Journal of Molecular and Cellular Cardiology 89 (2015) 98–112

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Special series article

CARMEN, a human super enhancer-associated long noncoding RNA controlling cardiac specification, differentiation and homeostasis

Samir Ounzain [a,*], Rudi Micheletti [a], Carme Arnan [b], Isabelle Plaisance [a], Dario Cecchi [b], Blanche Schroen [c], Ferran Reverter [b], Michael Alexanian [a], Christine Gonzales [a], Shi Yan Ng [d,e], Giovanni Bussotti [f], Iole Pezzuto [a], Cedric Notredame [f], Stephane Heymans [c], Roderic Guigó [b], Rory Johnson [b,*], Thierry Pedrazzini [a,*]

[a] *Experimental Cardiology Unit, Department of Medicine, University of Lausanne Medical School, Lausanne, Switzerland*
[b] *Bioinformatics and Genomics Group, Centre for Genomic Regulation, Barcelona, Spain*
[c] *Centre for Heart Failure Research, Cardiovascular Research Institute, Maastricht University, The Netherlands*
[d] *Stem Cell and Developmental Biology Group, Genome Institute of Singapore, Singapore*
[e] *NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore*
[f] *Comparative Bioinformatics Group, Centre for Genomic Regulation, Barcelona, Spain*

ARTICLE INFO

Article history:
Received 20 June 2015
Received in revised form 24 September 2015
Accepted 25 September 2015
Available online 28 September 2015

Keywords:
Cardiac development
Heart failure
Cardiac precursor cells
Gene regulation
Super enhancer
Long noncoding RNA

ABSTRACT

Long noncoding RNAs (lncRNAs) are emerging as important regulators of developmental pathways. However, their roles in human cardiac precursor cell (CPC) remain unexplored. To characterize the long noncoding transcriptome during human CPC cardiac differentiation, we profiled the lncRNA transcriptome in CPCs isolated from the human fetal heart and identified 570 lncRNAs that were modulated during cardiac differentiation. Many of these were associated with active cardiac enhancer and super enhancers (SE) with their expression being correlated with proximal cardiac genes. One of the most upregulated lncRNAs was a SE-associated lncRNA that was named *CARMEN*, (CAR)diac (M)esoderm (E)nhancer-associated (N)oncoding RNA. *CARMEN* exhibits RNA-dependent enhancing activity and is upstream of the cardiac mesoderm-specifying gene regulatory network. Interestingly, *CARMEN* interacts with SUZ12 and EZH2, two components of the polycomb repressive complex 2 (PRC2). We demonstrate that *CARMEN* knockdown inhibits cardiac specification and differentiation in cardiac precursor cells independently of *MIR-143* and *-145* expression, two microRNAs located proximal to the enhancer sequences. Importantly, *CARMEN* expression was activated during pathological remodeling in the mouse and human hearts, and was necessary for maintaining cardiac identity in differentiated cardiomyocytes. This study demonstrates therefore that *CARMEN* is a crucial regulator of cardiac cell differentiation and homeostasis.
© 2015 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The postnatal heart has minimal regenerative potential, and heart failure typically develops following injury [1]. Currently, heart transplantation remains the only viable therapeutic option for end-stage heart failure. Within this context, cell replacement therapy via injection of precursor cells into the damaged myocardium to promote cardiac regeneration and prevent pathological remodeling represents an attractive therapeutic approach [2]. However, the main challenge for utilizing cell therapies for heart disease in a clinical setting is the identification of a suitable source of human cardiac precursor cells (CPCs) that give rise to functionally integrated cardiomyocytes (CMs). The existence of resident CPCs in the adult mammalian heart, including the human heart, capable of differentiating into mature CMs has been demonstrated [2]. However, the number of CPCs in the adult heart is low and expansion of human CPCs in culture is a mandatory step to produce sufficient numbers of precursors for cell therapies. Furthermore, our relative inefficiency to induce robust differentiation of CPCs into functional CMs in vivo is a severe limitation to clinical application. Therefore, to promote cardiac regeneration, it is imperative to improve our understanding of the molecular pathways and regulatory circuitry that controls recruitment and differentiation of CPCs toward the cardiac lineage.

At the molecular level, cardiac specification and differentiation is a complex biological process dictated by the activity of gene regulatory networks (GRNs). Cardiac GRNs are under the control of the core transcription factors (TFs), including NKX2.5, GATA4, MESP1, MEF2C and TBOX proteins [3]. These factors interact in a combinatorial manner with chromatin remodeling complexes to target *cis*-regulatory sequences and elicit specific temporal and spatial gene expression

* Corresponding author at: Experimental Cardiology Unit, Department of Medicine, University of Lausanne Medical School, CH-1011 Lausanne, Switzerland.
E-mail addresses: samir.ounzain@chuv.ch (S. Ounzain), rory.johnson@crg.es (R. Johnson), thierry.pedrazzini@chuv.ch (T. Pedrazzini).

http://dx.doi.org/10.1016/j.yjmcc.2015.09.016
0022-2828/© 2015 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

programs. However, the notion that these networks are primarily protein-based regulatory systems has been somewhat premature [4]. Several recent studies have demonstrated that gene regulatory network activity is under the control of diverse noncoding RNAs (ncRNAs) [5,6]. These ncRNAs control numerous aspects of GRN activity including transcriptional control, post-transcriptional processing, and chromatin remodeling [6]. Currently, the best characterized ncRNAs in the heart are the microRNAs (miRNAs), which fine tune mRNA expression though post-transcriptional silencing [5]. However, the recent advent of unbiased high-throughput RNA-sequencing (RNA-Seq) has led to the discovery of the numerically greater and more diverse class of long noncoding RNAs (lncRNAs) [7,8]. LncRNAs are transcripts that are operationally defined as being larger than 200 nucleotides in size and that lack discernible protein coding potential. Latest estimates have more than 15,000 such transcripts in human gene collections (GENCODEv22), a number which continues to rise with accumulating data from cell-specific RNA-Sequencing (RNA-Seq) [4,7]. Less than 1% of human lncRNAs have so far been characterized experimentally [9]. However, an increasingly significant number have been shown to play diverse regulatory roles in the nucleus and cytoplasm. These include chromosome X inactivation, genomic imprinting, splicing, transcriptional regulation and targeting of epigenetic chromatin modifying complexes to specific genomic loci [4].

LncRNAs were originally annotated based on their genomic location. However, functional annotations based on underlying chromatin states have recently emerged. A class of lncRNAs has been associated with active enhancer states (H3K27Ac/H3K4me1/p300), and these are classified as enhancer-associated lncRNAs [10,11]. Interestingly, these transcripts are able to modulate gene expression both in *cis* and *trans*, expanding the canonical roles of enhancer sequences. Enhancers are an important but enigmatic class of regulatory sequences, which are the key information processing units that integrate temporal, spatial and environmental cues within the genome [12]. Importantly, chromatin looping and promoter pause/release, both important processes that mediate enhancer-promoter communication are dependent on the production of enhancer-derived ncRNAs [13]. Recently, a novel class of enhancers has been described, the class of super enhancers (SE) [14,15]. SEs are much larger than classical enhancers (typically 1–10 kbp in size) and are master regulators of cell identity genes, including developmental TFs and other components determining cell-specific biology. Their function is associated with increased production of enhancer-associated ncRNAs as well as enrichment of specific histone modifications and chromatin remodeling complexes [14,15]. Not surprisingly, a number of important lineage-determining lncRNAs are derived from tissue-specific SEs [16,17].

