Human Molecular Genetics, 2016, Vol. 25, No. 20 4369–4375

doi: 10.1093/hmg/ddw267
Advance Access Publication Date: 9 August 2016
Original Article

ORIGINAL ARTICLE

Vitamin B12 ameliorates the phenotype of a mouse model of DiGeorge syndrome

Gabriella Lania[1,†], Alberto Bresciani[2,†], Monica Bisbocci[2], Alessandra Francone[2], Vincenza Colonna[1], Sergio Altamura[2] and Antonio Baldini[1,3,*]

[1]Institute of Genetics and Biophysics, CNR, Via Pietro Castellino, Naples, Italy, [2]IRBM Science Park S.p.A, Via Pontina Km.30.600, Pomezia (RM) and [3]Department of Molecular Medicine and Medical Biotechnologies, University Federico II, Naples, Italy

*To whom correspondence should be addressed at: Antonio Baldini, MD, Institute of Genetics and Biophysics, Via Pietro Castellino 111, 80131 Naples, Italy. Tel: +39-0816132219; Fax: +39-0816132706; Email: antonio.baldini@igb.cnr.it

Abstract

Pathological conditions caused by reduced dosage of a gene, such as gene haploinsufficiency, can potentially be reverted by enhancing the expression of the functional allele. In practice, low specificity of therapeutic agents, or their toxicity reduces their clinical applicability. Here, we have used a high throughput screening (HTS) approach to identify molecules capable of increasing the expression of the gene *Tbx1*, which is involved in one of the most common gene haploinsufficiency syndromes, the 22q11.2 deletion syndrome. Surprisingly, we found that one of the two compounds identified by the HTS is the vitamin B12. Validation in a mouse model demonstrated that vitamin B12 treatment enhances *Tbx1* gene expression and partially rescues the haploinsufficiency phenotype. These results lay the basis for preclinical and clinical studies to establish the effectiveness of this drug in the human syndrome.

Introduction

Gene haploinsufficiency is a common cause of genetic disease. Potential treatment strategies include correcting the dysregulation of critical (pathogenic) genes targeted by the haploinsufficient gene, boosting the expression of the haploinsufficient gene to partially compensate for the reduced gene dosage, and targeting the pathological processes affected by haploinsufficiency. *TBX1* gene haploinsufficiency causes most of the clinical features associated with one of the most common segmental aneuploidies in humans, the 22q11.2 deletion syndrome (22q11.2DS) (1,2). The clinical phenotype, which is well recapitulated in *Tbx1* mouse mutants, includes congenital anomalies (e.g. heart and vascular defects) as well as adolescence/adult onset features (2,3). The latter is the most suitable to drug therapy, while the former would require diagnosis and treatment during early pregnancy.

To identify potential drugs for correcting the phenotype of *Tbx1* mutant mice, we have previously used genetic approaches, but results, albeit significant, have been of difficult practical application (4,5). Here, we have used a different approach that takes advantage of high throughput screening (HTS) to identify molecules capable of enhancing the *Tbx1* gene expression in mouse embryonic fibroblasts (MEFs). Surprisingly, we found

†These authors equally contributed to this work.
Received: January 26, 2016. **Revised:** July 26, 2106. **Accepted:** July 29, 2016

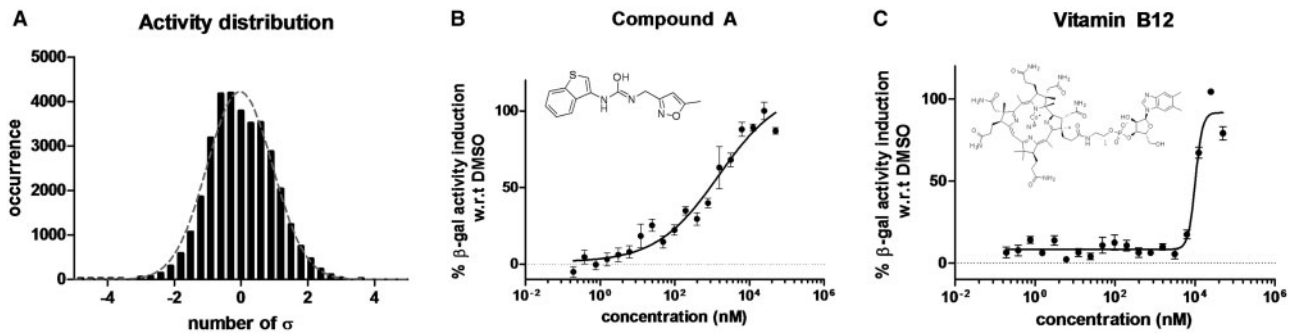

Figure 1. High throughput screening results. Occurrence distribution of compound screening activity (**A**). The bin width was set to +3 standard deviations. The grey dotted line represents the best fitted Gaussian distribution. Dose response of the *β*-gal activity for compound A (**B**) and vitamin B12 (**C**). The percentage of induction was calculated with relative to the average DMSO levels (0% induction).

that vitamin B12 increased expression of *Tbx1*. Validation in tissue culture using two different cell types confirmed the HTS results and showed that vitamin B12 treatment modifies the epigenetic profile of the gene. *In vivo* treatment also up regulated *Tbx1* gene expression in the haploinsufficient mouse model and ameliorated significantly the haploinsufficiency phenotype in mouse embryos. These findings open a completely new avenue for future studies directed at exploring potential clinical applications for this relatively frequent genetic syndrome using a very well tolerated drug.

