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Supplemental Information

VE-CADHERIN is expressed transiently in early ISL1^{+} cardiovascular progenitor cells and facilitates cardiac differentiation

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SUPPLEMENTAL INFORMATION

Experimental Procedures

Dissection of mouse embryos

This study was carried out housing facilities at the University of Ioannina. The experimental procedures followed the guiding principles of the Declaration of Helsinki, regarding ethical conduct of animal research. Embryos were collected from timed pregnancies. The day of a vaginal plug was considered as embryonic day 0.5 (E0.5). Embryos were dissected from females at various stages of pregnancy (E7.5-E11.5) and placed in cold PBS and fixed in 4% formaldehyde.

Immunocytochemistry

EBs were allowed to attach in gelatinized glass coverslips for 1-2 days and fixed in 4% formaldehyde for 10min at RT. For staining, EBs were incubated with 3% BSA containing 0.2% Triton-X 100 for 30min, and primary antibody labelling was performed at 4^0C O/N, followed by incubation with the secondary antibody for 1h. For whole-mount immunofluorescence, the procedure was carried out in 24-wells with the exception that incubation with BSA solution was 1-2h. For EBs mild dissociation, they were first treated with 0.25% trypsin-EDTA for 1-3min. Small cell clusters or single cells were subsequently allowed to adhere on plastic or glass chamber slides (Lab Tek, Permanox slides, Nunc) coated with fibronectin. Mouse embryos were fixed in 4% formaldehyde for 2h and then 30% sucrose over-night, and then embedded in OCT, sectioned, and stained using standard protocols. In brief, frozen tissue sections were permeabilized with 100% ice-cold methanol for 10min at –20°C and rinsed in PBS for 5min. Antibody labelling was carried out as above, with the exception that primary antibody was diluted in 0.2% fish skin gelatin and labeling was performed for 1h at RT. Whole mount immunostaining in embryos was performed according to

Abcam protocol. After fixation, clearing protocol was performed according to (Susaki et al., 2014; Tsata et al., 2020).

Flow cytometry

For Flow Cytometry EBs were harvested and washed twice with PBS. Then, they were treated with dissociation buffer (Gibco dissociation buffer, Cat. no. 13151014) for 3min at 37^{o}C. Single cell suspensions were prepared by gentle pipetting and resuspended in 10 ml of FACS buffer (5% FBS in PBS) followed by centrifugation at 300g for 5min, RT. Cells were stained with fixable live dead kit (Invitrogen, Cat. no. L34957) according to the manufacturer's instructions. Then, cells were washed with 10 ml of FACS buffer. Cells were stained for cell surface antigens using fluorochrome conjugated antibodies: PE anti-PDGFR alpha antibody (APA5, Abcam, ab93531), APC anti-mouse VEGFR2, FLK1 (clone 89B3A5, BioLegend), PE-Cyanine7 anti-mouse VE- CADHERIN monoclonal (clone BV13, BioLegend), anti-mouse ISL1 (clone 40.2D6, DSHB) and PE anti-mouse N- CADHERIN (130-116-273, Miltenyi Biotec), for 30min, RT, in a final volume of 100ul in FACS buffer. Following staining, cells were washed and fixed with 3.7% formaldehyde for 15min, RT. After fixation, cells were washed with FACS buffer and centrifuged at 500g for 5min. Cells were then permeabilized in 0.2% TX100-FACS buffer solution for 15min. Permeabilized cells were pelleted at 500g for 5min, and then intracellular staining was performed using ISL1 antibody (clone 40.2D6, DSHB) for 30min in permeabilization buffer, RT. Cells were washed with permeabilization buffer and stained with secondary fluorochrome conjugated antibody Alexa Flour 488 for 30 min, RT. Finally, stained cells were washed with FACS buffer and resuspended in 500ul for acquisition.

Antibodies

Primary antibodies: Rat monoclonals against VE- CADHERIN (1:50, clone 11D4.1, BD Biosciences), PECAM-1 (1:30, MEC 13.3, Santa Cruz), and E- CADHERIN (1:50, DECMA-1, Santa Cruz). Mouse monoclonals against E- CADHERIN (1:200 BD Bioscience, 610181) cardiac Troponin T (CTNT) (1:50, CT3, Iowa Hybridoma Bank),

ISL1 (1:1000, 39.4D5 and 40.2D6 Iowa Hybridoma Bank. N- CADHERIN (1:100, clone 3B9, Invitrogen) and Tbx5 (1:50, sc-515536 Santa Cruz). Goat polyclonals against GATA4 (1:100, C-20, Santa Cruz), BRACHYURY (1:100, sc-17745, Santa Cruz) and ISL1 (1:150, GT15051-100, Acris Antibodies). Rabbit monoclonals against MEF2c (1:400, D80C1), and VEGF receptor 2, FLK1 (1:100, clone 55B11, Cell Signaling Technology) and rabbit polyclonal anti-EGFP (kindly provided from Dr Boleti – Pasteur Institute, Athens).