Within this context, lncRNAs are emerging as an important class of regulatory mediators of cardiac lineage-specific commitment during development and of specialized cellular functions involved in maintaining cardiac integrity [18–24]. Two recently described lncRNAs, *Braveheart* (*Bvht*) and *Fendrr*, were shown to be key regulators of cardiac mesoderm specification and subsequent differentiation via their ability to epigenetically modulate the expression of important cardiac transcription factors [18,19]. Recent studies have also identified thousands of novel human and mouse heart enriched lncRNAs modulated in the adult heart post injury, implicating these in the pathological response [21–23,25], and highlighting their potential utility as biomarkers [21,22,24,26]. Moreover, considering the unique characteristics of SEs, cardiac lncRNAs associated with SE sequences are likely to be functionally important and relevant modulators of cardiac specification and differentiation. However, the roles of lncRNAs, and specifically SE-associated lncRNAs, in human CPCs have not been investigated. Therefore, there is a clear need to identify cardiogenic lncRNAs important in human development and CPC differentiation. In this study, we have identified and characterized *CARMEN*, a SE-associated lncRNA important for cardiac specification and differentiation in human CPCs.

2. Results

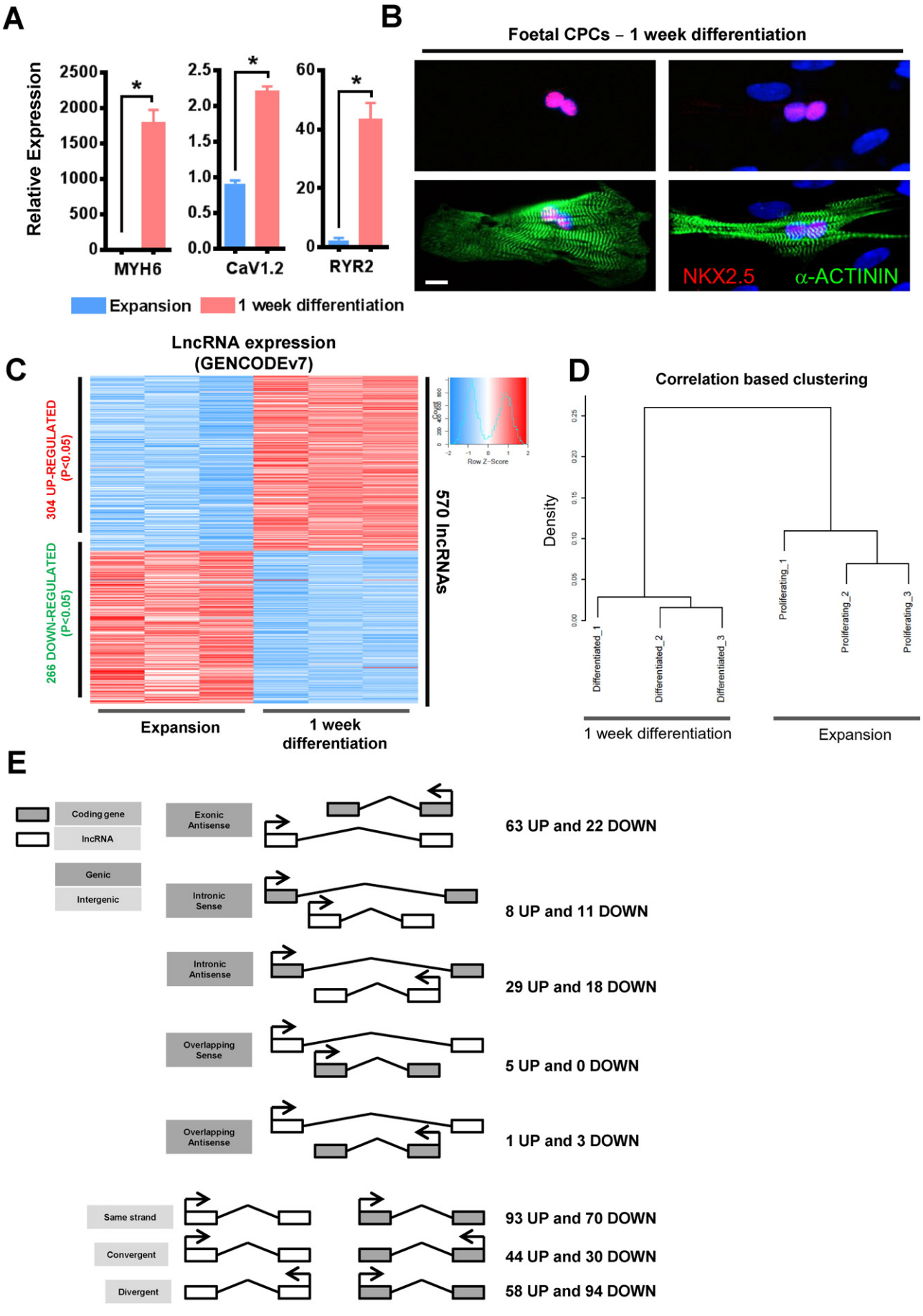
2.1. Profiling of the transcriptome in differentiating CPCs identifies differentially expressed lncRNAs

To address the role of lncRNAs in human CPC specification, differentiation and maturation, we first identified lncRNAs that were differentially expressed in human CPCs upon differentiation. CPCs were isolated from the fetal human heart as previously described [27]. These cells expressed early cardiac marker such as NKX2.5 and GATA4 but not proteins expressed by fully differentiated CMs, smooth muscle cells and endothelial cells (Suppl. Fig. 1A). CPCs were then induced to differentiate into CMs in vitro using an established method. Cardiac differentiation was associated with increased expression of mature CM marker genes including MYH6, CAv1.2 and RYR2 (Fig. 1A). This protocol typically resulted in efficient cardiac differentiation, with up to 16% of NKX2.5- and α-actinin-positive CMs presenting organized sarcomeres following seven days of differentiation (Fig. 1B). We therefore extracted RNA from undifferentiated (proliferating) and differentiated cell cultures and hybridized RNA samples to custom microarrays targeting essentially every transcript in the recently published GENCODEv7 lncRNA catalogue [7]. The majority of the lncRNAs that are in the GENCODEv7 catalogue are spliced, polyadenylated and primarily annotated based on ESTs and cDNAs from oligo-dT reverse transcribed cDNA libraries [7]. The array comprised four distinct, strand-specific probes against 14,880 transcripts, in addition to a set of 3231 randomly selected probes targeting human mRNAs.

