Setd5 is required in cardiopharyngeal mesoderm for heart development and its haploinsufficiency is associated with outflow tract defects in mouse

Michelle Yu-Qing Cheung1 | Catherine Roberts1,2 | Peter Scambler1
Athanasia Stathopoulou1

1Developmental Biology and Cancer, University College London Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, United Kingdom
2Institute of Medical and Biomedical Education, St. George’s, University of London, Cranmer Terrace, London, SW17 0RE, United Kingdom

Summary
Congenital heart defects are a feature of several genetic haploinsufficiency syndromes, often involving transcriptional regulators. One property of haploinsufficient genes is their propensity for network interactions at the gene or protein level. In this article we took advantage of an online dataset of high throughput screening of mutations that are embryonic lethal in mice. Our aim was to identify new genes where the loss of function caused cardiovascular phenotypes resembling the 22q11.2 deletion syndrome models, that is, heterozygous and homozygous loss of Tbx1. One gene with a potentially haploinsufficient phenotype was identified, Setd5, thought to be involved in chromatin modification. We found murine Setd5 haploinsufficiency to be associated with double outlet right ventricle and perimembranous ventricular septal defect, although no genetic interaction with Tbx1 was detected. Conditional mutagenesis revealed that Setd5 was required in cardiopharyngeal mesoderm for progression of the heart tube through the ballooning stage to create a four-chambered heart.

KEYWORDS
birth defects, early development, heart, mesoderm

1 INTRODUCTION
Syndromic congenital heart disease (CHD) encompasses several haploinsufficiency conditions. Haploinsufficient genes may be present at a “hub” of interacting networks (Huang, Lee, Marcotte, & Hurles, 2010), and phenotypic similarity has been used in human genetics to cluster syndromes based on the premise that these groupings inform biological relationships of the genes involved that is, the underlying genes may function within the same developmental “module” (Oti & Brunner, 2007). The candidate gene for 22q11.2 deletion syndrome (22q11DS) is Tbx1, and is haploinsufficient in mice (Jerome & Papaioannou, 2001; Merscher et al., 2001). In mice, Tbx1 heterozygotes present with hypo- or aplasia of the fourth PAA at embryonic day (E)10.5 which gives rise to great vessel defects such as interrupted aortic arch type B and aberrant right subclavian artery at later stages. Other cardiovascular defects include double outlet right ventricle (DORV) and ventricular septal defect (VSD) (Lindsay et al., 2001; Merscher et al., 2001).

An initiative entitled Deciphering the Mechanisms of Developmental Disorders (DMDD) aimed to take advantage of embryonic lethal mouse lines containing insertions that initially offer a gene-
that define the

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determination, this converts to a conditional allele offering the potential for
tissue-specific studies of heart and brain development. Thus, the
DMDD browser provides an online database of mutants where
standard phenotype ontology terms can be used to search for genes
with phenotypes related to known birth defect syndromes. The
database contains high-resolution episcopic microscopic (HREM)
images of embryos at E9.5 and E14.5 allowing local rephenotyping.
The database contained 209 genes analysed at E9.5 and E14.5.
Querying the online browser with key phenotype ontology terms
that define the Tbx1 haploinsufficient phenotype such as “DORV”
and “VSD” generated a list of genes that caused these defects
when mutated. Out of the 209 total genes, a mutation in only a single
gene caused DORV and VSD when heterozygous: Setd5 (MGI allele: 4432631). We concluded Setd5 was a strong candidate for
causing haploinsufficient cardiac defects that are similar to those
observed due to Tbx1 haploinsufficiency. Moreover, there was a
severe loss of function, lethal phenotype in Setd5 nulls (https://
dmddd.org.uk/).

Setd5, a ubiquitously expressed gene (Osipovich, Gangula, Vianna, &
Magnuson, 2016), is thought to act as a histone modifier. Unlike other
SET-domain proteins, SETD5 appears to lack histone methyltransferase
activity (Deliu et al., 2018; Osipovich et al., 2016). Immunoprecipitation
and mass spectrometry studies indicate SETD5 associated with HDAC3,
a protein complex containing histone-deacetylating activity; however,
there is conflicting evidence on whether SETD5 is required for
HDAC3 recruitment (Deliu et al., 2018; Nakagawa et al., 2020; Osipovich et al., 2016). Clinical reports reveal the implications of
SETD5 haploinsufficiency in human intellectual disability, with some
patients presenting with cardiac abnormalities (Fernandes et al., 2018;
Grozeva et al., 2014; Szczaluba et al., 2016). Furthermore, CHD is
present in up to 50% of patients with deleterious mutations in chromatin
modification proteins underlying neurodevelopmental or
psychiatric syndromes (Zaidi & Brueckner, 2017). Despite this, the
role of Setd5 in cardiac development has not been extensively
investigated.

