeal mesoderm (CPM). The latter contributes to a broad range of tissues and structures within the mediastinum, face and neck (Adachi et al., 2020). In the mouse, the CPM is well represented by the expression domains of the Tbx1Cre and the Mef2c-AHF-Cre drivers (Adachi et al., 2020; Huynh et al., 2007; Verzi et al., 2005). PhAp lineages have distinct origins and transcriptional profiles (Svedlund and Lescroart, 2020; Wang et al., 2019), and develop in close proximity or direct contact with each other, and their regionalization within the PhAp is mostly conserved across vertebrate evolution (Graham, 2001). However, the molecular code that governs regionalization has not been dissected in detail, although interactions between lineages are the subject of intense research (Calmont et al., 2009; Huang et al., 1998; Kodo et al., 2017; Mao et al., 2021; Sato et al., 2011; Shone and Graham, 2014; Warkala et al., 2020).

Loss of function of the Tbx1 gene in the mouse has profound and broad effects on the development of the PhAp (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001), and affects the expression of thousands of genes (Fulcoli et al., 2016; Ivins et al., 2005; Liao et al., 2008; Nomaru et al., 2021; Baba et al., 2012), making it difficult to identify the effectors/targets that are critical for specific developmental functions. Phenotypic rescue strategies represent an alternative approach to focus on genes associated with phenotypic improvement.

In a search for drugs that rebalance Tbx1 haploinsufficiency, we showed that high doses of vitamin B12 (vB12) rescued part of the mutant phenotype in vivo (Lania et al., 2016). Here, we show that the rescuing capacity of the drug extends to CPM-derived structures, such as the cardiac outflow tract and craniofacial muscles. Then, as a proof of principle of the usefulness of phenotypic rescue to provide insights into pathogenetic mechanisms, we leveraged vB12 treatment to identify genes and pathways that are critical for the expressivity of the rescued phenotype. This exposed a novel Tbx1 mutant phenotype through the identification of a SNAI2+ subpopulation of CPM cells. Specifically, we found that, in Tbx1 homozygous mutants, SNAI2+ cells were segregated from the NCCs rather than intermingled with them, suggesting a cell sorting defect. This abnormality was also evident in Tbx1 heterozygous mutants, albeit at a reduced expressivity. Thus, in the PhAp, TBX1 dosage is important, cell autonomously and non-autonomously,
for the regionalization of cell lineages. We propose that this TBX1-dependent function is part of the pathogenetic mechanism leading to severe abnormalities of the PhAp in the mouse mutants as well as in DiGeorge syndrome.

**RESULTS**

**vB12 reduces the severity of the intracardiac and craniofacial phenotypes in a hypomorphic Tbx1 mutant model**

High dosage of vB12 reduced the penetrance of the aortic arch phenotype and rebalanced Tbx1 expression in haploinsufficient mice (Lania et al., 2016). However, Tbx1+/− embryos rarely show second heart field (SHF)-related abnormalities such as outflow tract defects, which are commonly found in embryos that express low levels of Tbx1 (Liao et al., 2004; Zhang and Baldini, 2008) or in Tbx1−/− embryos (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). We asked whether vB12 treatment could modify the SHF-dependent phenotype on a Tbx1 reduced-dosage model. To this end, we exploited a hypomorphic Tbx1 allele (Tbx1neo2) (Zhang et al., 2006) that has a loxP-flanked neomycin resistance gene inserted into an intron. Tbx1neo2/− embryos exhibit heart defects similar to but less severe than those of null embryos (Zhang and Baldini, 2008). We crossed Tbx1neo2+/− and Tbx1−/− mice, and injected pregnant females daily from embryonic day (E)7.5 to E11.5 with vB12 (intraperitoneal injection, 20 mg/kg/day) or vehicle (PBS, controls). Embryos were harvested and dissected at E15.5 and E18.5. Table 1 summarizes the phenotyping results. We examined 21 Tbx1neo2/− embryos at E18.5 (ten controls and 11 treated with vB12). All control PBS-injected Tbx1neo2/− embryos had persistent truncus arteriosus (PTA) and ventricular septal defects (VSDs) (Fig. 1A,A′). In contrast, only two of the 11 vB12-treated Tbx1neo2/− embryos had typical PTA (Fig. 1B), while four had an incomplete PTA, in which there was an unseptated valve but a distal separation of the aorta and pulmonary trunk (Fig. 1B′), and two had double outlet right ventricle (DORV) (Fig. 1B″). All embryos had a VSD, with the exception of one embryo, which had an apparently normal heart (Fig. 1C; Fig. S1). In addition, we examined histologically a set of five control and three vB12-treated Tbx1neo2/− embryos at E15.5. All control embryos had VSD and PTA, whereas the vB12-treated embryos had VSD and overriding of the aorta, but no PTA (Table 1; Fig. S2).