We used a linear model approach to identify differentially expressed transcripts between proliferating and differentiated CPCs, taking advantage of the limma package for differential gene expression analysis [28]. This identified 570 (266 down- and 304 upregulated) differentially expressed lncRNAs during CPC differentiation at a false discovery rate (FDR) of <0.05 (Fig. 1C, Suppl. Table S2). Unsupervised hierarchical clustering of lncRNA and mRNA expression segregates the undifferentiated CPCs from differentiated CPCs (Fig. 1D), confirming that the differentiation process in vitro led to a robust change in the cardiac noncoding transcriptome. Consistent with previous reports in other tissues [7], lncRNAs in CPCs were detected at lower levels than mRNA (Suppl. Fig. 1B) and displayed a comparable lack of protein coding potential when compared to GENCODE mRNAs and lncRNAs (Suppl. Fig. 1C). Differentially expressed lncRNAs encompassed the various subtypes of lncRNAs (Fig. 1E) based on genomic geography with respect to proximal coding genes (i.e. intragenic vs. intergenic; exonic vs. intronic; sense vs. antisense; overlapping vs. non-overlapping). There were no significant differences in the conservation status of modulated lncRNAs with most being either highly or poorly conserved across 21 mammalian species (Suppl. Fig. 1D). However, as previously observed lncRNA exons were significantly less conserved than coding exons from mRNAs, although promoter sequences were equally well conserved between these two classes of transcripts (Suppl. Fig. 1E). In order to determine the expression patterns and tissue specificity of the modulated lncRNAs, we examined their expression in a wide variety of human tissues using available custom GENCODEv7 lncRNA array datasets [7]. Tissue-specific enrichment profiles were obtained for all modulated lncRNAs across tissues. Globally, their expression levels and breadth of expression across tissues was comparable to all GENCODEv7 lncRNAs (Suppl. Fig. 1F). Interestingly, many upregulated lncRNAs were more expressed in adult heart tissue as compared with downregulated and all GENCODEv7 lncRNAs. This supported the notion that upregulated lncRNAs could promote a cardiac differentiated state, which was comparable to that seen in the adult heart.

2.2. Identification of high priority candidate lncRNAs

We manually selected eleven lncRNAs as high priority candidates based on specific criteria, including the presence of an active


A
Relative Expression
MYH6
CaV1.2
RYR2
Expansion
1 week differentiation
B
Foetal CPCs – 1 week differentiation
NKX2.5
α-ACTININ
C
LncRNA expression
(GENCODEv7)
304 UP-REGULATED
(P<0.05)
266 DOWN-REGULATED
(P<0.05)
570 lncRNAs
Row Z-Score
Expansion
1 week
differentiation
D
Correlation based clustering
Density
Differentiated_1
Differentiated_2
Differentiated_3
Proliferating_1
Proliferating_2
Proliferating_3
1 week differentiation
Expansion
E
Coding gene
lncRNA
Genic
Intergenic
Exonic
Antisense
63 UP and 22 DOWN
Intronic
Sense
8 UP and 11 DOWN
Intronic
Antisense
29 UP and 18 DOWN
Overlapping
Sense
5 UP and 0 DOWN
Overlapping
Antisense
1 UP and 3 DOWN
Same strand
93 UP and 70 DOWN
Convergent
44 UP and 30 DOWN
Divergent
58 UP and 94 DOWN

enhancer-associated epigenetic signature (p300 enrichment) in fetal or adult human hearts, proximity to important coding and noncoding genes, significant modulation during CPC differentiation (log2FC > 0.5, <− 1.0) (Fig. 2A) and classification subtype (Suppl. Fig. 2A). First of all, we utilized a previously executed genome-wide ChIP-Seq screen [29], and determined the occupancy profiles of enhancer-associated co-activator proteins at candidate lncRNA loci in fetal and adult human hearts. A number of candidates were associated with significant p300/CBP enrichment, and specifically within the fetal heart (Lnc-869, Lnc-866, Lnc-518, Suppl. Fig. 2B), consistent with their expression in fetal CPCs. These data also suggested that some of these lncRNAs were representative of the previously described enhancer-associated lncRNAs [10,11]. Furthermore, using previously published data sets, we assessed the nuclear and cytoplasmic enrichment profiles of high priority candidates in ENCODE validated subcellular fractions (Fig. 2B) [7]. The majority of our candidates exhibited nuclear enrichment (e.g. Lnc-866). Interestingly, several lncRNAs displayed different subcellular localization depending on the cell type in which they were expressed (e.g. S-KANK). Finally, we validated differential expression of all high priority candidates in fetal CPCs one-week post differentiation using quantitative RT-PCR (qRT-PCR) (Fig. 2C). Of note, three candidate lncRNAs that were proximal to or overlapping coding genes (i.e. S-KANK1, AS-TNIK, AS-LHX1) exhibited highly correlated expression patterns with their corresponding coding genes, indicative of potential *cis*-regulation (Fig. 2D).