Confocal Microscopy

Confocal images were taken in a (LCS SP5) Leica confocal microscope using the LAS AF Lite software. Pictures were further manipulated with Fiji (NIH Image) and/or Adobe Photoshop (Adobe) software.

Quantitative rt-PCR

Total RNA extraction was performed using NucleoSpin RNA Plus according to manufacturer's protocol (Macherey-Nagel). To synthesize cDNA 1μg of purified RNA was used in 20ul reaction, using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). Quantitative real time PCR analysis was performed with one twelfth or one-sixth of the cDNA reaction as template, using KAPA SYBR® FAST qPCR Kit Master Mix (Kappa) in a Bio-Rad CFX96 for 45 cycles. All samples were analyzed in triplicates.

Control of genomic contamination was measured for each sample by performing the same procedure with or without reverse transcriptase. All values were normalized with respect to GAPDH and β-actin expression levels, translated to relative values. Analysis was performed by Qbase+ V3.4 software. Primer sequences are shown in Supplementary Table S1.

Statistical analysis

Statistical Analysis was performed with GraphPad Prism 8 Software. qPCR data represents the mean ±SEM from three independent biological experiments The statistical significance of difference was determined

by two-way ANOVA with Dunnett's multiple comparisons as post hoc analysis (Fig. 3C) and with Sidak's multiple comparisons test (Fig. 4E).

For all the other graphs data represent mean ± SD. Specifically, the data from graphs in Fig. 4F and 4I were evaluated by Repeated-measures two-way ANOWA followed by Bonferroni's multiple comparisons as post hoc analysis. Ordinary one-way ANOWA with Dunnett's multiple comparisons test was used to determine significance of quantitative data in Fig. 4C and in Fig.4H one-way ANOVA followed by Tukey's Multiple Comparisons Test. Probability values P <0.05 were considered significant (****P< 0.0001, ***P<0.001, **P<0.001, *P<0.05).

Generation of genetically modified ESCs

Stable ES cell lines: $5x10^6$ ESCs were electroporated with 20µg of DNA in 600µl PBS at 200V and 960uF in a 0.4cm cuvette using BTX-ECM600 electroporator (Harvard Apparatus). After 24h and for the next 14 days, cells were selected with Hygromycin (150ug/ml to 120ug/ml). Resistant clones were either isolated and propagated individually or pooled and expanded.

Plasmids

Pvec: An ~2.5kb fragment containing mouse *ve-cadherin* promoter elements and the first non-translated exon was derived by PCR using primers AGCAGAAACAAGGTCCTCTGGAAGAG (sense), TCACTTACCTTGTCCGTGAGC (antisense) from a mouse BAC library as template, further subcloned in Topo-XL vector (Invitrogen). pCDNA3-ΔEXD-VEC was described in (Kouklis *et al.*, 2003)

pPvec-VEC-ΔEXD: the following subcloning steps were performed: Construct A: the chimeric gene and stuffer fragment of pPyCAGIP (an episomal vector, kind gift from Prof. A. Smith, Wellcome Trust Centre for Stem Cell Research, University of Cambridge, UK) was inserted in the Topo XL vector downstream of the mouse Pvec by SchI/EcoRI-blunt ligation. Construct B: the ΔEXD-VEC fragment from pCDNA3 was

excised and ligated to pPyCAGIP using BstXI. Construct C: the *ve-cadherin* promoter and the chimeric gene from construct A was excised and ligated to construct B in SpeI/XhoI (partial for construct B). Finally, pPvec-VEC-ΔEXD was obtained after insertion of the hygromycin-resistance gene, under *thymidine kinase* (TK) promoter from vector pCEP4 (Invitrogen), at the NruI/SalI sites of construct C at the opposite transcriptional orientation.

pPvec-mock: the SpeI/NotI fragment from construct A (containing the *ve-cadherin* promoter, the chimeric gene and the stuffer fragment) was ligated to pPyCAGIP and subsequently the hygromycin cassette (from pCEP4) was inserted at NruI/SalI at the opposite transcriptional orientation.

pPvec-EGFP: the SpeI/XhoI fragment from construct A was ligated to pPyCAGIP and EGFP coding sequence (from pEGFP-N) digested with Xho/NotI was ligated to the same sites. Finally, the hygromycin cassette excised from pPvec-VEC-ΔEXD by HindIII blunt/XhoI was inserted at NdeI blunt/SalI.