Results

High throughput screening for molecules enhancing Tbx1 gene expression

To identify compounds that enhance the expression of *Tbx1*, we used *Tbx1*$^{lacZ/+}$ mouse embryonic fibroblasts (MEFs) in an HTS assay. In these cells, the expression of *Tbx1* can be evaluated using an automatable β-galactosidase assay. We plated these cells in 384-well plates and assayed them against a library of about 35,000 small molecules. The library collection contained a high percentage of drug-like and lead-like molecules, with over 90% of the compounds having a molecular weight below 500 (mean molecular weight is 348 Da) and with cLogP (calculated partition coefficient) and TPSA (total polar surface area) in the range generally accepted as being suitable for orally bioavailable compounds. The average number of rotatable bonds was 5. Screened for diversity by both MACCS166 fingerprints (6) and by methods measuring the maximum common substructure of the central scaffolds, the entire collection clustered into approximately 4500 unique clusters together with over 1000 singleton clusters. The average number of molecules per cluster was around 6. Drugs were used at a concentration of 5 μM, which is the highest possible concentration compatible with a viable DMSO concentration. In addition, due to the absence of a known strong inducer of *Tbx1* expression, the HTS was run without a positive control. Therefore, the response to the compounds was calculated as the number of standard deviations from the mean of the whole sample. The distribution of HTS results was found to be grossly normal (Fig. 1A) ; thus the hit threshold was set at 3 standard deviations over the mean. With these criteria, 22 compounds were selected as active (i.e. inducers of *Tbx1* expression) and further tested in a dose-response manner, to confirm their activity in the same induction assay. Only two compounds resulted consistently active (Fig. 1B and C). One of the two was vitamin B12, which showed a steep induction and a potency (EC_{50}) of 10 μM. The other compound, hereafter referred to as 'compound A' (see Materials and Methods) was found to have an EC_{50} of 1 μM.

Compound validation

We tested whether vitamin B12 and compound A enhance *Tbx1* gene expression in independent cell culture systems. To this end, we used the embryonic carcinoma cell line P19Cl6 (7) and four different MEF clones from WT mouse embryos. Cells were treated with 10μM of vitamin B12 or 1μM of compound A for 48 or 72h., and then RNA was extracted and subjected to quantitative assay (qRT-PCR) to determine the expression of the endogenous *Tbx1* gene. In P19Cl6 cells, *Tbx1* gene expression increased significantly after 48 h. treatment with compound A (Fig. 2A and A'), while for vitamin B12 treatment, significant up regulation was detected at 72h. (Fig. 2B and B'). In MEF cells, *Tbx1* expression increased after 72h. of treatment with compound A (Fig. 2C and C') and after 48h. of treatment with vitamin B12 (Fig. 2D and D'), but at 72h. there was no significant increase (Fig. 2D and D').

Further validation studies were focused on the use of vitamin B12 rather than compound A because the vitamin has a high potential to be quickly translated to the clinic, whereas for compound A there are no chemical characterization nor pharmacological studies.

We asked whether vitamin B12 treatment altered the chromatin state of the *Tbx1* gene. To this end, we performed quantitative chromatin immunoprecipitation (qChIP) assays using antibodies to H3K27Ac and H3K4me3, two histone marks that are generally associated with active enhancers or promoters, respectively. We tested for enrichment in three sites of the *Tbx1* gene: the upstream enhancer known as the Fox-responding element (FOX-RE) (8–10), the promoter region (-490bp from the transcription start site), and intron 1, which contains a regulatory element (10). The FOX-RE site was not enriched in H3K4me3 and there was no change after treatment. In contrast, H3K27Ac increased significantly at this site after treatment ($P = 0.00035$). Similarly, at the Intron 1, H3K27Ac enrichment was enhanced by vitamin B12 treatment ($P = 0.006$). We did not find statistically significant changes with H3K4me3 enrichment (Fig. 2E'), although we found a consistent upward trend in repeated experiments at the Intron 1 site (Fig. 2E', right panel). The two histone modifications at the promoter region did not change in response to treatment (Fig. 2E). Thus, vitamin B12 treatment is associated with H3K27Ac enrichment at regulatory regions, suggesting enhancer activation.