Osipovich et al showed that mice lacking Setd5 (MGI allele: 5576778) die before E10.5 and were developmentally delayed; embryos
were severely underdeveloped with many presenting with hemorrhaging compared to littermate heterozygous controls
(Osipovich et al., 2016). Interestingly, unlike the heterozygous
embryos presented in the DMDD browser, Osipovich et al
reported heterozygous mice as viable and indistinguishable from
the wild-type embryos. Furthermore, the embryonic lethality of
Setd5 nulls by E10.5 limits the full assessment of the cardiac
phenotype. The purpose of our study was, primarily, to more fully
delineate the cardiac phenotype caused by the disruption of
Setd5 expression, and secondarily to explore whether there was
any genetic interaction between Setd5 and Tbx1. Currently,

2.2 | Tissue processing, whole-mount analysis, and histological analysis

Whole embryos were fixed in 4% paraformaldehyde overnight at 4°C
and then stored in PBS until required. For histological analyzes, whole
embryos or hearts were dehydrated in ethanol, and then processed in
Histoclear and several washes in wax. Samples were embedded in paraffin
and cut into 10 μm sections, air-dried overnight and then
rehydrated through a decreasing gradient of ethanol and stained with
hematoxylin and eosin. Whole-mount embryo pictures were taken
using the Zeiss Lumar V12 Stereoscope. Histological sections were
imaged using the Zeiss Axioplan or the slide scanning facility at UCL IQPath.
Stage-matching was performed by assessment of the limb
buds and pharyngeal arches (Boehm et al., 2011; Musy et al., 2018).

2.3 | Calculation of double heterozygote expected frequencies

In the absence of a synergistic genetic interaction, the expected frequency
of defects in a double heterozygote in which two genes of
interest are haploinsufficient is calculated as follows:

\[ (A + B(1 - A))^n \]

A = percentage of defects observed in one single heterozygote.
B = percentage of defects observed in alternate single heterozygote.
n = total number of double heterozygotes collected.

2.1 | Mouse strains, breeding, and genotyping

All animal husbandry, maintenance, and procedures were carried out
in accordance to the UK Home Office regulations. All mice were
maintained on the C57Bl/6 background.

The Setd5<sup>Frt</sup> line was derived from the targeted mouse line
(Setd5tm1a[EUCOMM]Wtsi MGI:4432631) (Wellcome Trust Sanger
Institute) and kindly donated by the Basson laboratory (KCL).
Setd5 heterozygotes were generated by crossing the floxed line with mice expressing Actin-
Cre (Lewandoski, Meyers, & Martin, 1997). F1 mice harboring the Actin-Cre
were backcrossed with wild-type mice in order to generate mice without the
Actin-Cre for use in downstream experiments. Generation of double heterozygotes
involved breeding Setd5<sup>Frt</sup> heterozygotes, described previously (Lindsay et al., 2001). To generate Tbx1-cKOs, Setd5<sup>Frt</sup> mice were
crossed with Tbx1<sup>Frt</sup> heterozygous mice, described previously (Huynh, Chen, Terrell, & Baldini, 2007). To delete Setd5 in the cardiopharyngeal
mesoderm, Setd5<sup>Frt</sup> mice were bred with mice expressing Mesp1-Cre, described previously (Saga et al., 1999). Timed matings for embryo collection
involved pairing sexually mature mice overnight and checking the presence of
a copulation plug the following morning, which indicated 0.5 days post
copulation. The PCR strategies for genotyping are available upon request.

Materials and methods

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involved pairing sexually mature mice overnight and checking the presence of
a copulation plug the following morning, which indicated 0.5 days post
copulation. The PCR strategies for genotyping are available upon request.
Taking the example of VSDs presented in Table 1, \( A = 75\% = 0.75; B = 31\% = 0.31; n = 14 \).

\[(0.75 + 0.31[1-0.75])^* 14 = 12.\]

### 2.4 Statistical analysis

To determine whether genetically modified mice or embryos presented at Mendelian ratios, Chi-squared analysis was performed. To determine the association of genotype with the frequency of defects observed, Fisher’s exact test was performed. A \( p \) value less than .05 was taken to be significant.