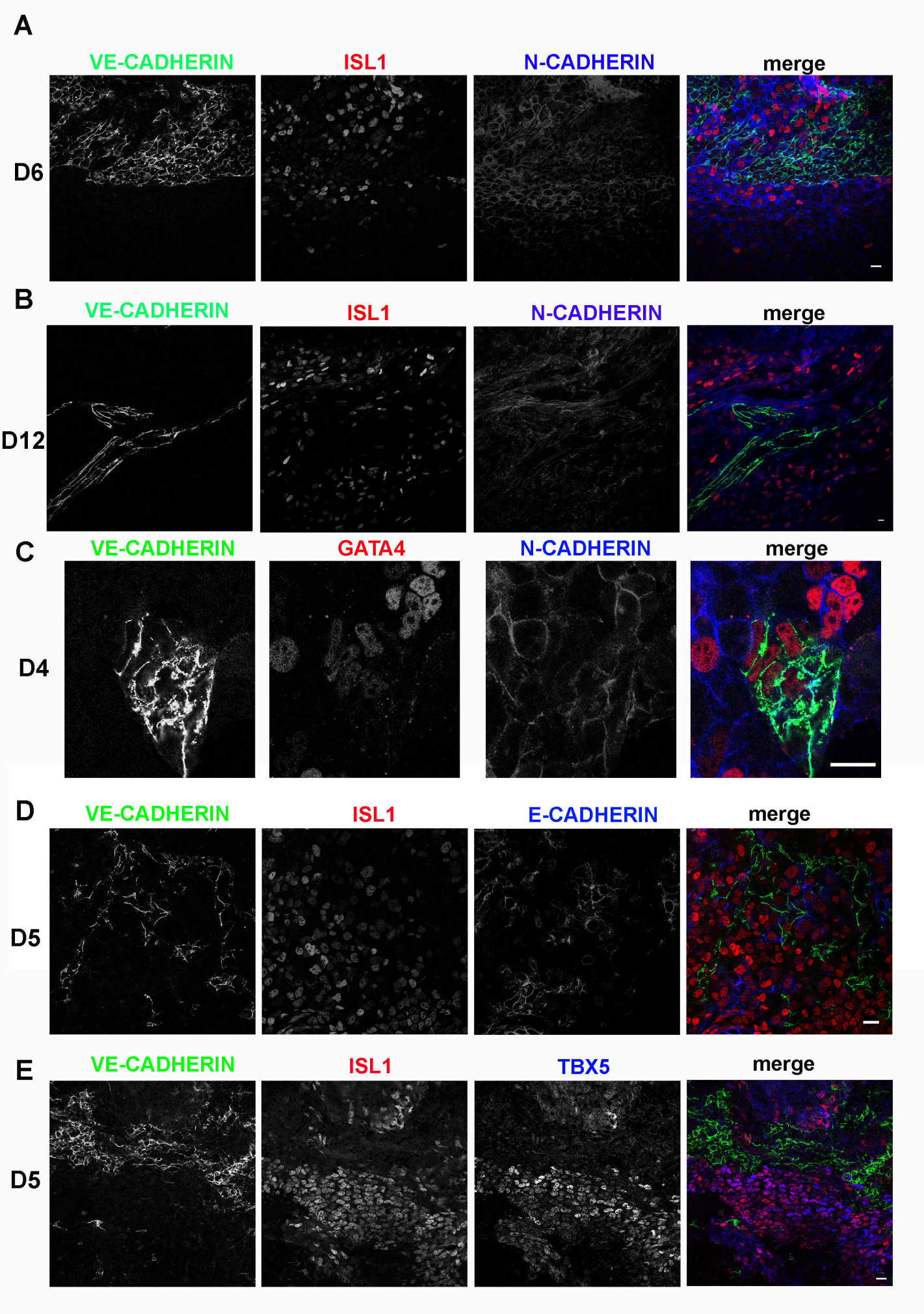
REFERENCES

Kouklis, P., Konstantoulaki, M., and Malik, A.B. (2003). VE-cadherin-induced Cdc42 signaling regulates formation of membrane protrusions in endothelial cells. J Biol Chem *278*, 16230-16236. 10.1074/jbc.M212591200

M212591200 [pii].

Susaki, E.A., Tainaka, K., Perrin, D., Kishino, F., Tawara, T., Watanabe, T.M., Yokoyama, C., Onoe, H., Eguchi, M., Yamaguchi, S., et al. (2014). Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell *157*, 726-739. 10.1016/j.cell.2014.03.042.