**Table 1. Summary of heart morphology phenotyping data**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Treatment</th>
<th>Normal</th>
<th>VSD</th>
<th>OvAo</th>
<th>DORV</th>
<th>IncPTA</th>
<th>PTA*</th>
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<tr>
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<td></td>
</tr>
<tr>
<td>Tbx1neo2/−</td>
<td>10</td>
<td>PBS</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>Tbx1fneo2/−</td>
<td>11</td>
<td>vB12</td>
<td>1 (9%)</td>
<td>8 (72%)</td>
<td>2 (18%)</td>
<td>2 (18%)</td>
<td>4 (36%)</td>
<td>2 (18%)</td>
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<td>0</td>
<td>0</td>
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<td>5 (100%)</td>
</tr>
<tr>
<td>Tbx1fneo2/−</td>
<td>3</td>
<td>vB12</td>
<td>0</td>
<td>3 (100%)</td>
<td>3 (100%)</td>
<td>3 (100%)</td>
<td>3 (100%)</td>
<td>3 (100%)</td>
</tr>
</tbody>
</table>

DORV, double outlet right ventricle; IncPTA, incomplete PTA; OvAo, overriding aorta; PTA, persistent truncus arteriosus; vB12, vitamin B12; VSD, ventricular septal defect. *P<0.001 (Chi-square test).
Reduced dosage of \textit{Tbx1} causes specific craniofacial muscle anomalies (Adachi et al., 2020; Dastjerdi et al., 2007; Kelly et al., 2004). We tested a set of five controls and three vB12-treated \textit{Tbx1}neo2/− embryos at E15.5 and scored the craniofacial muscle phenotype (Fig. 2; Fig. S3 and Fig. S4A). Results showed that vB12 treatment reduced the severity of anomalies of the muscles originating from the 1st pharyngeal arch (PA); bilateral defects of the anterior digastric muscles in \textit{Tbx1}neo2/− embryos reduced from 60% to 33% after vB12 treatment. Defects of 2nd PA-derived branchiomeris muscles were also rescued by vB12 treatment.
downregulated genes in Fig. 3. (47%) were upregulated and 2092 (53%) were downregulated by vB12 treatment (Fig. 3B; Table S2). GO analyses revealed that the upregulated genes were enriched for genes involved in RNA processing, whereas the downregulated genes were enriched for genes involved in developmental processes (Table 3). Details of the GO analyses are provided in Table S3. The intersection of the two groups of DEGs identified 468 shared genes (Fig. 4A), which is a significantly higher number than that expected by chance (P=1.4×10−7, hypergeometric test). Of these, 344 changed their expression in opposite directions in the two groups, i.e. the mutation changed expression in one direction whereas vB12 treatment rebalanced it (Fig. 4B; genes listed in Table S2); we define these genes as rescued by vB12. In the left column of the heat map shown in Fig. 4B are represented genes dysregulated by the mutation; the right column shows their expression after vB12 treatment (compared to WT). Thus, the dark color in the right column indicates genes that were expressed at or near WT level after treatment. Of these 344 genes, 85 (24.7%, shown in green) were downregulated, and 259 (75.3%, shown in red) were upregulated, relative to WT (Fig. 4B, left column).

We then applied a hypergeometric test to ask whether rescue of gene expression imbalances by vB12 could have occurred by chance, given the high number of genes affected by the treatment. Interestingly, we found that the rescue of the 259 upregulated genes was extremely significant (P<10−10), while the rescue of the 85 downregulated genes was borderline with a chance event (P=0.052). In addition, the number of genes that were further dysregulated by vB12 (468−344=124), which were almost equally distributed among upregulated and downregulated (59 and 65, respectively) genes, was not significantly different from that expected from a chance event. Thus, the most significant rescue effect of vB12 was on genes that were upregulated by Tbx1 heterozygosity and downregulated by vB12 treatment. GO of the 344 rescued genes showed enrichment of heart development genes (P=0.005) and transcription regulator genes (P=0.003) (Fig. 4C). Full results from the GO analysis are provided in Table S3.

SNAI2 identifies a mesodermal population partially overlapping with but distinct from the TBX1⁺, ISL1⁺ and Mef2c-AHF-Cre⁺ populations

Among the rescued genes, we identified 55 genes known to be expressed in the CPM, or its derivatives, and to a lesser extent in other tissues of the PhAp (Fig. 4D; Table S2). Among these, we...
noted a set of genes known to be involved in the Tgfβ1 pathway. Specifically, Snai1, Snai2, Twist2, Msx1 and Tgfb1 were all upregulated in Tbx1+/− mutant embryos and downregulated by vB12 treatment (blue underlined in Fig. 4D). We selected Snai2, encoding a transcriptional repressor also known as SLUG, which has not been previously associated with TBX1 biology. We performed immunofluorescence (IF) with an anti-SNAI2 antibody (Hultgren et al., 2020) to determine its expression relative to markers of the CPM in E9.5 WT embryos. The anti-SNAI2 and anti-TBX1 antibodies are raised in the same species; therefore, we used them in sequence on the same sections. Similar results were obtained by inverting the order of the antibodies. As shown in Fig. 5A,B, at all section levels considered, there was a very similar distribution of the two proteins, with notable exceptions. Specifically, in the 1st PA, both proteins were present in the core mesoderm, but SNAI2+ cells were fewer in the core, and there were more of them scattered in the body of the PA (arrow in Fig. 5B); TBX1+ cells were more evident in the proximal region of the arch (Fig. 5A,B). In the 2nd PA, the SNAI2 domain extended more distally towards the OFT, compared to the TBX1 domain (Fig. 5A′,B′). At the other two levels analyzed, namely, posterior to the OFT (Fig. 5A″,B″) and immediately anterior to the inflow tract (IFT) (Fig. 5A‴,B‴), the distribution of the two proteins was very similar in the lateral aspects of the dorsal pericardial wall and splanchnic mesoderm.