### 3 RESULTS

#### 3.1 Setd5 haploinsufficiency leads to outflow tract rotational defects and VSDs

Setd5^{Fl/Fl} mice were derived from the targeted mouse line (Setd5^{tm1a [EUCOMM][Wtsi]}). The floxed line was then bred with Tmem163^{+/-}[ACTB-cre]^2Mrt (Actin-Cre) mice in order to generate Setd5 heterozygotes (Setd5^+/−) for use in downstream embryo experiments. At E14.5, normal heart development results in the septation of the outflow tract (OFT) into the pulmonary trunk and aorta, with an intact interventricular septum dividing the left and right ventricles. A rotational defect, such as DORV or overriding aorta, occurs when the OFT fails to align with the two future ventricles at the looping stage. Correct looping of the heart tube is necessary to align the two definitive outflow vessels with their corresponding ventricular chambers (Christoffels et al., 2000; Moorman & Christoffels, 2003). If the OFT is misaligned such that an abnormal amount of aortic blood originates from the right ventricle, then this is known as an overriding aorta. If more than 50% of the aortic blood arises from the right ventricle, then this is known as an overriding aorta. We found that wild-type hearts displayed correctly OFT septation with the outflow vessels originating from separate ventricles (Figure 1a-a”). In contrast, despite correct OFT septation, 50% of Setd5 heterozygotes presented with rotational defects which included DORV or overriding aorta (Figure 1b-b”) (\( p < .05 \), Fisher’s exact test, Table 1). DORV is always accompanied by a VSD, since the fusion of the interventricular septum with the OFT cushions is necessary to complete ventricular septation. If the outflow vessels of the OFT are misaligned with the ventricular chambers, as in DORV, then this fusion cannot take place which leads to a perimembranous VSD (Anderson, 2003; Lin, Lin, Chen, Zhou, & Chang, 2012). Figure 1b” shows a perimembranous VSD, which was observed in 75% of Setd5 heterozygotes (\( p < .05 \), Fisher’s exact test, Table 1).

Therefore, in alignment with the DMDD browser, our results show that Setd5 haploinsufficiency causes OFT rotation defects and VSDs.

#### 3.2 No detectable genetic interaction between Setd5 and Tbx1, a well-established haploinsufficient gene

Because some of the phenotypes observed in Setd5 heterozygotes overlap with those observed in Tbx1 heterozygotes, the genetic interaction between Setd5 and Tbx1 was investigated by generating double heterozygotes, in which both genes of interest are haploinsufficient. Setd5 heterozygotes were mated with Tbx1^{lacZ/−} heterozygotes (Tbx1^{tm1Bld}) and embryos were harvested at E14.5. An exacerbation of defects, that is, an increased frequency of defects over that expected by additive effects, or an increase in severity of defects, would be observed in the double heterozygotes in the case of synergistic genetic interaction.

There was no difference in the number of embryos at E14.5 based on the genotypes (Table S1, Chi-squared, \( p > .05 \)). Gross morphological analysis during embryo dissection and internal assessment by histological methods revealed no exacerbation or increased frequency of defects in double heterozygotes, suggesting that there was no detectable synergistic genetic interaction between Setd5 and Tbx1 and that these two genes are not functionally interdependent (Table 1).

### Table 1: Summary of heart defects observed in WT, Setd5 heterozygotes (Setd5^{+/−}), Tbx1 heterozygotes (Tbx1^{lacZ/-}) and double heterozygotes (Setd5^{+/−}; Tbx1^{lacZ/-}) at E14.5

|                         | WT(n = 8) | Setd5^{+/−}(n = 12) | Tbx1^{lacZ/-}(n = 13) | Setd5^{+/−}; Tbx1^{lacZ/-}(n = 14) |
|-------------------------|----------|---------------------|-----------------------|-----------------------------------|
|                         |          | Observed            | Expected n number     |                                   |
| CAT                     | 0        | 0                   | 0                     | 0                                 |
| Rotational defects      | 0        | 6 (50%)*            | 1 (8%)                | 8 (57%)                           |
| VSD                     | 1 (13%)  | 9 (75%)*            | 4 (31%)               | 12 (86%)                          |
| Great vessel defects    |          |                     |                       |                                   |
| AbRS                    | 0        | 0                   | 1 (8%)                | 3 (21%)                           |

Note: A Fisher’s exact test was performed to determine whether the defects observed in Setd5^{+/−} were statistically significant compared to wild-type control embryos (\( * p < .05 \) compared with WT). The expected frequency of defects in double heterozygotes (Setd5^{+/−}; Tbx1^{lacZ/-}) was calculated as described in the methods. Rotational defects encompass both double outlet right ventricle and overriding aorta.