2.3. CARMEN is a human super enhancer-associated lncRNA associated with cardiac differentiation

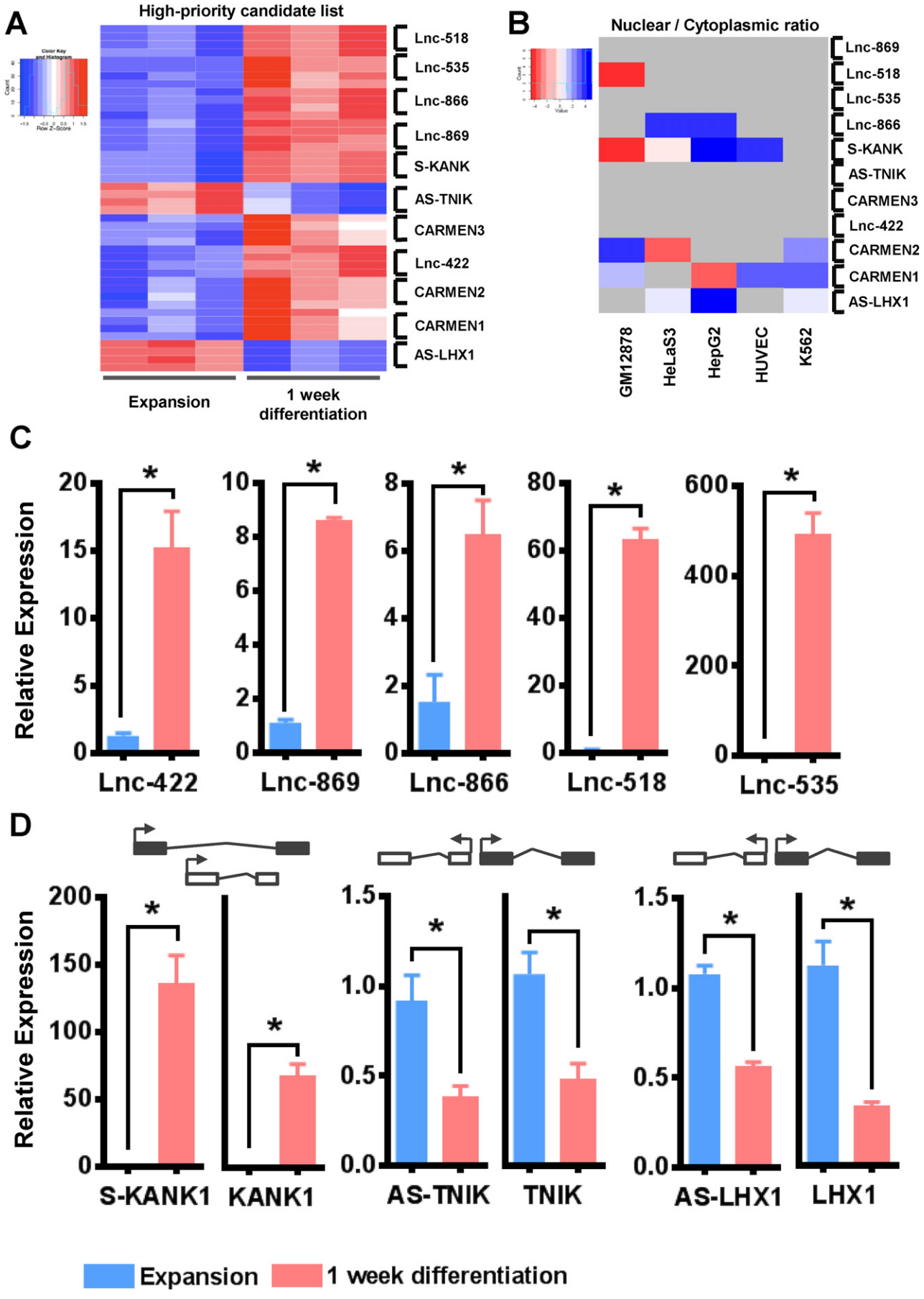
Amongst the modulated lncRNAs, three transcripts, namely ENST00000519898, ENST00000518014 and ENST00000509909, represent distinct annotated isoforms of a single lncRNA gene (Fig. 3A and B). Importantly, *CARMEN* isoforms were predicted to have no apparent coding potential using a variety of computational tools for (Suppl. Fig. 3A). In particular, we tested *CARMEN* using PhyloCSF (Suppl. Fig. 3A) [30]. The resulting score of −44.5 (using default settings), below the accepted threshold of 28.34 for coding transcripts, indicates no evidence for translation of any possible ORF. To further experimentally validate this observation, we searched two experimental datasets investigating the human proteome, neither of which record a peptide originating from *CARMEN* [31,32]. This locus was of particular interest for several reasons. Firstly, it was directly upstream of MIR-143 and -145, two important developmental miRNAs implicated in CPC specification and differentiation (Fig. 3A). Specifically, these two miRNAs were previously shown to promote differentiation toward the smooth muscle cell lineage [33,34]. Secondly, ChIP-analysis demonstrates that these transcripts mapped to a highly active cardiac enhancer in both fetal and adult human hearts (Fig. 3B). Notably, this region was shown to harbor a cardiac SRF/NKX2.5-bound enhancer required for cardiac-specific expression of miR-143 and -145 during cardiac development [34]. Furthermore, this enhancer has been previously demonstrated to be a Notch-responsive enhancer, capable of modulating miR-143 and -145 expressions in various developmental contexts [35]. Accordingly, the enhancer was able to drive robust cardiac-specific activity in a mouse transgenic reporter assays at E11.5 p.c. (Fig. 3C; images available using enhancer ID: Hs1752 at the VISTA enhancer browser, http://enhancer.lbl.gov/). Finally, using super enhancer (SE) tracks generated from data obtained in the adult human left ventricle [14], we demonstrated that this locus encompassed a cardiac SE. These findings indicated that this genomic locus and its associated lncRNAs were potentially important regulators of cardiac identity genes and pathways. We named this gene *CARMEN* for (CAR)diac (M)esoderm (E)nhancer-associated (N)oncoding RNA, and its individual isoforms *CARMEN1*, *CARMEN2* and *CARMEN3* (Fig. 3B). We proceeded to quantify the absolute expression of *CARMEN* isoforms using primers designed to distinguish individual transcripts (Suppl. Fig. 3B). All three isoforms were expressed at relatively high levels and significantly induced in differentiating fetal CPCs (Fig. 3D), supporting a role during cardiac specification and differentiation for these SE-associated lncRNAs. Interestingly, *MIR-143* and *-145* were modulated in an opposing manner during differentiation (Fig. 3E).

Evolutionary conservation supports the importance of CARMEN in cardiac precursor cell differentiation. A long noncoding RNA, (AK087736), is transcribed from the orthologous region of the mouse genome, which we designate as the mouse ortholog (Fig. 4A). Interestingly, the promoter region is highly conserved, supporting an important role in the regulation of transcription at this locus. In the mouse, another annotated convergent lncRNA is present on the opposite strand. This lncRNA, AK143260, has recently been demonstrated to be a cardiac lncRNA named *Braveheart* (*Bvht*), critically required for progression of nascent mesoderm toward the cardiac fate during cardiogenesis [18]. It is important to note that no ortholog of *Bvht* has been identified in human.

We proceeded to quantify the expression of both *Carmen* and *Bvht* during cardiogenesis in two independent murine models of cardiac specification and differentiation in vitro. We first induced mouse embryonic stem (ES) cells to differentiate using the hanging drop model [36]. This model recapitulates embryonic cardiac development in vitro, generating all appropriate cardiac lineages. We first examined the temporal gene expression patterns associated with pluripotency (ES), cardiac mesoderm (MES), cardiac precursors (CPC) and differentiated cardiomyocytes (CM) (Suppl. Fig. 4A). Upon differentiation, the pluripotency markers *Oct4* and *Nanog* were rapidly downregulated (Suppl. Fig. 4B). This occurred concomitantly with the transient induction of cardiac mesoderm specifying TFs, *Mesp1* and *Eomes* (Fig. 4B; Suppl. Fig. 4B). Three core cardiac TFs, *Nkx2.5*, *Gata4* and *Islet1*, which specify CPCs and initiate the cardiac gene program, were significantly upregulated at the CPC stage (Fig. 4B; Suppl. Fig. 4B). Finally, this was followed by the robust expression of cardiac differentiation and structural proteins, *Myh6*, *Myh7* and *Myl2* (Suppl. Fig. 4B). We then assessed *Carmen* and *Bvht* expression. *Carmen* was induced between the MES and CPC stages with maximal expression occurring in CPCs (Fig. 4C). *Bvht* was initially downregulated between ES and MES stages, before subsequently being upregulated in CPCs and CMs (Fig. 4C), in agreement with previously published observations [18]. Altogether, these different kinetics of expression supported a role for *Carmen* during cardiac specification and differentiation of mouse ES cells. Importantly, *Carmen* expression in the adult mouse heart was comparable to expression in differentiated ES cell-derived CMs (Suppl. Fig. 4D).

