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# A novel cluster of putative regulatory sequences modulates Tbx1 gene expression

Background: Tbx1 function is involved in cardiac and pharyngeal development. Information regarding the genetic elements and molecular mechanisms that regulate the gene expression is still incomplete. We used in vitro differentiation, single cell biology, and bioinformatic tools to identify and validate regulatory elements of the gene.

Material and methods: We employed simultaneous single cells RNA-seq and ATAC-seq data from mouse ES cells (mESCs) differentiating into precardiac organoids; on these, we correlated chromatin accessibility and Tbx1 gene expression in distinct cell clusters and identified differentially accessible regions. We applied a machine-learning approach to score the probability of being enhancers using logistic regression. Finally, we manipulated putative enhancers by CRISPR-Cas9 to test their requirement for Tbx1 gene expression.

Results and Conclusion: We identified 14 putative regulatory sequences (PRS) on the Tbx1 locus, through integration of scRNAseq with scATACseq datasets. Using ATAC datasets in public repositories, we confirmed that ATAC peaks corresponding to the PRSs were also present in mouse embryo tissues. We focused on a cluster that includes 3 PRSs, named PRS10, 11, and 12, located approx. 10Kb upstream of the gene. PRS10 and PRS12 had a positive predictive score, while PRS11 had a lower score. With CRISPR-Cas9 technology, we generated mESC lines deleted for the entire cluster and we also deleted the 3 PRSs individually. We then differentiated the engineered clones into precardiac organoids to test the Tbx1 gene expression profile. Loss of the entire cluster and of PRS10 and PRS12 individually, led to strong, significant reduction of Tbx1 expression compared to the parental WT line. These results demonstrate that the enhancer cluster is required for Tbx1 gene regulation. Gene expression analyses of clones lacking only PRS11 gave variable results, but for the majority of clones a reduction in Tbx1 expression was observed. In order to determine whether these PRSs are cell-type specific, we performed a scRNA-seq experiments on precardiac organoids derived from deletions of individual PRSs. Preliminary analysis shows that Tbx1 expression is mostly in a distinct cell cluster and shows a significant reduction of Tbx1 expression in cells deleted for PRS10 and PRS12 and a significant increase in PRS11, compared to WT. These results suggest that the enhancer cluster that we have identified has both positive and negative regulatory sequences of the Tbx1 gene.

## Department

Depatment of Molecular Medicine and Medical Biotechnology, University Federico II, Naples, Italy

**Primary author(s) :** ALLEGRETTI, Sara (PhD program in Molecular Medicine and Medical Biotechnology, University Federico II, Naples, Italy)

**Co-author(s) :** LANZETTA, Olga (Institute of Genetics and Biophysics, National Research Council, Naples, Italy); FERRENTINO, Rosa (Institute of Genetics and Biophysics, National Research Council, Naples, Italy); AURIGEMMA, Ilaria (Department of Chemistry e Biology, University of Salerno, Fisciano, Italy); BALDINI, Antonio (PhD program in Molecular Medicine and Medical Biotechnology, University Federico II, Naples, Italy)

**Presenter(s) :** ALLEGRETTI, Sara (PhD program in Molecular Medicine and Medical Biotechnology, University Federico II, Naples, Italy)