Abbreviations: AbRS, aberrant right subclavian artery; CAT, common arterial trunk; VSD, ventricular septal defect.
3.3 | Conditional mutagenesis of Setd5 reveals a role in cardiogenic mesoderm

Our results show that the haploinsufficiency of Setd5 led to rotational defects and VSD. These defects are reminiscent of the second heart field (SHF)-specific defects seen in Tbx1 mutants. To investigate whether Setd5 has a specific role in the SHF, and/or pharyngeal epithelia, Setd5 was deleted using a Tbx1-Cre driver known to be active in these tissues (Huynh et al., 2007). Figure 2 presents the gross morphology of control and several Tbx1 conditional knockout (Tbx1-cKO)
hearts at E15.5. Minor abnormalities such as retroesophageal right subclavian artery were observed (one in both Tbx1 conditional heterozygote and knockout); this was likely due to the effect of the Cre knock-in to the Tbx1 locus. Similarly, assessment of internal heart morphology revealed no obvious differences between the genotypes (Figure S1). Therefore, we concluded Setd5 likely exerts its role earlier during cardiac development, or in other lineages, or both.

Indeed, the DMDD browser revealed an embryonic lethality of homozygous Setd5 mutation (no surviving embryos at E14.5), and Osipovich et al., showed that mice constitutively null for Setd5 die before E10.5, and are severely underdeveloped. Hearts at E9.5 were swollen with a single ventricular chamber (Osipovich et al., 2016). The embryonic lethality of Setd5 constitutive null embryos by E10.5 limits analysis of the importance of Setd5 in cardiac development. Lineage tracing experiments show that the majority of cardiac cells are derived from Mesp1-expressing progenitor cells (Saga et al., 1999). Therefore, we used the Mesp1-Cre driver to delete Setd5 in mesoderm encompassing both the first heart field (FHF) and SHF, as well as the

**Figure 2**  No obvious heart malformations were observed when Setd5 was homozygously deleted using the Tbx1-Cre at E15.5. No great vessel defects were observed across the genotypes (n = 19 conditional mutants analysed). AA, aortic arch; LA, left atria; LCC, left common carotid; LSA, left subclavian artery; LV, left ventricle; RA, right atria; RCC, right common carotid; RSA, right subclavian artery; RV, right ventricle; T, thymus. Scale bars = 1 mm
pharyngeal mesoderm through which the (non-Mesp1-expressing) neural crest migrates. Homozygous deletion in this cardiopharyngeal mesoderm lineage led to embryonic lethality before E12.5. At E10.5, Mesp1-cKO embryos were harvested at the expected Mendelian ratio (Table S2) but exhibited varying degrees of abnormal cardiac morphogenesis compared to stage-matched control embryos (p < .05, Table 2). 90% of Mesp1-cKOs displayed abnormal cardiac chamber ballooning, with 25% of these exhibiting a dumbbell-shaped heart (Figure 3b), while others displayed right ventricular hypoplasia and abnormal atrial ballooning (p < .05, Figure 3d). We assessed the internal heart morphology by histological techniques and found that 100% of Mesp1-cKO embryos presented with a short OFT, indistinct ventricular chambers, and poorly developed atria, revealing the importance of Setd5 in OFT elongation and cardiac chamber ballooning (Figure 4). These defects were accompanied by a statistically significant growth delay, determined by the crown-to-rump length (p < .05, Figure 5). The OFT length was further assessed by quantifying the number of paraffin sections containing the OFT and expressing this as a ratio to the crown-to-rump length, confirming the shorter OFT in Mesp1-cKO compared to control embryos (Figure 5). Morphological analysis of Mesp1-Cre conditional heterozygotes (n = 6) did not reveal any OFT phenotype, and histological analysis revealed only a single embryo as having poorly developed cardiac chambers.

4 | DISCUSSION

Our results indicate that haploinsufficiency of Setd5, a gene implicated in human intellectual disability (Fernandes et al., 2018; Grozeva et al., 2014; Szczaluba et al., 2016) with reported histone-modifying activity (Deliu et al., 2018; Nakagawa et al., 2020; Osipovich et al., 2016), leads to cardiac defects in mice. It is worth noting that only one Mesp1-Cre conditional heterozygote presented with poorly developed cardiac chambers. The remaining embryos exhibited normal cardiac morphology, highlighting the fact that deleting one allele of Setd5 in the Mesp1 lineage does not fully recapitulate the global heterozygotes and that haploinsufficiency of Setd5 requires constitutive heterozygosity. Since haploinsufficient genes exist in hubs or networks and are more likely to interact with other haploinsufficient genes (Huang et al., 2010), and because haploinsufficiency of Setd5, and of Tbx1 lead to similar cardiac defects, one of our initial aims were to investigate their genetic interaction. However, our findings did not reveal a detectable synergistic genetic interaction, suggesting that Setd5 and Tbx1 are not functionally interdependent. Nevertheless, homozygous deletion of Setd5 in the cardiopharyngeal mesoderm revealed a novel role of Setd5 in cardiac development: its importance in OFT elongation and cardiac chamber ballooning.