We sought further support for these findings in another model of cardiac differentiation using P19CL6 cells. These can readily be induced to differentiate into beating cardiomyocytes upon induction by dimethyl sulfoxide (DMSO) [37]. Indeed, differentiating P19CL6 cells recapitulated cardiac differentiation, and demonstrated the expected change in gene expression in pluripotency (*Oct4*, *Nanog*), MES (*Mesp1*, *Eomes*), CPC (*Gata4*, *Islet1*) and CM (*Myh6*, *Myl2*) marker genes (Fig. 5). Mesodermal specification occurred specifically between day 0 and day 2 while CPCs and CMs emerged between day 2 and day 4. Comparable to their expression kinetics during ES differentiation, both *Carmen* and *Bvht* were maximally expressed between the MES and CPC stages.

Fig. 1. Global identification of differentially expressed lncRNAs during cardiac differentiation. (A) Quantitative RT-PCR analysis of differentiation marker genes in proliferating (blue) and differentiated (pink) CPCs. (B) Human CPCs derived from the fetal heart express differentiation markers 1 week after culture in differentiation medium. (C) Microarray heatmap of differentially expressed lncRNAs during cardiac differentiation. (D) Correlation based clustering of individual samples based on lncRNA expression. (E) Number of differentially modulated lncRNAs in orientations relative to proximal coding genes. *P < 0.05, SEM. Scale bars: 20 μm (n = 4).


A
High-priority candidate list
Lnc-518
Lnc-535
Lnc-866
Lnc-869
S-KANK
AS-TNIK
CARMEN3
Lnc-422
CARMEN2
CARMEN1
AS-LHX1
Expansion
1 week differentiation
B
Nuclear / Cytoplasmic ratio
Lnc-869
Lnc-518
Lnc-535
Lnc-866
S-KANK
AS-TNIK
CARMEN3
Lnc-422
CARMEN2
CARMEN1
AS-LHX1
GM12878
HeLaS3
HepG2
HUVEC
K562
C
Relative Expression
Lnc-422
Lnc-869
Lnc-866
Lnc-518
Lnc-535
D
Relative Expression
S-KANK1
KANK1
AS-TNIK
TNIK
AS-LHX1
LHX1
Expansion
1 week differentiation

Considering that human *CARMEN* maps to a cardiac super enhancer, we determined stage-specific activation of chromatin states at the locus in the mouse. We took advantage of publicly available chromatin state maps, generated using chromatin immunoprecipitation followed by sequencing (ChIP-Seq) in differentiating ES cells [38]. Analyses were executed in pluripotent mouse ES cells (ES, i.e. *Oct4*-positive cells), at the cardiac mesoderm stage (MES, i.e. Mesp1-positive cells), the cardiac precursor stage (CPC, i.e. *Nkx2.5*-positive cells) and in differentiated cardiomyocytes (CM, i.e. *Myl2*-positive cells). Assessment of H3K27me3 and H3K4me3 (associated with inactive and active canonical promoters respectively), and H3K4me1 and H3K27Ac (associated with poised and active enhancers respectively) allowed us to analyze chromatin state transitions at the *Carmen* locus during cardiogenic differentiation (Suppl. Fig. 4C). First of all, *Carmen* was associated with a super enhancer-like signature as judged by the broad and high H3K27Ac occupancy levels during cardiac differentiation. Maximal enhancer activity, as indicated by the peak of H3K27Ac enrichment, occurred between the MES and CPC stages. *Carmen* expression therefore correlated with enhancer activity, as recently observed for other fetal cardiac enhancers active during cardiac differentiation [11]. Interestingly *Bvht* was associated with a canonical promoter signature at its transcriptional start site (i.e. H3K4me3), which was absent at the *Carmen* locus. This suggests that *Bvht* should be classed as a promoter-associated intergenic lncRNA. Altogether these data strongly support *Carmen* being a bona fide SE-associated lncRNA, whose expression correlates with both the chromatin state activation of its associated enhancer in CPCs as well as with specification and subsequent differentiation of CPCs into cardiomyocytes.

2.4. CARMEN controls cardiac specification and differentiation in mouse CPCs

To determine if *Carmen* actively regulated cardiac specification and differentiation, we stably transfected P19CL6 cells with a shRNAi targeting *Carmen*. Cardiogenesis was induced in transfected cells by DMSO addition. Four days following induction, loss-of-function resulted in a complete block of *Carmen* expression (Fig. 5). Interestingly, *Bvht* induction was also completely blocked upon *Carmen* silencing, suggesting that *Bvht* was controlled by *Carmen*. In accordance with the known roles of *Bvht* in regulating the cardiogenic program, the expression of Mesp1, the downstream cardiac TFs *Gata4* and *Islet1*, and the CM-specific genes *Myl2*, *Myh6* were severely inhibited by *Carmen* loss-of-function. In addition, *Eomes*, a key TF upstream of *Mesp1* in the cardiac mesoderm specification pathway and the pluripotency regulators *Oct4* and *Nanog* were also affected by *Carmen* depletion. Previous work demonstrated that *Eomes* and the pluripotency genes were not affected by *Bvht* knockdown. These data therefore suggest the existence of feedback mechanisms able to affect pluripotency (*Oct4*, *Nanog*) and early mesodermal specification (*Eomes*) that are dependent on *Carmen* expression.

To extend these findings in human cells, fetal CPCs were transfected with modified antisense oligonucleotides (GapmeRs) targeting the three human *CARMEN* isoforms. After 72 h, transfected proliferating CPCs were cultured under differentiation conditions. Efficient depletion was observed in GapmeR-treated cells 10 days post induction of cardiac differentiation (Fig. 6A). This depletion was associated with a significant reduced expression of cardiac transcription factors and differentiation makers, including *GATA4*, *NKX2.5*, *TBX5*, *MYH6*, *MYH7*, and *TNNI* (Fig. 6B). Furthermore CARMEN depletion was associated with decreased expression of the smooth muscle cell marker, MYH11. Importantly, CARMEN depletion was not associated with decreased expression of miR-143 and -145, further supporting the notion that CARMEN isoforms are not the precursor transcripts for these miRNAs (Fig. 6C). As a consequence, *CARMEN* silencing impaired the capacity of human CPCs to produce differentiated cardiomyocytes (Fig. 6D). To further dissect the roles of *CARMEN* and the proximal miRNAs in this differentiation process, *CARMEN*-depleted cells were also co-transfected with MIR-143 and MIR-145 mimics. This led to increased expression of both miRNAs in control and *CARMEN*-depleted cells. The predicted downregulation of the prototypic miRNA targets *ELK1* and *KLF4* demonstrated efficient MIR mimic transfection (Fig. 6C). Importantly, and confirming a miRNA-independent role for C*ARMEN* in cardiogenic differentiation, MIR mimics were not able to restore cardiogenic differentiation induced by *CARMEN* knockdown. In contrast, expression of the smooth muscle cell marker *MYH11* was induced by MIR-143 and MIR-145 mimics, in agreement with the known roles of the two miRNAs as promoters of smooth muscle cell differentiation. Altogether, this indicates that *CARMEN* is able to control cardiac specification and differentiation independently of MIR-143/145.