Next, we compared the expression of SNAI2 to that of the Mef2c-AHF enhancer using an anti-CRE antibody (also raised in the same species as the anti-SNAI2 antibody) on sections of Mef2c-AHF-Cre embryos (Verzi et al., 2005). Also in this case, the expression patterns of the two proteins were very similar (Fig. 5C,D), except for two substantial differences: in the 2nd PA, Mef2c-AHF-Cre was expressed more distally towards the OFT, including the myocardial layer of the OFT, (Fig. 5C‴,D‴), more extensively in the dorsal pericardial wall (Fig. 5C″) and more extensively in the splanchnic mesoderm of the posterior region of the embryonic pharynx (Fig. 5C‴,D‴).

Table 3. GO analysis of downregulated and upregulated DEGs from comparison of Tbx1+/−+vB12 and Tbx1+/++PBS

<table>
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<td>cellular nitrogen compound metabolic process</td>
<td>GO:0034641</td>
<td>5.27×10^{-20}</td>
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<td>GO:0032502</td>
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We then compared SNAI2 expression to that of ISL1, which is expressed throughout the CPM (Fig. 6). The two markers had a very similar, mostly overlapping expression in the 1st PA (Fig. 6A,B) of WT embryos. In the 2nd arch, SNAI2 was more highly expressed in the distal portion of the arch, where it overlapped with ISL1, whereas in the proximal portion, there was a high expression of ISL1 but not of SNAI2. Double-labeled cells were detected in a mediolateral region of the dorsal pericardial wall, where ISL1 expression was more extensive (Fig. 6A″,B″); in addition, the more posterior expression of ISL1 in the splanchnic mesoderm (arrow in Fig. 6B″) is similar to the TBX1 expression domain (Fig. 6C,D). Fig. S5 shows a higher-magnification image of a section adjacent to that shown in Fig. 6A. Because of the scattered expression of SNAI2 in the body of the 1st PA, which is heavily populated by NCCs, we co-stained E9.5 embryos (WT) with TFAP2A, which labels migrating NCCs at this stage (as well as ectodermal cells) (Mitchell et al., 1991). With very few exceptions, we did not observe double-labeled cells in the entire embryo (examples in Fig. 7A,C,E), but we observed extensive intermingling of SNAI2+ and TFAP2A+ cells. Thus, SNAI2 is expressed in mesodermal cells,

A

B

C

D

<table>
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Fig. 4. vB12 rescues the expression of 344 genes involved in gene regulation and heart development. (A) Venn diagram plot representing the intersection of two groups of differentially expressed genes (Tbx1+/− versus Tbx1+/+, and Tbx1+/−+vB12 versus Tbx1+/−+PBS). (B) Heat maps of rescued genes in Tbx1+− embryos (treated with vB12 or PBS) based on their fold change relative to WT. (C) Gene ontology analysis by the gProfiler tool. (D) Heat map of 55 genes known to be expressed in the pharyngeal apparatus, relative to WT.
mainly in the distal 2nd PA, in the mediolateral dorsal pericardial wall, in the posterior lateral splanchnic mesoderm and, to a minor extent, in the 1st PA.

**Tbx1 mutants have lineage regionalization abnormalities**

We next investigated whether *Tbx1* loss of function affected the SNAI2+ population. We first examined this population in comparison to NCCs (TFAP2+; *TBX1−/−*). At the level of the 1st PA, we found a striking pattern in which SNAI2+ cells in *Tbx1−/−* embryos were tightly grouped in the core mesoderm, forming a large area surrounded by, but not mixed with, TFAP2A+ cells (Fig. 7B), whereas in WT embryos the two cell types were intermingled (Fig. 7A). A similar pattern was evident in the head mesoderm/proximal 1st PA (Fig. 7A,B, arrows). At the level of the 2nd PA, which is severely hypoplastic in *Tbx1−/−* embryos, the mixing of the two populations was substantially reduced, although here the TFAP2A+ population appeared smaller than in WT (Fig. 7C,D, arrows). More posteriorly (caudal to the OFT), this segregation phenotype was not apparent; of note is a relative expansion of the SNAI2+ population at this level in the splanchnic mesoderm of the mutant embryo (Fig. 7E,F, arrows).