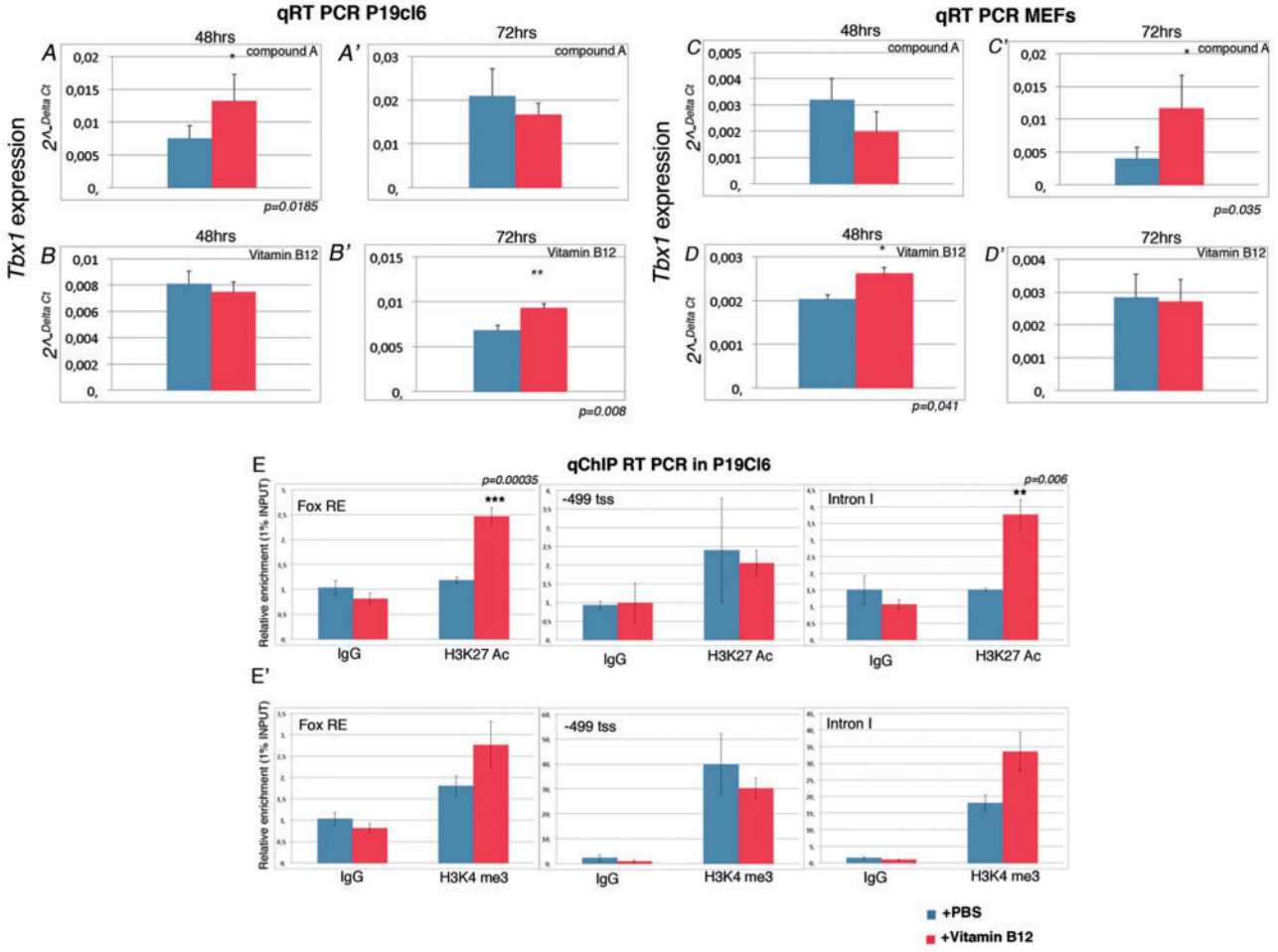

Figure 2. Compound A and vitamin B12 enhance *Tbx1* expression in P19Cl6 and MEF cells. (**A-D'**) Quantitative RT-PCR evaluation of *Tbx1* expression in P19Cl6 cells treated for 48h and 72h with the compound A (A-A') and vitamin B12 (B-B'). Quantitative RT-PCR evaluation of *Tbx1* expression in MEFs cells treated for 48h and 72h with chemical Compound A (C-C') and vitamin B12 (D-D'). (**E-E'**) Quantitative ChIP (qChIP) results for three loci, Fox responsive element (FOX-RE) (mm9 chr16:18601587-18601697), promoter (-499 fro transcription start site; mm9 chr16:18587346-18587459), and intron I of *Tbx1* gene (mm9 chr16:18587346-18587459). qChIP assays were performed on P19Cl6 cells with and without vitamin B12 treatment. The histograms represent the mean of 6 independent experiments. Data are presented as mean ± SEM. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Vitamin B12 treatment partially rescues Tbx1$^{lacZ/+}$ pharyngeal arch artery anomalies

We tested whether vitamin B12 is able to up-regulate *Tbx1* expression in mouse embryos *in vivo*. To this end, we used a mouse model of *Tbx1* gene haploinsufficiency, *Tbx1*$^{lacZ/+}$ (11). We crossed *Tbx1*$^{lacZ/+}$ mice with WT mice and injected intraperitoneally pregnant females with vitamin B12 (or PBS) at E7.5 and E8.5 (2mg/kg body weight, dissolved in PBS (12)). We harvested embryos at E9.5, extracted RNA and performed quantitative real time reverse-transcription PCR (qRT-PCR) to evaluate *Tbx1* gene expression. Results, demonstrated that vitamin B12-treated *Tbx1*$^{lacZ/+}$ embryos had a significantly higher *Tbx1* gene expression compared to PBS-treated embryos ($P = 0.0045$, Fig. 3A). We also tested, in the same embryos, the expression of a Tbx1 target gene known to be involved in the *Tbx1* haploinsufficiency phenotype, *Fgf8* (9,13,14). Consistently with an increase of *Tbx1* expression, we found that *Fgf8* is also up-regulated in vitamin B12-treated *Tbx1*$^{lacZ/+}$ embryos ($P = 0.031$, Fig. 3B). Next, we asked whether the effect of vitamin B12 on *Tbx1* gene expression could be exploited to modify the *Tbx1* mutant phenotype. We crossed *Tbx1*$^{lacZ/+}$ mice with WT mice and injected pregnant females with the same dosage of vitamin B12 or PBS at E7.5, E8.5, and E9.5, and we have harvested embryos at E10.5 for phenotypic analysis. Specifically, we have scored the presence of 4th pharyngeal arch artery (PAA) defects that are typical of heterozygous mutants (11,15,16). In PBS-treated embryos, the incidence of 4th PAA defects was 90% ($n = 21$), while in the treated group it was 61% ($n = 26$); this is a significant reduction of incidence in the treated group ($P = 0.04$) (Fig. 4A–A'). To confirm that the observed rescue has a significant impact on the definitive remodelling of the aortic arch later in development, we tested the phenotype at E17.5, when remodelling is complete. For this test, we injected vitamin B12, or PBS, at E7.5, E8.5, E9.5, and E10.5, and harvested fetuses at E17.5. Fetuses were dissected and examined for the presence of 4th PAA-derived anomalies, i.e. aberrant origin of the right subclavian artery, interrupted aortic arch type B, and right aortic arch. Results are summarized in Table 1 and Fig. 4B'', examples are shown in Fig. 4B and B'. Overall, the incidence of defects in PBS-treated *Tbx1*$^{lacZ/+}$ fetuses was 46% ($n = 26$), while in the vitamin B12-treated group was 19% ($n = 26$, $P = 0.038$). Thus, vitamin B12 has a significant effect on the incidence of anomalies after the remodelling was complete.