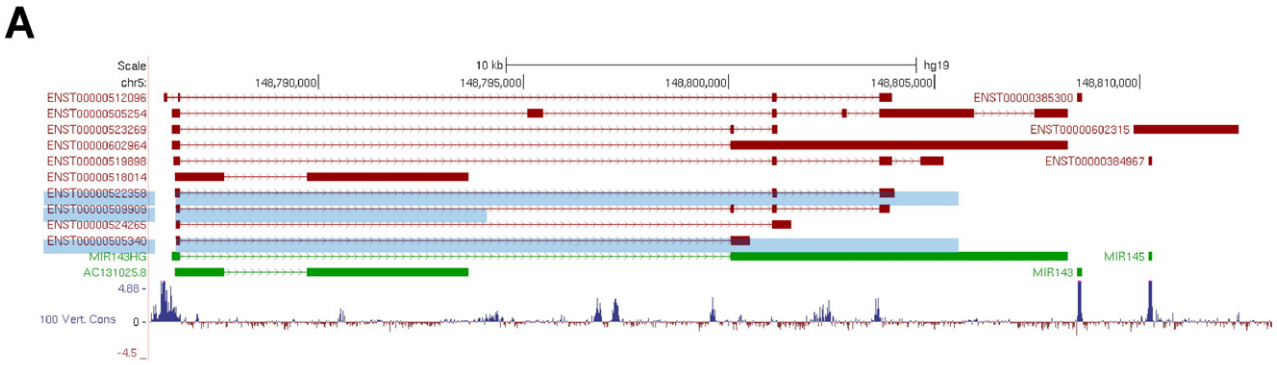
2.5. CARMEN exhibits cis-acting function, and interacts with SUZ12 and EZH2

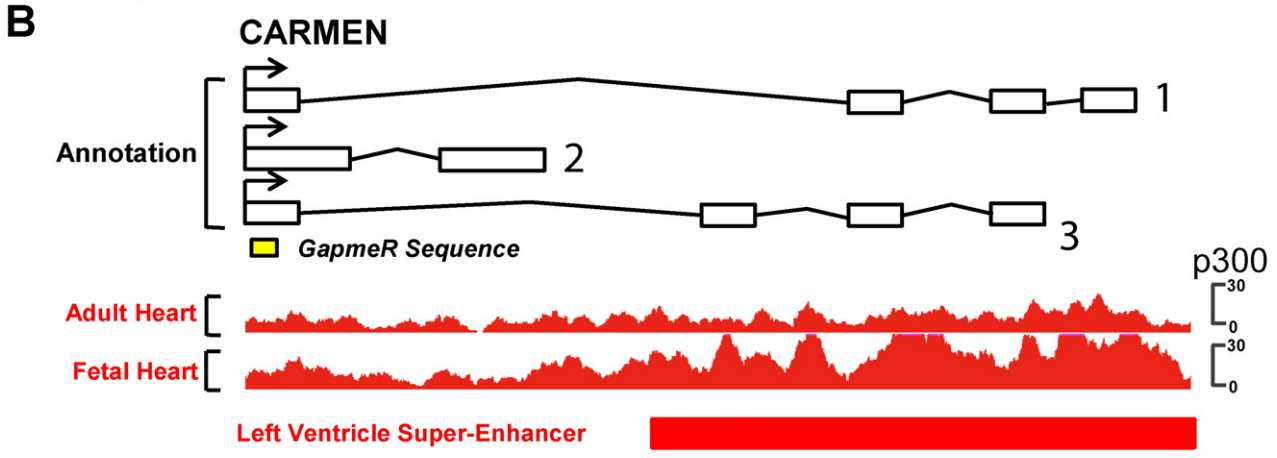
Based on the genomic overlap with bona fide *cis*-acting enhancer sequences and the lack of a canonical promoter signature at the *CARMEN* transcriptional start site, we suspected that *CARMEN* enacted regulatory activity akin to other recently described enhancer associated lncRNAs [16–19]. In order to test this hypothesis, we constructed a plasmid, in which *CARMEN* expression was under the control of a Doxycycline (Dox)-inducible promoter (Fig. 7A). This promoter is completely inactive in the absence of Dox. A firefly luciferase reporter gene under the control of a basal SV40 promoter was then inserted directly downstream of the *CARMEN* expression cassette. A control vector was also constructed containing a GFP sequence instead of *CARMEN*. The presence of CARMEN DNA in the construct resulted in significant luciferase activity, even in the absence of CARMEN transcription (Dox neg) indicating the intrinsic enhancer activity of the locus. Crucially, induction of CARMEN transcription by addition of Doxycycline caused additional activation of luciferase activity. Thus, RNA from the *CARMEN* enhancer contributed to stimulate luciferase expression (Fig. 7B). These results support the idea that *cis*-enhancing activity of this enhancer locus is dependent on the production of an associated lncRNA, i.e. *CARMEN*. Importantly, a number of recent reports have indicated that enhancer *cis*-acting lncRNAs can also enact *trans* functions through interaction with ubiquitously expressed chromatin-modifying complexes [19]. In particular, many lncRNAs have been shown to associate with le polycomb repressive complex 2 (PRC2). We therefore investigated if *Carmen* could physically interact with components of this complex in mouse embryonic stem cells. We performed RNA immunoprecipitation (RIP) assays, in which RNA-protein complexes were immunoprecipitated with antibodies specific to EZH2 and SUZ12 (Fig. 7C and D). We found that *Carmen* was associated with both proteins, suggesting that *Carmen* may have *trans*-repressive roles during cardiac differentiation.

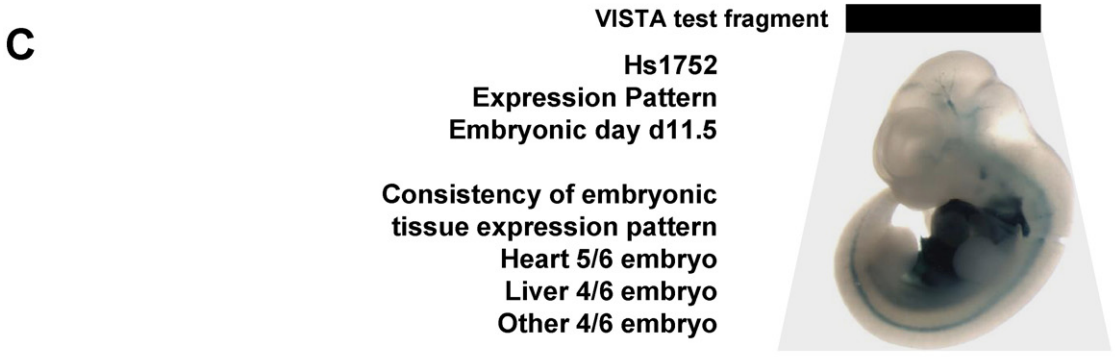
2.6. CARMEN is modulated in cardiac disease and required for maintaining a differentiated cardiac fate