Tsata, V., Kroehne, V., Wehner, D., Rost, F., Lange, C., Hoppe, C., Kurth, T., Reinhardt, S., Petzold, A., Dahl, A., et al. (2020). Reactive oligodendrocyte progenitor cells (re-)myelinate the regenerating zebrafish spinal cord. Development *147*. 10.1242/dev.193946.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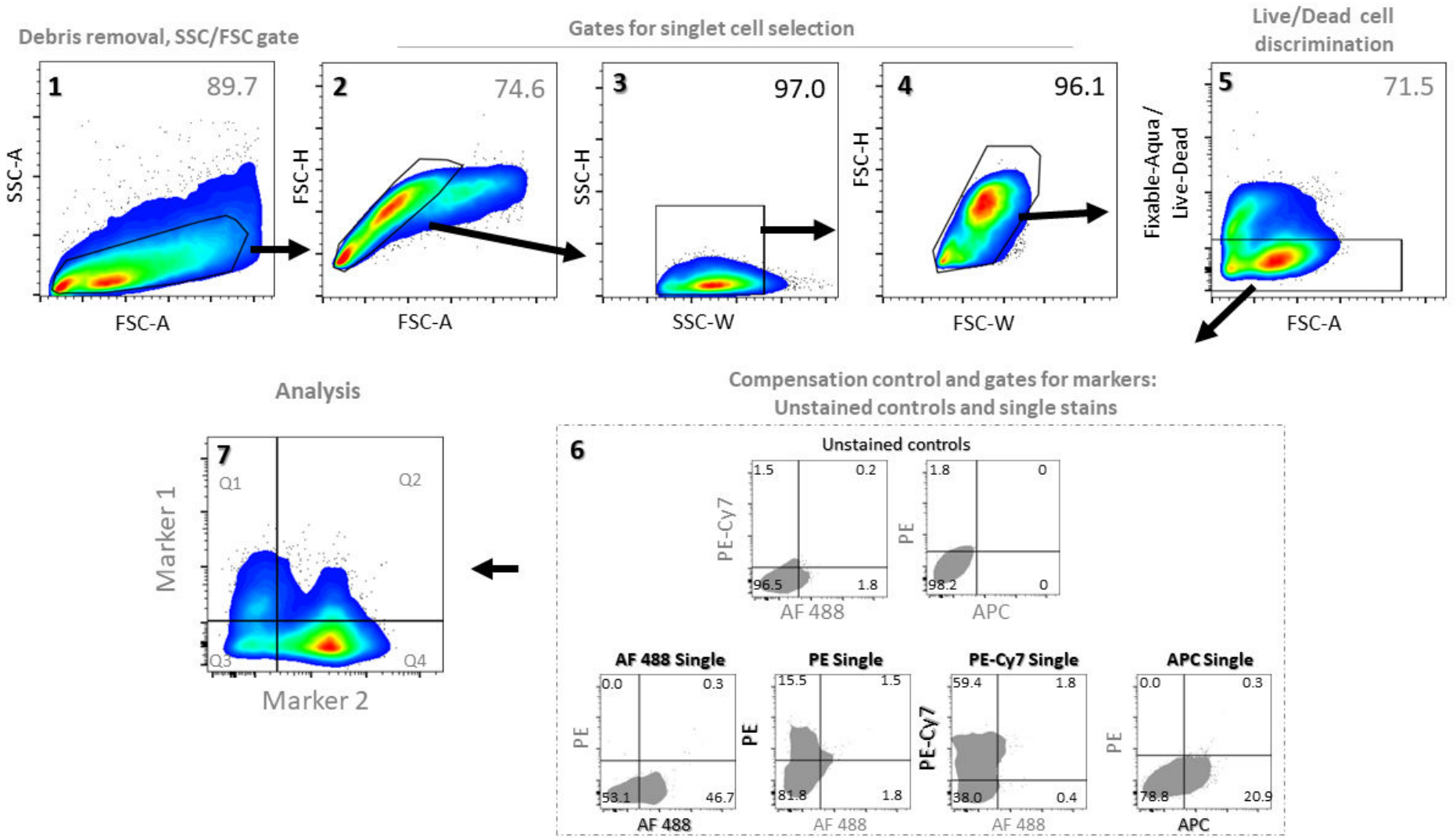
VE-CADHERIN
ISL1
N-CADHERIN
merge
D6
B
VE-CADHERIN
ISL1
N-CADHERIN
merge
D12
C
VE-CADHERIN
GATA4
N-CADHERIN
merge
D4
D
VE-CADHERIN
ISL1
E-CADHERIN
merge
D5
E
VE-CADHERIN
ISL1
TBX5
merge