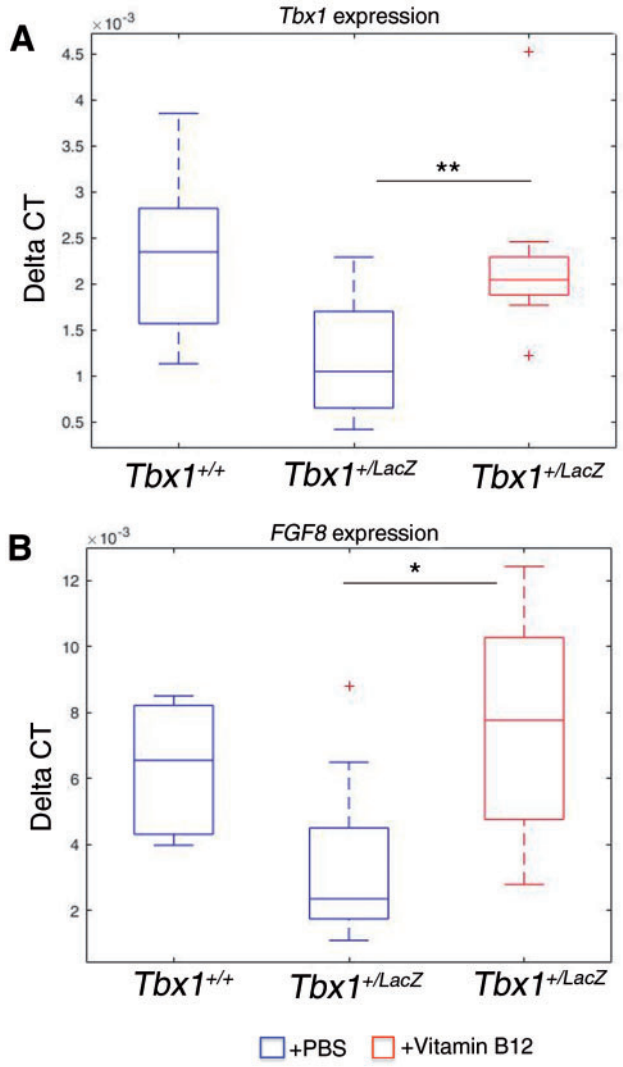

Figure 3. *Tbx1* and *Fgf8* gene expression is upregulated by vitamin B12 *in vivo*. Box plots of qRT-PCR evaluation of *Tbx1* (**A**) and *Fgf8* (**B**) gene expression in E9.5 embryos ($n = 11$ for each point), with or without vitamin B12 treatment. The error bars represent the minimal and maximal values of relative expression. *** $P = 0.0045$; * $P = 0.031$.

Discussion

We present the results of an HTS study that led to the identification of vitamin B12 as a positive regulator of *Tbx1* gene expression. We have validated the results in cultured cells and *in vivo*, and shown that the vitamin can partially rescue the haploinsufficiency phenotype in a mouse model of 22q11.2DS. The phenotypic anomaly that we measured in our *in vivo* study is a sensitive indicator of *Tbx1* dosage because even a modest reduction of *Tbx1* RNA in hypomorphic mutants can induce it (17,18).

Vitamin B12 functions as a cofactor for two enzymes: methylmalonyl CoA mutase, which catalyses the isomerization of methylmalonyl-CoA to succinyl-CoA, and methionine synthase, which catalyses the synthesis of methionine (19), the direct precursor of S-adenosylmethionine (SAM). SAM is the general donor of methyl groups for methyltransferase activity, including that involved in epigenetic coding, such as DNA and histone methylation. This mechanism provides a general link between vitamin B12 and gene regulation (20,21). However, how exactly vitamin B12 treatment (at the doses used here) up regulates the *Tbx1* gene is not easily explained by a general increase of histone methylation because we did not detect a significant increase in H3K4me3 at the loci tested. We did see significant enhancement of H3K27 acethylation, suggesting that the vitamin is inducing epigenetic changes of the *Tbx1* gene. However, we cannot exclude that the partial rescue of the *Tbx1* haploinsufficiency phenotype is due to or aided by mechanisms other than *Tbx1* up-regulation, for example metabolic changes or expression changes of Tbx1 target genes. Indeed, we have shown an increase of the important target *Fgf8* gene. Such increase could be due to *Tbx1* up-regulation, but we cannot exclude that it may be due to a direct effect of vitamin B12 on the expression of this gene. Nevertheless, our data open a viable window of opportunity for future treatments of at least some features of the 22q11.2DS.