In humans, loss of function mutations in SETD5 lead to moderate or severe intellectual disability (Fernandes et al., 2018; Grozeva et al., 2014; Powis et al., 2018; Rawlins, Stals, Eason, & Turnpenny, 2017; Szczaluba et al., 2016). It is also one of three genes within the candidate region of 3p25 microdeletion syndrome which is characterized by developmental delay, intellectual disability, low birth weight, microcephaly and craniofacial abnormalities, and some observations of CHD (Grozeva et al., 2014; Kuechler et al., 2015; Szczaluba et al., 2016). Mutations of SETD5 have been reported in other conditions. For instance, one deletion and two mutations were described in patients clinically diagnosed with KBG syndrome without a mutation in ANKRD11, which is usually observed in these cases (OMIM 148050). One such patient presented with mitral stenosis (Crippa et al., 2020). Interestingly, ANKRD11 recruits HDACs and is mutated in autism (Gallagher et al., 2015). Recently, SETD5 has been implicated in moyamoya angiopathy, which is the occlusion of large cerebral arteries leading to childhood stroke (Pinard et al., 2020). Our results

| Table 2 | Summary of defects observed in Setd5Fl/FI, Setd5Fl/WT, Setd5Fl/WT; Mesp1Cre/+, and Setd5Fl/FI; Mesp1Cre/+ embryos at E10.5 |
| Abnormal external morphology | Setd5Fl/FI (n = 17) | Setd5Fl/WT (n = 23) | Setd5Fl/WT; Mesp1Cre/+ (n = 17) | Setd5Fl/FI; Mesp1Cre/+ (n = 19) |
| Hemorrhage | 0 | 0 | 0 | 3 (16%) |
| Pericardial effusion* | 0 | 3 (13%) | 0 | 14 (74%) |
| Abnormal cardiac chamber ballooning* | 0 | 0 | 0 | 18 (95%) |

| H&E analysis | Setd5Fl/FI (n = 2) | Setd5Fl/WT (n = 4) | Setd5Fl/WT; Mesp1Cre/+ (n = 6) | Setd5Fl/FI; Mesp1Cre/+ (n = 6) |
| Short OFT* | 0 | 0 | 0 | 6 (100%) |
| Abnormal ventricular ballooning* | 0 | 0 | 1 (17%) | 6 (100%) |
| Abnormal atrial ballooning* | 0 | 0 | 0 | 6 (100%) |

Note: The upper part of the table describes the defects observed during the dissection stage. The abnormal external cardiac morphology was further delineated by sectioning and H&E analysis. Fisher’s exact test was used to determine the association between the genotype and the frequency of any defects observed.

*p < .05.
reveal that Setd5, a gene implicated in neurodevelopmental disorders, is also important in cardiac development, and may provide some explanation to the clinical observations of CHD when SETD5 is haploinsufficient in humans. Taken together, these data suggest considerable pleiotropy of SETD5 function.

The genetic underpinnings of CHD remain unknown in over 50% of cases, highlighting the importance of identifying novel genes that may be involved in orchestrating cardiac development. Furthermore, genome sequencing CHD studies present an overrepresentation of genes involved in neurodevelopmental disorder (Sigmon, Kelleman,
Susi, Nylund, & Oster, 2019; Watkins et al., 2019). As such, screening for mutations in \textit{SETD5} during genetic testing could potentially provide some knowledge to families to better understand if there could be recurrent risks of CHD to other family members, or provide information for neurodevelopmental outcome.

Thus, our results provide the basis for further exploration of \textit{Setd5} in cardiac development. It will be interesting to investigate how this histone modifier plays a role in orchestrating cardiac development in conjunction with other known cardiac-relevant genes. \textit{Setd5} can now be placed within a growing number of genes encoding chromatin modifiers whose alleles are associated with both neurodevelopmental defects on the one hand, and heart dysmorphogenesis on the other.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Michelle Yu-Qing Cheung https://orcid.org/0000-0002-6387-347X
Catherine Roberts https://orcid.org/0000-0001-5568-8752
Peter Scambler https://orcid.org/0000-0002-1487-4628
Athanasia Stathopoulou https://orcid.org/0000-0001-8579-7706

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