D5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Gates for singlet cell selection
Live/Dead cell discrimination
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SSC-A
FSC-A
2
74.6
FSC-H
FSC-A
3
97.0
SSC-H
SSC-W
4
96.1
FSC-H
FSC-W
5
71.5
Fixable-Aqua / Live-Dead
FSC-A
Analysis
7
Q1
Q2
Q3
Q4
Marker 1
Marker 2
Compensation control and gates for markers:
Unstained controls and single stains
6
Unstained controls
1.5
0.2
96.5
1.8
PE-Cy7
AF 488
1.8
0
98.2
0
PE
APC
AF 488 Single
0.0
0.3
53.1
46.7
PE
AF 488
PE Single
15.5
1.5
81.8
1.8
PE
AF 488
PE-Cy7 Single
59.4
1.8
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APC Single
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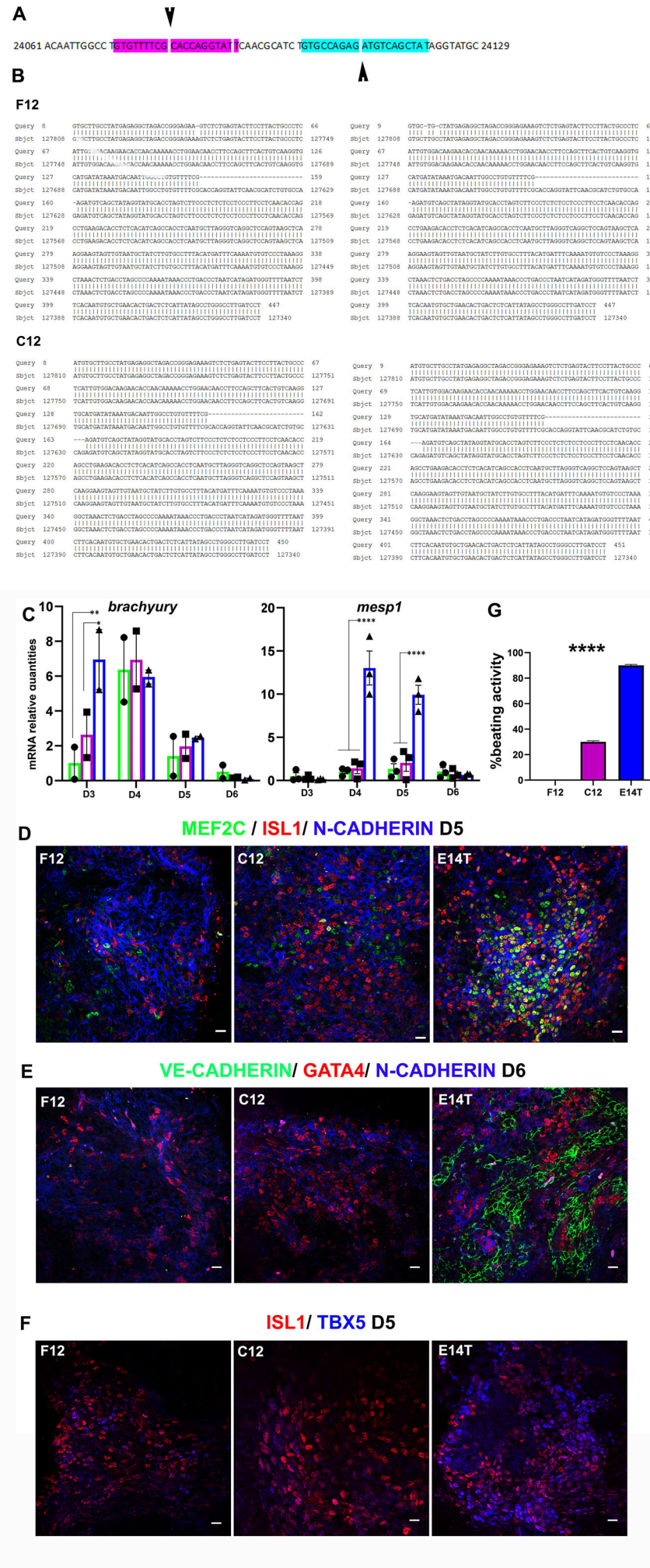
PE
APC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B
F12
C12
C
brachyury
mesp1
mRNA relative quantities
D3
D4
D5
D6
G
%beating activity
F12
C12
E14T
D
MEF2C / ISL1/ N-CADHERIN D5
F12
C12
E14T
E
VE-CADHERIN/ GATA4/ N-CADHERIN D6
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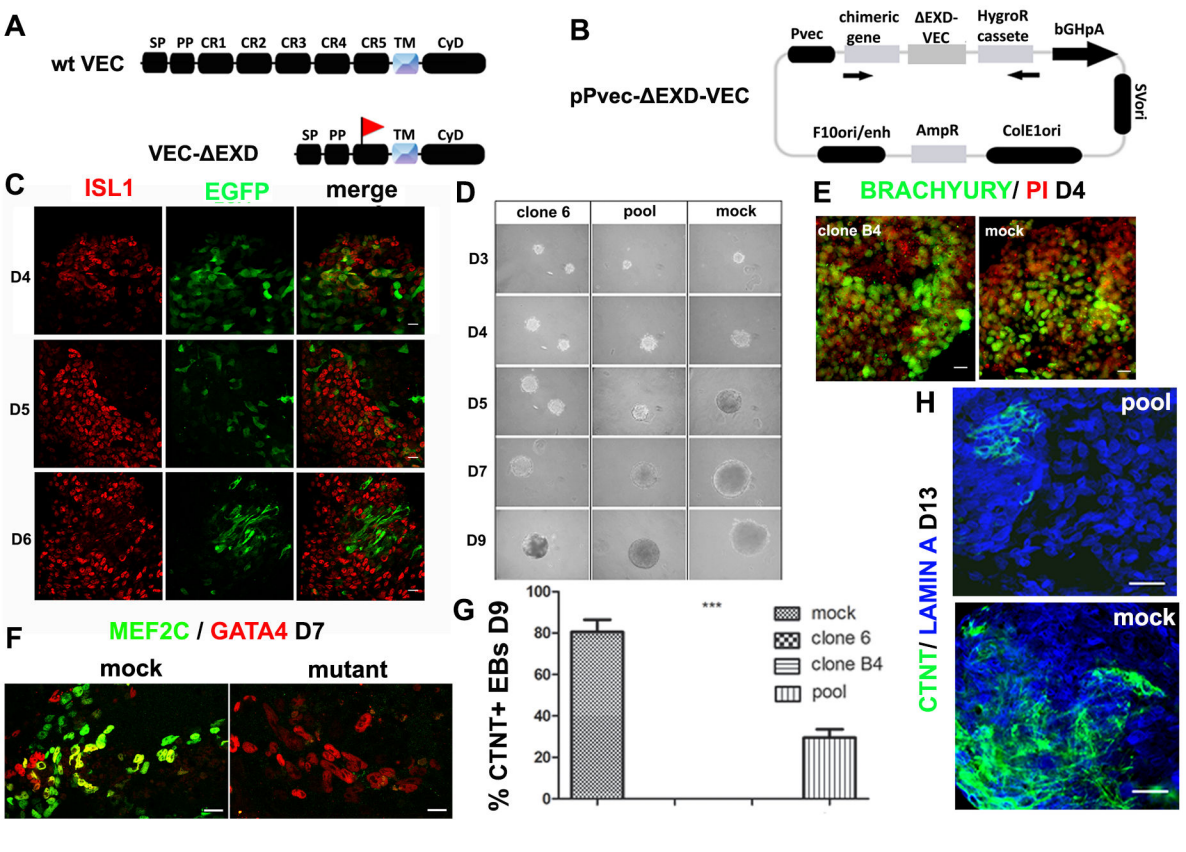
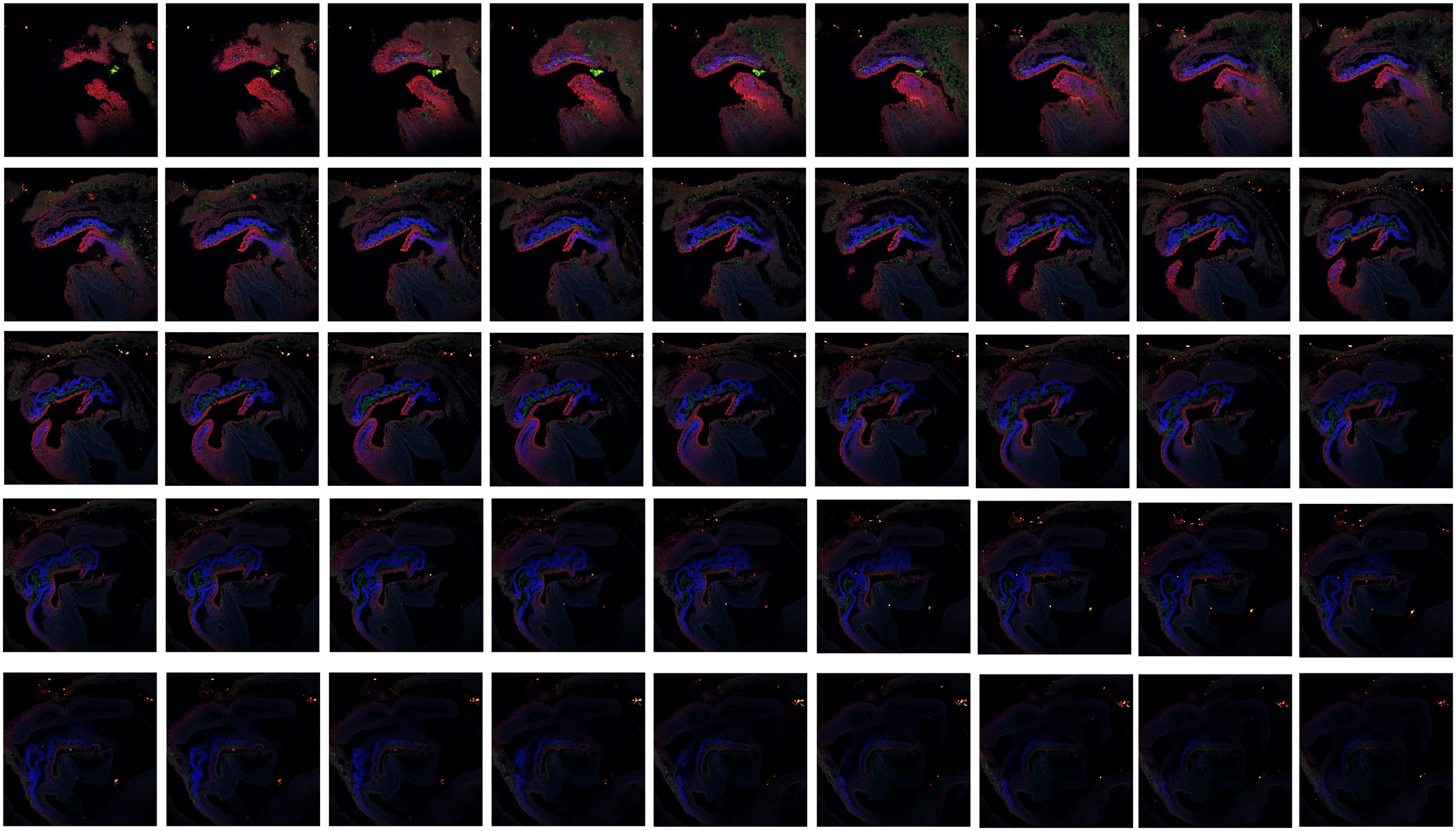
F
ISL1/ TBX5 D5
F12
C12
E14T