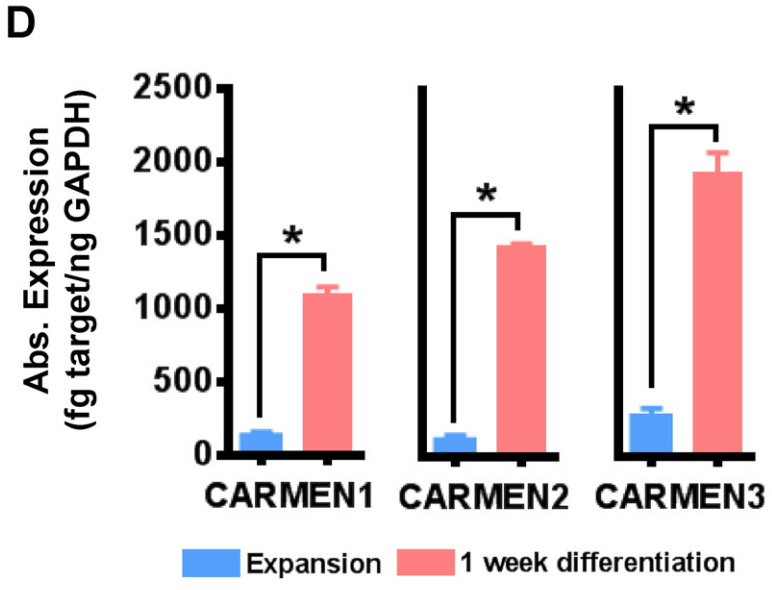
Our data indicated that *CARMEN* was required for the specification and cardiac differentiation of pluripotent and cardiac precursor cells. Furthermore, utilizing data from previous RNA-Seq based studies demonstrated that this transcript was highly expressed in the adult mouse

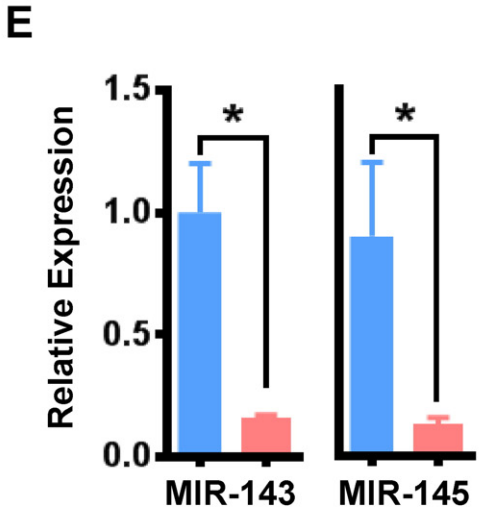
Fig. 2. Identification of high priority cardiac lncRNAs. (A) Microarray heatmap of differentially expressed high priority candidate lncRNAs. (B) Subcellular location of high priority candidate lncRNAs. Blue indicates nuclear while red indicates cytoplasmic enrichment. (C) qRT-PCR analysis of high priority candidates in proliferating (blue bar) and differentiated (pink bar) CPCs. (D) Expression analysis of lncRNA candidates with proximal coding gene. LncRNA/protein coding gene genomic orientation is illustrated above each graph. *$P < 0.05$, SEM ($n = 4$).


A
Scale
chr5:
10 kb
hg19
148,790,000
148,795,000
148,800,000
148,805,000
148,810,000
ENST00000512086
ENST00000505254
ENST00000523269
ENST00000602964
ENST00000519898
ENST00000518014
ENST00000522358
ENST00000509909
ENST00000524265
ENST00000505340
MIR143HG
AC131025.8
ENST00000385300
ENST00000602315
ENST00000384967
MIR145
MIR143
4.88
100 Vert. Cons
0
-4.5


B
CARMEN
Annotation
1
2
3
GapmeR Sequence
p300
Adult Heart
Fetal Heart
30
0
Left Ventricle Super-Enhancer


C
VISTA test fragment
Hs1752
Expression Pattern
Embryonic day d11.5
Consistency of embryonic
tissue expression pattern
Heart 5/6 embryo
Liver 4/6 embryo
Other 4/6 embryo


D
Abs. Expression
(fg target/ng GAPDH)
2500
2000
1500
1000
500
0
*
CARMEN1
CARMEN2
CARMEN3
Expansion
1 week differentiation