The clinical presentation of 22q11.2DS is characterized by broad variability, so it would be of interest to test whether there is a link between clinical presentation at birth or after, and vitamin B12 intake during pregnancy, as vitamin B12 is often included in multivitamin complexes recommended as dietary supplements. In addition, genome wide data on 22q11.2DS patients' cohorts (22) could be interrogated for variants of genes related to vitamin B12 pathways, and tested for correlation with phenotypic presentation. Finally, 22q11.2DS is associated with an adolescence/adult onset phenotype, at least some of which appears to be related to *Tbx1* haploinsufficiency (3,2). While it is still unclear whether this 'late' phenotype also originates from embryonic or fetal damage, it is possible that post-natal treatment might help in preventing the late-onset phenotype.

Materials and Methods

Mouse lines

Animal research was conducted according to EU and Italian regulations. The animal protocol has been approved by the animal welfare committee of the Institute of Genetics and Biophysics (Organismo per il Benessere Animale), protocol 0002183-04062013, and protocol 257/2015-PR of the Italian Ministry of Health. We have used $Tbx1^{lacZ/+}$ mice (11) bred into the C57/Bl6 strain, to generate heterozygous and wild type embryos. Administration of vitamin B12 (cyanocobalamin Sigma-Aldrich Prod. Number V2876) was performed by intraperitoneal injections of pregnant females at embryonic days (E) 7.5, 8.5, and 9.5 (2μg/kg body weight dissolved in PBS, one injection per day). We also injected at E10.5 for the study of phenotype at E17.5. Developmental staging was established by considering the morning in which the vaginal plug was seen as day E0.5. Control mice were injected with the same volume of PBS. To evaluate the impact of vitamin B12 treatment on *Tbx1* gene expression, pregnant females were treated at E7.5 and E8.5, and sacrificed at E9.5. Twelve embryos for each genotype, $Tbx1^{lacZ/+}$ and $Tbx1^{+/+}$, were collected at E9.5 and RNA isolated as described below.

Statistical methods

Statistical significance of differences between treated and untreated samples was determined by parametric and non-parametric tests. In quantitative chromatin immunoprecipitation (qChIP) assays, significance of fold change differences was assessed using a 2-tailed Student's t test. Gene expression differences in quantitative real time PCR experiments were