A
wt VEC
SP PP CR1 CR2 CR3 CR4 CR5 TM CyD
VEC-ΔEXD
SP PP TM CyD
B
pPvec-ΔEXD-VEC
Pvec
chimeric gene
ΔEXD-VEC
HygroR cassete
bGHpA
SVori
F10ori/enh
AmpR
ColE1ori
C
ISL1
EGFP
merge
D4
D5
D6
D
clone 6
pool
mock
D3
D4
D5
D7
D9
E
BRACHYURY/ PI D4
clone B4
mock
F
MEF2C / GATA4 D7
mock
mutant
G
% CTNT+ EBs D9
***
mock
clone 6
clone B4
pool
H
CTNT/ LAMIN A D13
pool
mock

A

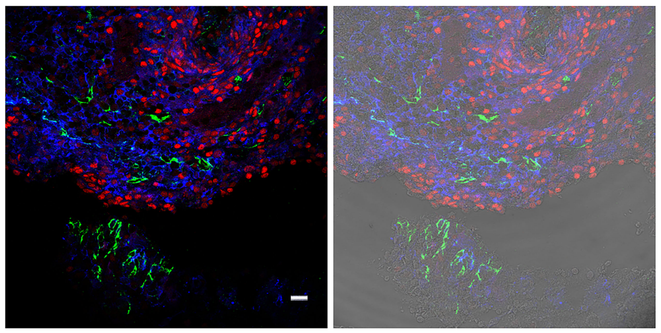
VE-CADHERIN / ISL1/ MEF2c E8.5



B

VE-CADHERIN/ ISL1/ N-CADHERIN

E11.5



Supplemental Figure an Video Legends

Supplementary Figure 1: Separate immunostainings and merged confocal micrographs: VE-CADHERIN, ISL1, N-CADHERIN at D6 (A) and D12 (B), VE-CADHERIN, GATA4, N-CADHERIN at D4 (C), VE-CADHERIN, ISL1, E-CADHERIN at D5 (D) and VE-CADHERIN, ISL1, TBX5 (E). Scale bars: 20µm.

Supplementary Figure 2: Gating strategy for flow cytometric analysis of extracellular and intracellular markers in single cell suspensions. Analysis of acquired events was performed with pre-gating steps for (1) Forward Scatter (FSC), Side Scatter (SSC), (2,3,4) doublet exclusion and (5) live-dead discrimination. Unstained cells and Single stain controls (6) for all fluorochromes were used for compensation and gating of extracellular and intracellular markers (7).

Supplementary Figure 3: (A) The gRNAs used to create the *ve-cadherin* KO clones are represented in the *ve-cadherin* genome sequence. (B) Sequence alignment of homozygous clones F12 and C12 with wild type mouse *ve-cadherin*. (C) *brachyury* and *mesp1* mRNA expression levels in KO and control EBs quantified by qPCR during differentiation days indicated. Data represent Mean ± SEM. Statistical analysis was performed by two-way ANOWA with Dunnett's multiple comparisons test as a post-hoc analysis $^{****}P<0.0001$, $^{**}P<0.01$, $^{*}P < 0.05$. (D) Immunofluorescence staining in KO and E14T clones at D5 showing down-regulation of ISL1 and MEF2c, but not N-CADHERIN. (E) GATA4, VE- and N-CADHERIN expression in KO and control EBs at D6. (F) $TBX5^{+}$ cells were reduced in KO EBs compared to control at D5. (G) Statistical analysis of beating activity at D10 (an EB was considered as beating if contained one or more beating areas). The numbers of EBs counted were clone F12: 195, clone C12: 218 and control: 205. Data represent Mean ± SD. (n=3). Statistical analysis was performed by Ordinary one-way ANOWA with Dunnett's multiple comparisons test. $^{****}P<0.0001$. (D-E, Scale bars: 20µm).