We next examined the distribution of SNAI2+ cells compared to ISL1+ cells in *Tbx1−/−* embryos. In the 1st PA of *Tbx1−/−* embryos, and in contrast to WT embryos, we observed a large, well-defined cluster of SNAI2+ cells that were mostly ISL1+ in the core mesoderm and appeared to extend posteriorly, as if it resulted from merging with the core of the 2nd PA, which is severely hypoplastic in these mutants (arrowheads in Fig. 8A,B). In a more medial sagittal plane, the aggregate is also clearly visible (Fig. 8C,D). This aggregation phenotype was observed in all the homozygous embryos examined (*n*=11).

We next tested whether cells of the *Tbx1* genetic lineage are mislocalized, relative to SNAI2+ cells, in the absence of *Tbx1* function. To this end, we performed anti-SNAI2 and anti-GFP IF on *Tbx1Cre−/+;R26RmT-mG* (*Tbx1* null) and *Tbx1Cre+/+;R26RmT-mG* (heterozygous, control) E9.5 embryos (*Tbx1Cre* is a null allele). Results showed that, in control embryos, GFP+ cells (shown in red in Fig. 9A,B) were more prominent in the proximal and lateral aspects of the 1st PA relative to SNAI2+ cells, with only a limited overlap. Moreover, the relationship between the markers was largely conserved in *Tbx1* null embryos (Fig. 9B), indicating that the SNAI2+ aggregate in the 1st PA is mostly made of cells that did not activate *Tbx1* gene transcription.

To support the qualitative observations reported above, we performed quantitative evaluation of SNAI2+ cell number and distribution in WT, *Tbx1+/−* and *Tbx1−/−* mutant embryos. For these
tests, we used an additional set of five embryos/genotype after SNAI2 IF. Consecutive images of transverse sections were collected along the entire 1st PA. Using computer-generated 5×4 grids placed on the arch images, we divided each section into 20 subregions, and we counted the SNAI2+ cells for each subregion and for each 10 µm-thick section. Subsequently, we evaluated the numerosity (absolute number of positive cells) and density (number of cells per 100 µm²) for each region and genotype. Because of differences in the spatial distribution of SNAI2+ cells in the WT, we divided the arch into two segments along the anterior–posterior axis. The anterior (A) segment is defined from the early invagination of the first branchial pouch (upper limit of the arch) through the buccopharyngeal membrane (lower limit); the rest of the arch is the posterior (P) segment. A graphic representation of the results is shown in Fig. 10. In the A segment, the cell density distribution is significantly affected by Tbx1 dosage (WT versus Tbx1+/+, $P<0.001$; WT versus Tbx1−/−, $P<0.001$). Furthermore, we observed subregions of high SNAI2+ cell density in the Tbx1−/− embryos that were not observed in other genotypes (Bin 12, $P=0.0003$; Bin 13, $P=0.0034$; Bin 17, $P=0.005$; Bin 18, $P=0.0005$; Bin 19, $P=0.007$). In addition, the numerosity of cells was significantly different across genotypes (WT versus Tbx1+/+, $P=0.048$; WT versus Tbx1−/−, $P=0.004$). In the P segment, the SNAI2+ cell density increased significantly with the reduction of Tbx1 dosage (WT versus Tbx1+/+, $P<0.001$; WT versus Tbx1−/−, $P<0.001$) (Fig. 10A,B,C). Unlike in the A segment, in the P segment, we found subregions in which cell densities were significantly higher in Tbx1 heterozygous and null embryos (in Tbx1+/+: Bin 13, $P=0.0047$; Bin 14, $P=0.003$; in Tbx1−/−: Bin 12, $P=0.002$; Bin 16, $P=0.0012$) (Fig. 10A′,B′,C′). Furthermore, cell counts revealed that, overall, the A segment of both mutants had a higher number of SNAI2+ cells compared to WT, while there were no significant differences in the number of cells in the P segment (Fig. 10E).

Thus, quantitative data revealed regional differences along the anterior–posterior axis of the 1st PA in the response to Tbx1 dosage and confirmed the visual evidence that the SNAI2+ cell population tends to be denser as the Tbx1 gene dosage decreases.

To understand how the aggregation of SNAI2+ cells in the 1st PA of Tbx1−/− mutants arises, we examined earlier developmental stages: 11-, 15- and 20-somite stage (st), immunostained with anti-SNAI2 and anti-ISL1 antibodies. In the WT embryo, at 11st, the 1st PA was mostly populated by compacted mesoderm (ISL1+ and SNAI2+) and by a very limited non-mesodermal mesenchymal population (Fig. S6A,A′). At this stage, the NCCs have not populated the arch in a substantial manner (Grenier et al., 2009). As NCCs populate the arch at 15st, they mostly surround the mesodermal core, but a subpopulation invades the core, resulting in the dispersal of SNAI2+ and ISL1+ cells within the arch mesenchyme (Fig. S6B,B′). This process of dispersion continues at 20st (Fig. S6C,C′). In the Tbx1−/− embryo, this process of dispersion does not occur at any stage (Fig. S6D–F′), and, as a result, the SNAI2+ and ISL1+ cells remain compacted. These observations suggest that, in Tbx1 mutants, NCCs fail to penetrate the pre-existing mesodermal core so that the two lineages remain segregated.