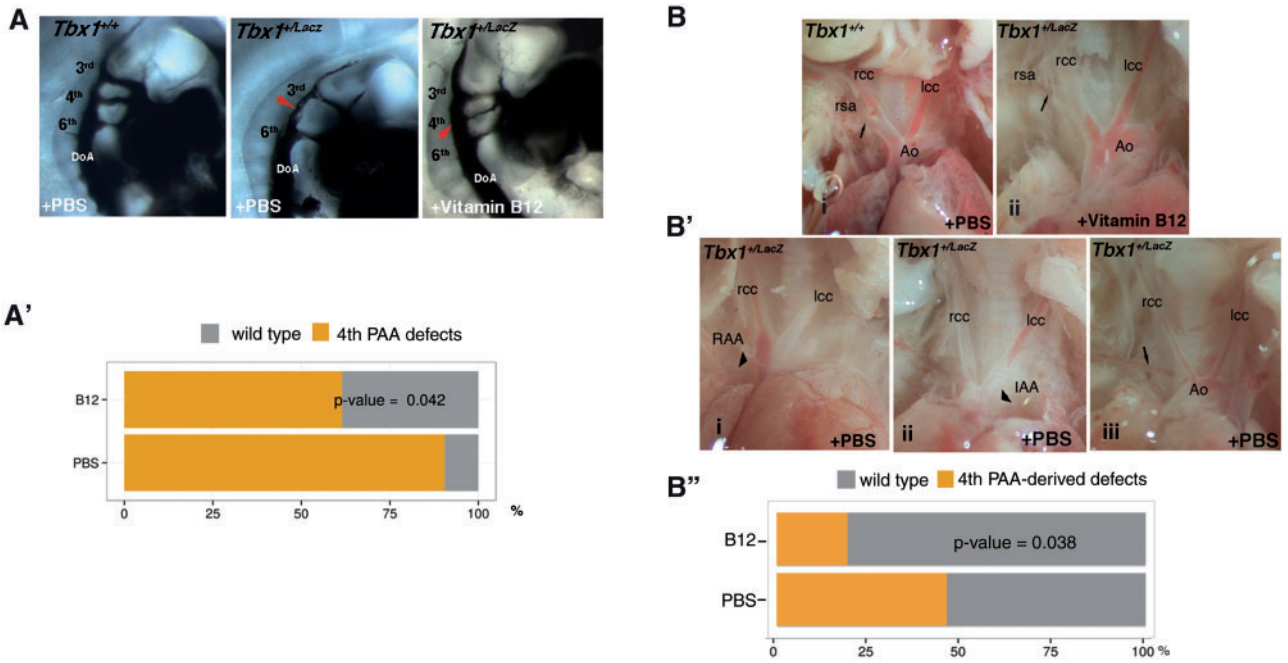

Figure 4. Vitamin B12 treatment partially rescues the *Tbx1* haploinsufficiency phenotype. (**A**) Pharyngeal arch arteries of E10.5 embryos visualised by ink injection. Lateral view of PBS-treated WT (left panel), PBS-treated $Tbx1^{lacZ/+}$ (centre), and vitamin B12-treated $Tbx1^{lacZ/+}$ (right) embryos. (**A'**) Graphic representation of the percentage of embryos with 4th PAA defects (PBS sample $n = 21$; vitamin B12-treated sample $n = 26$). (**B**) Representative immages of aortic arch and great vessels of E17.5 fetuses. (i) WT pattern; (ii) normal pattern in a vitamin B12-treated $Tbx1^{lacZ/+}$ fetus; (**B'**) Examples of aortic arch patterning defects in $Tbx1^{lacZ/+}$ fetuses: (i) right aortic arch (RAA), the arrowhead indicates the retropositioned arch; (ii) interrupted arch aortic (IAA) type B, the arrowhead indicates the interruption; (iii) retroesophageal right subclavian artery. Arrows indicate the right subclavian artery. Ao: aorta; lcc: left common carotid artery; rcc: right common carotid artery; rsa: right subclavian artery. (**B''**) Graphic representation of the percentage of fetuses with aortic arch patterning abnormalities derived from 4th PAA defects (PBS sample $n = 26$; vitamin B12-treated sample $n = 26$).

Table 1. Summary of phenotypic analysis of E17.5 fetuses

Genotype Treatment	Number of fetuses	AbRSA	IAA-B	RAA
$Tbx1^{+/+}$ PBS	18	0	0	0
$Tbx1^{+/+}$ Vitamin B12	16	0	0	0
$Tbx1^{lacZ/+}$ PBS	26	8	3	1
$Tbx1^{lacZ/+}$ Vitamin B12	26	3	2	0

AbRSA: aberrant origin of the right subclavian artery; IAA-B: interruption of the aortic arch type B (between the left common carotid artery and the ductus arteriosus);. RAA: right aortic arch.

evaluated using the two-sample Kolmogorov-Smirnov test. The rescue of the 4th pharyngeal arch artery defects was tested using the Fisher's exact test. Data are presented as mean ± SEM. P values of less than 0.05 were considered statistically significant.

Ink injection and phenotype scoring

To visualize the pharyngeal arch arteries (PAAs), $Tbx1^{lacZ/+}$ and $Tbx1^{+/+}$, treated and untreated E10.5 embryos were injected intracardially with India ink. Embryos were then dehydrated with 70% EtOH and cleared in 1:2 benzyl benzoate:alcohol benzylic.

The PAA phenotype was scored blind to genotype on cleared embryos under a stereo microscope, by at least two experienced observers. 'Normal' vessels were defined as completely patent to ink (from the aortic sac to the dorsal aorta) and with a size comparable to the 3rd and/or 6th PAAs of the same embryo. 'Defective' vessels were defined as having one of the following phenotypes: aplasia (no section of it was filled by ink), or hypoplasia (very thin ink filling, often interrupted between the aortic sac and dorsal aorta). Embryos with technically defective injection, i.e. when the aortic sac, 3rd PAA and 6th PAA were not well filled, were discarded.

E17.5 fetuses were dissected and phenotyped by direct observation under the stereo microscope by at least two experienced observers blind to genotype.

Tissue culture experiments

Primary mouse embryonic fibroblasts (MEFs) were isolated from WT and $Tbx1^{lacZ/+}$ (11) mouse E12.5-13 embryos, using standard methods. Briefly, embryo tissue was disaggregated with 0.25% trypsin for 25–30 min at 37 °C in a 5% CO_2 incubator, and then resuspended in DMEM with 10% FBS, 1% non essential amino acids, 1U/ml penicillin, 1μg/ml streptomycin (Life Technologies, Carlsbad, CA, USA), by pipetting with a 1000μL tip and generate a single cell suspension.

For HTS experiments we used a MEF clone isolated from $Tbx1^{lacZ/+}$ embryos. This clone, Cl72 after 15-passages showed an immortalized phenotype. For compound, validation, we used independent MEF clones obtained from WT embryos. These clones were used at the second or third passage in tissue culture.

P19Cl6 cells were cultured in alpha-MEM,10% FBS,1% Glutamine, 1U/ml penicillin, 1μg/ml streptomycin. $1.25x10^5$ cells/well were plated and were treated after 24h with 10 μM vitamin-B12 and collected at 48 hrs. and 72 h. to RNA isolation and ChIP experiments.

HTS assay

50 nl of test compounds (2 mM solution in DMSO) were transferred to tissue culture-treated, 384-well white plates (Greiner Bio One, Frickenhausen, Germany) by acoustic transfer (EDC biosystems, Milmont, CA, USA). 20 μl of a suspension of 10^4 *Tbx1*$^{lacZ/+}$ MEF cells were added to assay plates. After 48 h incubation at 37 °C, 5% CO_2 in humidified atmosphere and 30 min incubation at RT, we added 20 μl/well of Beta Glo (Beta Glo Assay System, Promega). After 1 h incubation at RT, the signal intensity was quantified by the ViewLux uHTS microplate imager (PerkinElmer, USA). Data analysis was performed using the Dotmatics suite (Dotmatics, Bishops Stortford, UK).