Supplementary Figure 4: (A) Schematic representation of wild type (wt) and mutant *ve-cadherin* (ΔEXD-VEC) expressing the FLAG epitope for detection (SP: signal peptide sequence; PP: pre-peptide sequence; TM: transmembrane domain; CR: cadherin repeat) (B) and pPvec-ΔEXD-VEC, the episomal construct used for ΔEXD-VEC expression. (C) EBs expressing ΔEXD-VEC under Pvec show suppression of growth from D4 and onwards**.** Typical images of live, mutant (clone 6 and pool) and mock EBs between differentiation days 3 and 9 (representative of >15 experiments and >500 EBs). Note that clone 6-derived EBs have smaller size than the pool EBs. Photos were taken in a phase microscope, using the same magnification. (D) Whole-mount immunofluorescence of clone B4 and mock EBs with a-BRACHYURY at D4. Nuclei were counterstained with PI. Representative images of 83 mock EBs and 75 clone B4 EBs were analyzed in 2 independent differentiation experiments. Scale bars: 20μm. (E) Immunofluorescence staining in mock and mutant EBs at D7 shows absence of $MEF2c^+$ cells in mutant EBs, whereas GATA4 could be detected, possibly in non-cardiac cell types. Scale bars: 20μm. (F) Statistical analysis of $CTNT^+$ EBs at D9, by whole-mount IF staining. The numbers of EBs counted were: mock: 193, clone B4: 198, clone 6: 185 and pool: 210. Statistical analysis was performed by one-way ANOVA followed by Tukey's Multiple Comparison Test ***P <0,001 vs mock. (G) Whole-mount double-IF staining in mock and pool EBs for CTNT and LAMIN A, as a nuclear marker, at D13. Scale bars: 40μm.

Supplementary Figure 5: (A) VE-CADHERIN, ISL1 and MEF2c whole-mount immunostaining of a representative embryo at E8.5. All stacks (5μm/stack) presented. For quantification of VE-CADHERIN $^+$/ $MEF2c^+$ cells at E8.5, two whole-mount embryos were examined at the heart area as indicated by ISL1 and MEF2C staining (220μm depth/ 5μm step size and 195μm depth/6μm step size respectively). In each case, $MEF2c^+$ cells and VE-CADHERIN $^+$/$MEF2c^+$ cells measurement was performed to 45 and 30 sections (appr 15 and 16 μm apart) respectively to avoid measurement of one cell two times. Then the confocal micrographs were counted with Fiji cell-counter. (B) anti- ISL1, N-CADHERIN and VE-CADHERIN triple staining of frozen sections from mouse embryos at E11.5. Scale bar: 20μm.

Supplementary Video 1: Video compiled from stacks shown in Fig. S5 using the Imaris Image Analysis Software.

Supplemental Table S1

Primer Table

Gene name	**Forward primer 5->3**	**Reverse primer 5->3**	**Product Size cDNA**
ve-cadherin	GTAACCCTGTAGGGAAAGAGTCCATT	GCATGCTCCCGATTAAACTGCCCATA	260bp
flag-ΔEXD-vec	GAGTCGCAAGAATGCCGACTACAAGG ACGACGATGACAAGACCTTCTGCGAG GATATGG	AAGGAAGTCGTAATCCACGTCAG	560bp
isl-1	GCAGCTCCAGCAGCAGCAACCCA	TGGGAGCTGCGAGGACATCGATGC	253bp
nkx2.5	AGCCGCCCCCACATTTTACCCG	GCGAGAAGAGCACGCGTGGCT	178bp
flk1	TTTGGCAAATACAACCCTTCAGA	GCAGAAGATACTGTCACCACC	133bp
brachyury	GGAACAGCTCTCCAACCTATGCG	CTGAGCTCCCAGCCCGTTGGAC	212bp
fgf8	TCCGGACCTACCAGCTCTAC	GAACTCGGACTCTGCTTCC	147bp
tbx5	TCTCCTTCTGGTTTGGTTCTGCCT	TGTCACCCAGGGCTCTTTCAGTTT	221bp
mesp-1	GCAGTCGCTCGGTCCCCGTT	CTGCGGCGGCGTCCAGGTTT	223bp
n-cadherin	TTGCTTCTGACAATGGAATCCCGC	AGGGAAGATCAAACGCGAACG	201bp
ctnt	AGCCCACATGCCTGCTTAAA	TCTCGGCTCTCCCTCTGAAC	115bp
mlc2v	ACTTCACCGTGTTCCTCACGATGT	TCCGTGGGTAATGATGTGGACCAA	254bp
mlc2a	AAGGGAAGGGTCCCATCAAC	AACAGTTGCTCTACCTCAG	202bp
b-actin	GTGACGTTGACATCCGTAAAG	GCCGGACTCATCGTACTC	244bp
gapdh	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	94bp