We next asked whether the segregation of SNAI2+ cells may be explained by differential cell–cell adhesion mediated by cadherins. CDH2 (also known as N-cadherin) is expressed in many mesodermal tissues and is involved in collective cell migration (reviewed in Alimberti and Andreadis, 2015). We performed IF with an anti-CDH2 antibody along with an anti-SNAI2 antibody, and we found very low expression in the 1st PA of WT E9.5 embryos (Fig. 11A). However, in Tbx1−/− embryos, the compacted SNAI2+ mesodermal core of the 1st PA was clearly CDH2+, well above the level of expression in the surrounding mesenchyme (Fig. 11B).

In summary, our expression analysis indicates that TBX1 has cell-autonomous, and perhaps more extensive non-cell-autonomous, functions in regulating the regionalization of cell lineages that are critical for the development of the PhAp.

**SNAI2 identifies a novel haploinsufficiency phenotype rescued by vB12**

Tbx1−/− embryos exhibit significant anatomical anomalies, thus raising the question of whether some of the regionalization differences may be due to anatomical constraints. However, quantitative data shown in Fig. 10 indicate that heterozygous embryos, which have no gross anatomical abnormalities (with the
exception of hypoplasia of the 4th PA artery and parathyroids), also have regionalization anomalies. Furthermore, visual IF analysis showed that, in Tbx1+/− embryos, SNAI2+ cells were grouped in the core mesoderm (Fig. 12A′; additional examples shown in Fig. S7). A similar result was obtained using Mef2c-AHF-Cre-driven deletion of Tbx1 in Tbx1floox−/−;Mef2c-AHF-Cre embryos (Fig. S8), indicating that this anomaly is dependent upon Tbx1 haploinsufficiency in the mesoderm. This phenotype is reminiscent of, but less severe than, that noted in Tbx1−/− embryos (compare with Fig. 7A,B and Fig. 8C,D). Interestingly, vB12 treatment re-established a staining pattern similar to WT (Fig. 12A′) in three independent experiments. Quantitative evaluation of the SNAI2 staining on an additional set of four embryos revealed that the number of SNAI2+ cells increased modestly, but significantly, in the A segment of PBS-treated heterozygous mutants, compared to PBS-treated WT embryos (Fig. 10E). vB12 treatment improved the numerosity phenotype, as it became closer to the WT phenotype (because there is no significant difference with the WT embryos), but not sufficiently to reach significance when tested against the untreated heterozygous embryos (P=0.5, Mann–Whitney one-tailed test). We think that this is because our quantitative test is not sufficiently sensitive. Therefore, the phenotypic improvement after vB12 treatment is documented in part by qualitative observations and in part by quantitative evidence that treated embryos are closer to the WT phenotype than the untreated ones.

**DISCUSSION**

Gene haploinsufficiency is a frequent cause of birth defects and postnatal morbidity. Counterbalancing haploinsufficiency is possible, but it is challenging in the clinical setting and may lack sufficient precision to rescue the full spectrum of phenotypic changes. Pharmacological rescue would be particularly useful in the clinics if it could be precisely targeted.

High doses of vB12 can partially rescue the 4th PA artery phenotype associated with Tbx1 gene haploinsufficiency in the mouse (Lania et al., 2016); in this study, we tested additional phenotypic recoveries by crossing a hypomorphic allele, which, combined with a null allele, causes a more complex phenotype than the one exhibited by heterozygous mutants. Indeed, we observed that vB12 treatment improved the septation process of the outflow tract in Tbx1neo2/− embryos. In addition, we found that branchiomeric muscle defects were also diminished after treatment.

Having shown that the treatment improves a range of phenotypic abnormalities, we sought to leverage this property to identify genes and pathways dysregulated by Tbx1 haploinsufficiency and rebalanced by vB12; these genes may be critical for the pathogenesis of the rescued phenotypes. We found that, in E9.5 Tbx1+/− embryos, 24% of the genes dysregulated by Tbx1 haploinsufficiency were rescued by vB12, and GO analysis of the rescued genes revealed enrichment of genes involved in heart development, thus providing a transcriptional correlate of the phenotypic observations. Among the rescued genes, we noted several that are implicated in epithelial–mesenchymal transition, and we selected Snai2 for further studies. At E9.5, SNAI2 identifies a mesodermal population in the PhAp that partially overlaps with other markers of the CPM, such as TBX1, ISL1 and Mef2c-AHF-Cre. This suggests that the SNAI2+ cell population may include SHF cardiac progenitors and branchiomeric muscle progenitors of the cardiopharyngeal lineage. Importantly, the SNAI2 expression pattern changes in heterozygous and homozygous Tbx1 mutants. This could be due to ectopic expression of the Snai2 gene, or to mislocalization of SNAI2+ cells. However, the second hypothesis, i.e. defective regionalization, is supported by the finding that the expression of other genes also follows similar pattern changes. In addition, quantitative analyses of SNAI2+ cells confirmed significant distribution and density changes, and revealed an
absolute increase in the number of SNAI2+ cells in the 1st PA of Tbx1−/− embryos at E9.5. In addition, the finding that NCCs, as identified by TFAP2A staining, are also mislocalized supports the hypothesis that the Tbx1 mutation is associated with anomalous regionalization of multiple cell lineages. It is unlikely that regionalization anomalies are due to morphogenetic defects because some of these anomalies, along with gene expression dysregulation, are also evident in the heterozygous mutants that do not show major morphogenetic defects.