'Compound A' (1-(benzo[b]thiophen-3-yl)-3-((5-methylisoxazol-3-yl)methyl)urea) was purchased from Maybridge (Product Code: HTS12348, ACD Code: MFCD04110438).

The purity of the compound was assessed by UPLC analysis to be 91%. A Waters UPLC system with both diode array detection and electrospray (+'ve and –'ve ion) MS detection was used. The stationary phase was a Waters Acquity UPLC BEH C_{18} 1.7um 2.1x50mm column. The mobile phase was H_2O containing 0.1% formic acid or MeCN containing 0.1% formic acid. Flow rate 0.5 mL/min. Sample concentration: 1 mg/mL. Injection volume 2 μl.

Quantitative gene expression analyses

Total RNA was isolated with TRIZOL (Invitrogen) and reverse-transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystem catalog. n. 4368814). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green PCR master mix (Applied Biosystem). Relative gene expression was evaluated using the $2^{-\Delta\Delta Ct}$ method, and *Gapdh* expression as normalizer. Primers are *Tbx1*-F: 5'-CTGACCAATAACCTGCTG GATGA-3'; *Tbx1*-R: 5'-GGCTGATATCTGTGCATGGAGTT-3'; *FGF8*-F: 5'-CAGGTCCTGGCCAACAAG-3'; *FGF8*-R: 5'-GGTCTCCACA ATGAGCTTCG-3'; *GAPDH*-F: 5'-TGCACCACCAACTGCTTAGC-3'; *GAPDH*-R: 5'-TCTTCTGGGTGGCAGTGATG-3'. Expression data are shown as the mean ± SEM. Statistical analyses were performed using the Student's *t*- test.

Chromatin immunoprecipitation (ChIP)

For ChIP assay, cells were fixed with 1% formaldehyde in PBS at room temperature for 10 min. The cross-linking reaction was stopped using 0.125 mol/l glycine at room temperature. Cells were lysed and chromatin was sonicated in sharing buffer (0.1%SDS, 15mM Tris pH 7.6, 1mM EDTA) using S2 Covaris System (Duty Cycle: 2%, Intensity: 3, Cycles/Burst: 200, Cycles: 6, Cycles time: 60 s, Temperature: 4 °C). Sonicated chromatin (12 μg for IP) was immunoprecipitated with an anti-H3K4me3 (Millipore) or anti-H3K27 acetylated (Abcam) antibody or Rabbit Control IgG (Abcam). The incubation was performed in sharing buffer containing 0.1%BSA, 1% Triton, 150mM NaCl, 5μg of antibody and preblocked protein A/G coated beads at 4 °C overnight. After incubation, immunoprecipitation, samples were extensively washed with low salt, high salt and LiCl washing solution. The reverse cross-linked was performed in ChIP elution buffer (0.1M $NaHCO_3$ 1% SDS) with 0.4 μg/μl of PK. DNA was purified using AM pure beads (Beckman Coulter) and subjected to quantitative PCR amplification.

ChIP primer sequences and genomic location

FOX-RE: genome assembly mm9 cordinates chr16:18601587-18601697. Primers: 5'-TCAGCACAGCCAGCCGCTTT-3' 5'-ATT TCCTTTGGCCCCGCCCC-3'.
-499 TSS: chr16:18587346-18587459. Primers: 5'-TTTACGA TTGAAAGGGCAAAG-3' 5'- TTTCTCGGTGTCACTCTCTCC-3'.
Intron I: chr16:18586010-18586171. Primers: 5'-GAGAAG GCTTTGCAAACAGG-3' 5'- GCCAGTGCCTGGTTATTTGT-3'.

Acknowledgements

We thank Emanuela Nizi for her technical assistance.

Conflict of Interest statement. Alberto Bresciani, Monica Bisbocci, Alessandra Francone, and Sergio Altamura receive a salary from a private company, IRBM Science Park S.p.A.

Funding

This work was funded in part by the CNCCS Scarl Initiative (to S.A. and A. Ba) and by a grant from the Italian Telethon Foundation GGP14211 (to A. Ba).