The aggregation of SNAI2+ cells, particularly evident in the 1st PA, and the segregation of these cells from the neural crest lineage, suggest that the mutation is altering mechanisms of cell sorting, a crucial process in embryonic morphogenesis. This problem may occur for a number of reasons. For example, the differential adhesion hypothesis (Steinberg, 1962) predicts that cells tend to group together if they have higher affinity with each other, compared with other neighboring cell populations. This possibility is supported by the finding that aggregated cells express higher levels of CDH2, a cell adhesion molecule, compared to the surrounding NCC-derived mesenchyme in the 1st PA of Tbx1−/− embryos. Our observations in the 1st PA of early embryos indicate that, in Tbx1 mutants, incoming mesenchymal cells fail to mix with core mesodermal cells, consistently with a differential cell adhesion hypothesis. Intermingling of Myf5+ myogenic core cells and incoming TFAP2A+ NCCs in the 1st PA of WT embryos has previously been described (Grenier et al., 2009), although the mechanisms that govern this process are not yet established. We show here that TBX1 function is part of these mechanisms, although the effectors remain to be identified. The association of Tbx1 heterozygosity and NCC distribution has been noted previously in the posterior pharynx (Calmont et al., 2009). Cell–cell adhesion and/or cell–extracellular matrix interactions may interfere with NCC migration, delaying proper localization, and TBX1 loss of function has been associated with alteration of these interactions.

Fig. 8. The core mesoderm of the 1st PA of Tbx1−/− embryos is populated by SNAI2+;ISL1+ cells. (A–D ″) Double IF using anti-SNAI2 (green) and anti-ISL1 (red) antibodies on lateral (A,B) and mediolateral sagittal (C,D) sections of the 1st PA from Tbx1+/+ (A,C) and Tbx1−/− (B,D) embryos. Note the colocalization of ISL1 and SNAI2 in the core mesoderm of the 1st PA in Tbx1−/−. In the WT, this is much less evident (compare regions indicated by arrowheads in A, B, C and D). In addition, SNAI2 expression is extended in the splanchnic mesoderm of the Tbx1−/− embryo (arrows in B and B ′). Scale bar: 100 µm.

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Treatment with vB12 could also target NCCs and modify their migratory behavior.

It is tempting to speculate that lineage regionalization abnormalities are part of the pathogenetic mechanism underlying the severe developmental defects of the PhAp associated with Tbx1 mutation. Mislocalization, even transient, of different cell types may expose them to different signaling cues (or different concentrations thereof), causing further developmental defects downstream.

In this work, we used Snai2 as a marker gene, but we did not address a potential role of Snai2 in the observed phenotypes. SNAI2 is a transcriptional repressor that targets genes encoding adhesion molecules (such as E-cadherin) in epithelial cells, thus supporting their mobilization and mesenchymalization (Zhou et al., 2019). It is difficult to directly apply these concepts to the pharyngeal mesenchymal cells that we have studied. However, SNAI2 has also been associated with a number of different functions, including skeletal muscle differentiation (Tang et al., 2016) and with upregulated CDH2 in some contexts (reviewed in Loh et al., 2019). Therefore, it would be of interest to determine, in the future, whether SNAI2 has a specific role in branchiomeric muscle differentiation or development, which is impaired in Tbx1 mutants (Grifone et al., 2008; Kelly et al., 2004). However, at this point we

Fig. 9. The positional relationship between TBX1+ and SNAI2+ cells is maintained in Tbx1 null embryos. (A–B″) IF using anti-GFP (red) and anti-SNAI2 (green) antibodies on transverse sections of Tbx1CreERT2;R26RmTmG (functionally heterozygous; A) and Tbx1CreERT2;R26RmTmG (null mutant; B) E9.5 embryos. A′, B′ and A″, B″ show the green and red, respectively, signals in separate channels. Note that Tbx1-activating cells and their descendants (in red) localize predominantly laterally in the core mesoderm (arrowheads) and in the proximal region of the PA, in both cases. Scale bar: 100 µm.
do not have evidence implicating Snai2 upregulation in the pathogenesis of the Tbx1 mutant phenotype.

In summary, we show that vB12 treatment is sufficient to rescue, in part, several anomalies of the PhAp. We leveraged this activity to identify a set of genes already known to be involved in heart development that may be part of or associated with the pathogenesis of TBX1-dependent phenotypes. Finally, one of the rescued genes, encoding SNAI2, has been instrumental in the discovery of a novel phenotype of lineage regionalization defects.

MATERIALS AND METHODS

Mouse lines
In this work, we used mouse lines previously described: Tbx1lacZ (referred to as Tbx1+/−) (Lindsay et al., 2001), Tbx1neo2 (Zhang et al., 2006), Tbx1Cre (Huynh et al., 2007), Tbx1floX (Xu et al., 2004), R26RmTmG (Muzumdar et al., 2007) and Mef2c-AHF-Cre (Verzi et al., 2005). We crossed Tbx1lacZ/+ mice with Tbx1lacZ/+ or Tbx1neo2 to generate heterozygous, WT, null or hypomorphic embryos. We crossed Tbx1Cre with R26RmTmG mice to map the distribution of Tbx1-expressing cells and their descendants. vB12 (cyanocobalamin; Sigma-Aldrich, V2876) was solubilized in PBS and injected intraperitoneally (20 mg/kg). The impact of vB12 on great vessels and ventricular septation defects was scored at E15.5 and E18.5. Pregnant females were injected daily from E7.5 to E11.5. Developmental stages were assessed by considering the morning of vaginal plug as E0.5. Control mice were injected with the same volume of PBS.

Animal studies were carried out under the auspices of the animal protocol 257/2015-PR (licensed to the A.B. laboratory) reviewed, according to Italian regulations, by the Italian Istituto Superiore di Sanità and approved by the Italian Ministero della Salute. All animals are bred into the C57/B6J strain for at least five generations. All the mouse lines generated in our laboratory are available through the European public repository EMMA/Infrafrontier.

Mouse phenotyping
E15.5 and E18.5 embryos were examined under the stereomicroscope and fixed overnight in 4% paraformaldehyde (PFA). E15.5 embryos were embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin. E18.5 hearts and great vessels were manually dissected and photographed under a stereomicroscope, and then embedded in paraffin, sectioned and stained.

IF
Embryos were fixed overnight in 4% PFA and embedded in wax. For IF analysis, embedded embryos were cut into 7 µm sections. Sections were deparaffinized in xylene, rehydrated, and, after antigen unmasking with citrate buffer, sections were incubated overnight at room temperature with primary antibodies (in 0.5% milk, 10% fetal bovine serum, 1%
bovine serum albumin in H₂O). Each experiment was repeated at least three times.

For antibodies raised in the same species (Fig. 5), we performed two rounds of IF on the same sections. After the first round, using the method described above, we acquired images of the sections and then stripped the antibody by boiling the slides in citrate buffer. We checked the absence of any residual signal and then performed a subsequent incubation with another antibody, before fluorescence detection. We then collected images of the same sections. The two sets of images were compared and merged to identify the overlapping signals. The stripping was considered ineffective if the signals were 100% overlapping, in which case samples were excluded from analysis.

We used the following primary antibodies: anti-GFP (Abcam, ab13970, diluted 1:1000), anti-SNAI2 (Cell Signaling Technology, 9585, diluted 1:100); anti-TFAP2A (Hybridoma Bank, clone 3B5, diluted 1:300); anti-ISL1, (Hybridoma Bank, clone 39.4D5, diluted 1:100); anti-CRE (Millipore, 69050-3, diluted 1:1000); anti-TBX1 (Abcam, ab18530, diluted 1:100).

Image acquisition
Most images were acquired using a Nikon A1 laser scanning confocal microscope with objective PLANAPO 10×. For SNAI2 quantitative analysis, the images were acquired using a Nikon Motorized Optical Microscope with 10× and 20× objectives. Digital images were saved using the acquisition software provided with the instruments. Microphotographs obtained from the microscopes were cut and labeled to build figures without further processing using Adobe Photoshop.
For SNAI2+ cell density analysis, the following numbers of embryos were evaluated: WT E9.5, n=5 embryos; Tbx1+/− E9.5, n=5 embryos; Tbx1−/− E9.5, n=5 embryos; Tbx1+/− B12-treated E9.5, n=4 embryos. Cell counts were performed on subsequent transverse sections spanning the entire region of interest (1st PA). Embryos in which immunostaining failed, or sections were damaged, were excluded from the analysis. The region of interest was divided into two segments along the anterior–posterior axis. We counted all the sections from the tag of ruptured buccopharyngeal membrane until the early invagination of the first pharyngeal pouch (8-10 sections for each genotype, anatomically matched). This portion has been identified as A segment. All the sections below the junction, until the end of the arch, were considered as P segment and were entirely analyzed (four to seven sections for each genotype, anatomically matched).

Density distribution heat maps were generated by counting SNAI2+ cells in both A and P segments, to record their distribution in the entire structure, on one side (left). Densities were mapped onto a schematic 4×5 grid of equidistant bins placed on the lateral and distal border of the arch and spanning toward the mediopropontal portion (Fig. 10D). Bin areas were calculated using the ImageJ software graphic pen by Analyze and Measure tool. The SNAI2+ cells were quantified by ImageJ software Cell Counter Pug-in, and the cell density was represented as number of SNAI2+ cells/100 µm2.