References

1. Yagi, H., Furutani, Y., Hamada, H., Sasaki, T., Asakawa, S., Minoshima, S., Ichida, F., Joo, K., Kimura, M., Imamura, S., *et al.* (2003) Role of TBX1 in human del22q11.2 syndrome. *Lancet*, **362**, 1366–1373.
2. Paylor, R., Glaser, B., Mupo, A., Ataliotis, P., Spencer, C., Sobotka, A., Sparks, C., Choi, C.H., Oghalai, J., Curran, S., *et al.* (2006) Tbx1 haploinsufficiency is linked to behavioral disorders in mice and humans: implications for 22q11 deletion syndrome. *Proc. Natl. Acad. Sci. U. S. A*, **103**, 7729–7734.
3. Swillen, A. and McDonald-McGinn, D. (2015) Developmental trajectories in 22q11.2 deletion. *Am. J. Med. Genet. C Semin. Med. Genet.*, **169**, 172–181.
4. Vitelli, F., Lania, G., Huynh, T. and Baldini, A. (2010) Partial rescue of the Tbx1 mutant heart phenotype by Fgf8: genetic evidence of impaired tissue response to Fgf8. *J. Mol. Cell. Cardiol.*, **49**, 836–840.
5. Caprio, C. and Baldini, A. (2014) p53 suppression partially rescues the mutant phenotype in mouse models of DiGeorge syndrome. *Proc. Natl. Acad. Sci. U. S. A*, **111**, 13385–13390.
6. Voigt, J.H., Bienfait, B., Wang, S. and Nicklaus, M.C. (2001) Comparison of the NCI open database with seven large chemical structural databases. *J. Chem. Inf. Comput. Sci.*, **41**, 702–712.
7. Mueller, I., Kobayashi, R., Nakajima, T., Ishii, M. and Ogawa, K. (2010) Effective and steady differentiation of a clonal derivative of P19CL6 embryonal carcinoma cell line into beating cardiomyocytes. *J. Biomed. Biotechnol.*, **2010**, 380561.
8. Yamagishi, H., Maeda, J., Hu, T., McAnally, J., Conway, S.J., Kume, T., Meyers, E.N., Yamagishi, C. and Srivastava, D. (2003) Tbx1 is regulated by tissue-specific forkhead proteins through a common Sonic hedgehog-responsive enhancer. *Genes Dev.*, **17**, 269–281.

9. Brown, C.B., Wenning, J.M., Lu, M.M., Epstein, D.J., Meyers, E.N. and Epstein, J.A. (2004) Cre-mediated excision of Fgf8 in the Tbx1 expression domain reveals a critical role for Fgf8 in cardiovascular development in the mouse. *Dev. Biol.*, **267**, 190–202.
10. Zhang, Z. and Baldini, A. (2010) Manipulation of endogenous regulatory elements and transgenic analyses of the Tbx1 gene. *Mamm. Genome*, **21**, 556–564.
11. Lindsay, E.A., Vitelli, F., Su, H., Morishima, M., Huynh, T., Pramparo, T., Jurecic, V., Ogunrinu, G., Sutherland, H.F., Scambler, P.J., *et al.* (2001) Tbx1 haploinsufficieny in the DiGeorge syndrome region causes aortic arch defects in mice. *Nature*, **410**, 97–101.
12. Mutti, E., Lildballe, D.L., Kristensen, L., Birn, H. and Nexo, E. (2013) Vitamin B_{12} dependent changes in mouse spinal cord expression of vitamin B_{12} related proteins and the epidermal growth factor system. *Brain Res.*, **1503**, 1–6.
13. Vitelli, F., Taddei, I., Morishima, M., Meyers, E.N., Lindsay, E.A. and Baldini, A. (2002) A genetic link between Tbx1 and Fibroblast Growth Factor signaling. *Development*, **129**, 4605–4611.
14. Castellanos, R., Xie, Q., Zheng, D., Cvekl, A. and Morrow, B.E. (2014) Mammalian TBX1 preferentially binds and regulates downstream targets via a tandem T-site repeat. *PloS One*, **9**, e95151.
15. Merscher, S., Funke, B., Epstein, J.A., Heyer, J., Puech, A., Min Lu, M.M., Xavier, R.J., Demay, M.B., Russell, R.G., Factor, S., *et al.* (2001) TBX1 Is Responsible for Cardiovascular Defects in Velo-Cardio-Facial/DiGeorge Syndrome. *Cell*, **104**, 619–629.
16. Jerome, L.A. and Papaioannou, V.E. (2001) DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. *Nat. Genet.*, **27**, 286–291.
17. Zhang, Z. and Baldini, A. (2008) In vivo response to high-resolution variation of Tbx1 mRNA dosage. *Hum. Mol. Genet*, **17**, 150–157.
18. Liao, J., Kochilas, L., Nowotschin, S., Arnold, J.S., Aggarwal, V.S., Epstein, J.A., Brown, M.C., Adams, J. and Morrow, B.E. (2004) Full spectrum of malformations in velo-cardio-facial syndrome/DiGeorge syndrome mouse models by altering Tbx1 dosage. *Hum. Mol. Genet.*, **13**, 1577–1585.
19. Shane, B. and Stokstad, E.L. (1985) Vitamin B12-folate interrelationships. *Annu. Rev. Nutr.*, **5**, 115–141.
20. Guéant, J.L., Caillerez-Fofou, M., Battaglia-Hsu, S., Alberto, J.M., Freund, J.N., Dulluc, I., Adjalla, C., Maury, F., Merle, C., Nicolas, J.P., *et al.* (2013) Molecular and cellular effects of vitamin B12 in brain, myocardium and liver through its role as co-factor of methionine synthase. *Biochimie*, **95**, 1033–1040.
21. Mentch, S.J., Mehrmohamadi, M., Huang, L., Liu, X., Gupta, D., Mattocks, D., Gómez Padilla, P., Ables, G., Bamman, M.M., Thalacker-Mercer, A.E., *et al.* (2015) Histone Methylation Dynamics and Gene Regulation Occur through the Sensing of One-Carbon Metabolism. *Cell Metab.*, **22**, 861–873.
22. Guo, T., Chung, J.H., Wang, T., McDonald-McGinn, D.M., Kates, W.R., Hawuła, W., Coleman, K., Zackai, E., Emanuel, B.S. and Morrow, B.E. (2015) Histone Modifier Genes Alter Conotruncal Heart Phenotypes in 22q11.2 Deletion Syndrome. *Am. J. Hum. Genet.*, **97**, 869–877.