Data from multiple embryos were merged in one schematic arch grid, and a color code was adopted to illustrate the density of SNAI2+ cells present in each region (method adapted from Francou et al., 2017). Data were statistically analyzed and graphically represented using Microsoft Office Excel and Prism software. Results were expressed as the mean±s.d. Appropriate statistical tests were used for each sample. Each region of interest was entirely analyzed. No serial sampling was adopted. No randomization or blinding was used. Analysis of data concerning density distribution of SNAI2+ cells was performed using the χ2 test followed by unpaired, one-tailed Student’s t-test. We applied the Benjamini–Hochberg procedure to correct for multiple testing. Statistics on SNAI2+ cell numbers were performed by Kruskall–Wallis one-way test, followed by unpaired one-tailed Mann–Whitney test for small samples.

RNA extraction and RT-PCR
Total RNA was isolated from E9.5 (22 somites) Tbx1+/− and Tbx1+/+ embryos with TRIZOL (Invitrogen) and reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystem, 4368814).

RNA-seq gene expression data analysis
For processing and analysis of RNA-seq data, raw data for the high-throughput sequencing of cDNA were generated with Illumina platform for strand-specific paired-end reads. These reads are 125 bp long. In total, eight RNA-seq samples were sequenced. The three biological replicates of RNA-seq for Tbx1+/− condition are indicated as Tbx1+/−_rep1, Tbx1+/−_rep2 and Tbx1+/−_rep3. The two biological replicates of RNA-seq for heterozygous with BV12 treatment are denoted as Tbx1+/−+(+BV12)_rep1 and Tbx1+/−+(+BV12)_rep2, and the three biological replicates of RNA-seq for WT condition are denoted as Tbx1+/−_rep1, Tbx1+/−_rep2 and Tbx1+/−_rep3. Quality control on raw reads was performed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

For alignment of sequence reads, first, the reads were mapped to the mouse genome (mm9) using TopHat2 (version 2.0.7) (Trapnell et al., 2009), with the following options: -G annotation_file.gff -transcriptome-index transcriptome. All other parameters were used with their default values. The annotation gene transfer format (GTF) file, Mus_musculus.NCBIM37.67.gtf, was downloaded from http://wwwensembl.org.

Gene count matrix was obtained as output using featureCount function from Rsubread R package (version 0.5.4) on ‘exon’ feature type, considering reverse strand for paired end reads with the annotation GTF file. We selected the total counts on 37,620 genes for differential expression analysis. The raw counts were first filtered by applying the Proportion test, which retained a total of 14488 genes, and then normalized with the upper-quartile normalization method using the RNA-SeqGUI R package (Russo and Angelini, 2014). Principal component analysis (PCA) was performed to separate biological conditions. PCA results showed that samples clustered for different library preparations and different times, and therefore raw data had to be corrected for batch effects. First, we performed a complex design, considering the presence of an unknown batch. We removed batch effects using ARSNeq function from filtered gene count matrix and considering reads per kilobase of exon per million reads mapped (RPKM) normalization approach. Then, we evaluated differential expression between pair conditions using the non-parametric NOISeqBIO function (Tanzona et al., 2015) after applying upper quartile as normalization method. A posterior probability greater than or equal to 0.95 was used to determine DEGs.

DEGs with absolute value of fold change greater than or equal to 1.2 were considered for pathway analysis. In addition, we performed pathway analysis using the g:Profiler tool (Raudvere et al., 2019), setting the organism to Mus musculus, choosing as custom background the list of 14,488 expressed genes in our system, setting the significance threshold for the multiplicity correction ‘fdr’ (i.e. Benjamini and Hochberg false discovery rate (FDR)) with the user threshold 0.05. We limited the sources to GO, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Phenotype Ontology databases to evaluate functional enrichment. To establish the statistical significance of the overlap between sets of genes, we performed the hypergeometric test using the R function phyper.R. Condition lines were as follows: Pval.Common=phyper(q=467, n=3954, m=(14488-3954),k=1409,lower.tail=FALSE); Pval.1=phyper(q=258, m=851, n=(14488-851),k=2092,lower.tail=FALSE) ## Up and down; Pval.2=phyper(q=84, m=558, n=(14488-558),k=1862,lower.tail=FALSE) ## down and up; Pval.3=phyper(q=58, m=851, n=(14488-851),k=1862,lower.tail=FALSE) ## up and down; Pval.4=phyper(q=64, m=358, n=(14488-558),k=2092,lower.tail=FALSE) ## down and down, where 14,488 is the number of expressed genes, and the other numbers are the different sets.

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Competition interests
The authors declare no competing or financial interests.

Author contributions

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Data availability
The RNA-seq datasets generated for this study have been deposited in the Gene Expression Omnibus (GEO) database under the accession number GSE206737.

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