E
Relative Expression
1.5
1.0
0.5
0.0
*
MIR-143
MIR-145

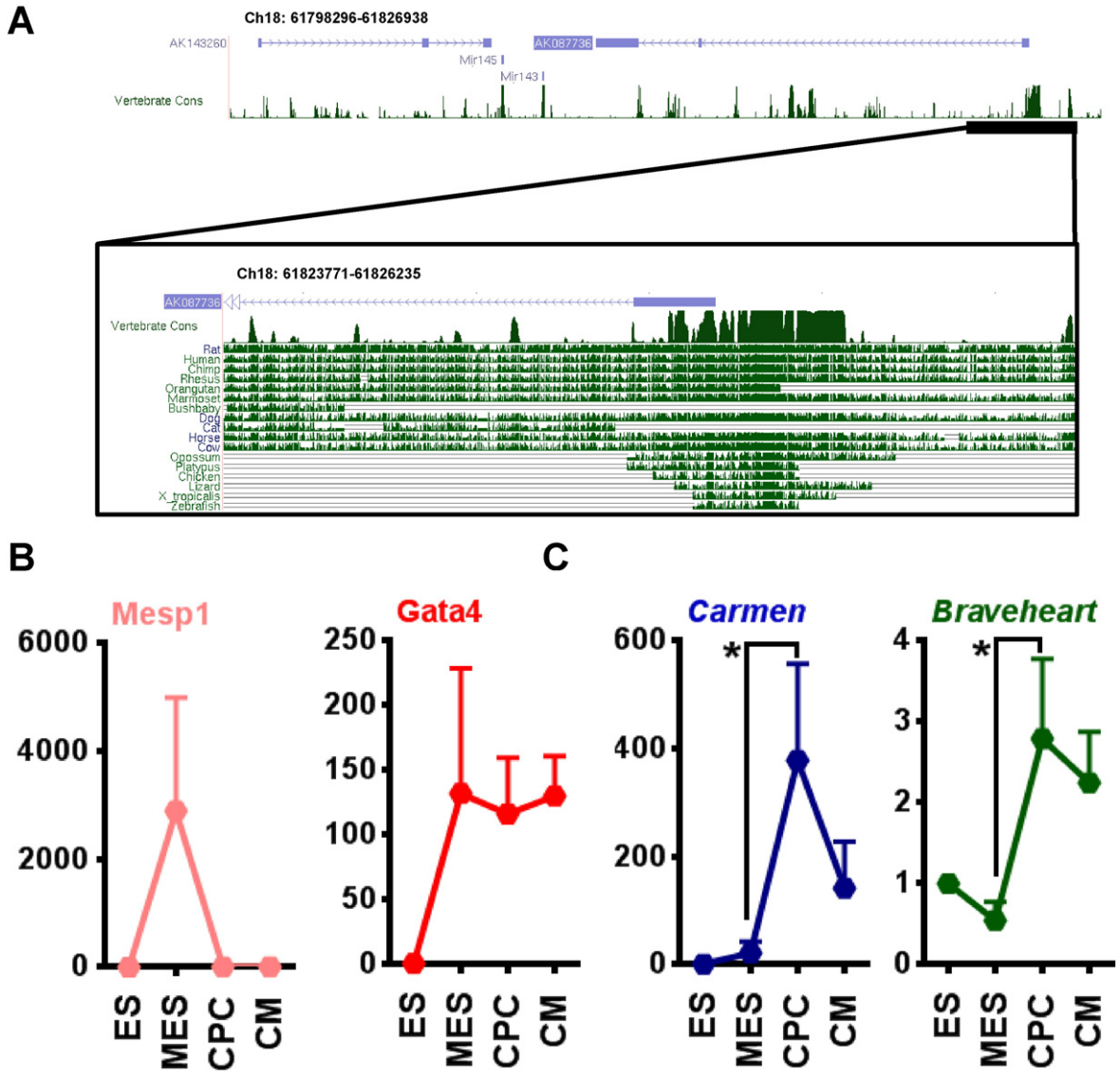


Fig. 4. *Carmen* is differentially expressed during murine cardiac differentiation. (A) *Carmen* is highly conserved across vertebrate species. Comparative genomic alignment of 17 vertebrate species to the mouse genome (mm9) using conservation tracks available on the UCSC browser. (B–C) *Mesp1*, *Gata4*, *Carmen* and *Braveheart* are differentially expressed during cardiac differentiation of mouse embryonic stem cells. *P < 0.05, SEM (n = 3).

and human heart (not shown) [22]. We therefore investigated whether *CARMEN* expression was modulated in response to cardiac stress in murine models of heart disease and in the heart of human patients suffering from two different cardiac pathologies. We first determined expression in a mouse model of myocardial infarction. Fourteen days after infarction, the myocardium was characterized by intense remodeling and decreased cardiac function (Suppl. Fig. 5A), and induction of cardiac markers of stress (Fig. 8A). *Carmen* was significantly upregulated post myocardial infarction (Fig. 8A), supporting a role for *Carmen* in adult heart homeostasis and cardiac remodeling.

To determine the potential roles of human *CARMEN* in cardiac disease, we examined its expression in two human heart pathologies, i.e. idiopathic dilated cardiomyopathy (DCM) and aortic stenosis (AOS). DCM patients presented reduced cardiac function whereas AOS patients demonstrated preserved function. In addition, cardiac dilation was evident in DCM patients and AOS patients were characterized by cardiac hypertrophy (Suppl. Fig. 5B and C; Suppl. Table S3). The stereotypical cardiac stress marker NPPA was also significantly upregulated in both pathologies (Fig. 8B and C). We then assessed the expression of all three human *CARMEN* isoforms. *CARMEN3* was significantly upregulated in both DCM and AOS whereas *CARMEN1* and *2* were only induced in AOS patients (Fig. 8B and C). We further searched for independent validation of the relationship between *CARMEN3* expression and cardiac disease state. The Gtex project has produced RNA-Seq transcriptome data from post mortem organs across a panel of human individuals with medical history [39]. We examined therefore two relevant heart tissues within Gtex, namely atrium and ventricle, for which 25 and 66 individual samples were respectively available. Of these two, *CARMEN3* expression was markedly higher in atria ($P = 1.9e-09$, Wilcoxon test) (Fig. 8D). Next, we used medical data to separate patients based on a diagnosis of cardiovascular disease. Interestingly, hypertensive patients demonstrated increased expression in atria ($P = 0.008$, Wilcox test)

Fig. 3. Identification of *CARMEN*. (A) UCSC screenshot of the Human *CARMEN* locus with *CARMEN* isoforms 1, 2 and 3 highlighted in blue. (B) The three *CARMEN* isoforms are associated with a human super-enhancer as indicated by p300 occupancy; yellow box indicates sequences targeted by *CARMEN* specific GapmeRs. (C) When assessed in a transgenic mouse reporter assay, the enhancer fragment indicated by a black box drives reproducible cardiac expression in E11.5 mouse embryos. (D); (E) Absolute qRT-PCR analysis of *CARMEN* isoforms and MIR-143 and -145 during cardiac differentiation in proliferating (blue bar) and differentiated (pink bar) CPCs. *P < 0.05, SEM (n = 4).

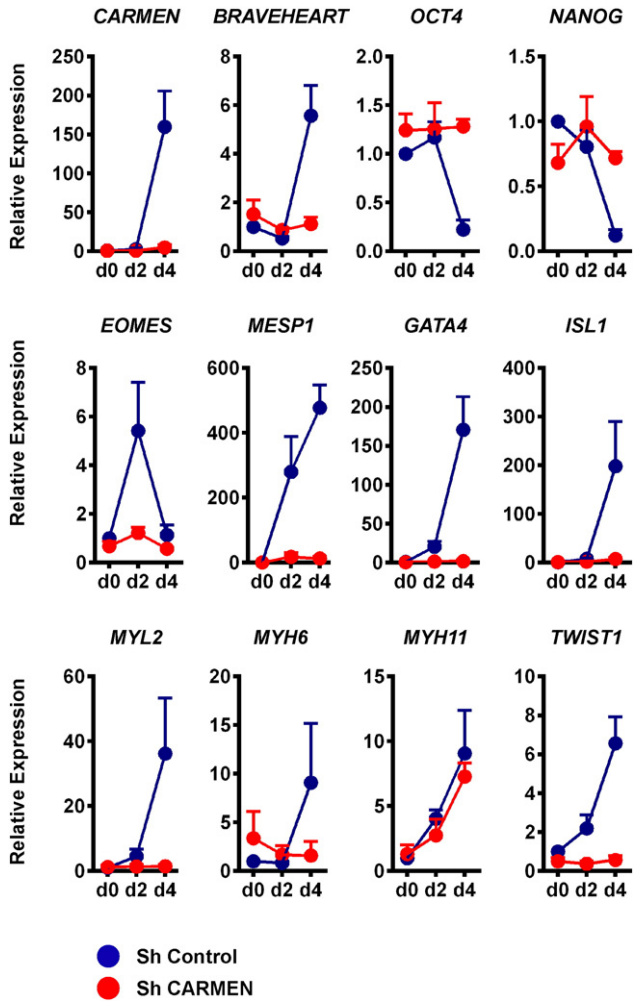


Fig. 5. Knockdown of *Carmen* impacts upon cardiac specification and differentiation. (A) P19CL6 were transfected with *Carmen*-shRNA and then differentiated for 4 days in 1% DMSO. Target gene expression levels were assessed by qRT-PCR. *P < 0.05, SEM. **P < 0.01, SEM (n = 3).

(Fig. 8E) while levels in ventricles was not significantly different (P = 0.1474, Wilcox test) (Suppl. Fig. 5D).

Finally, to further evaluate the importance of *Carmen* in cardiac homeostasis, we analyzed the effects of *Carmen* depletion in primary murine cardiomyocytes. Cardiomyocytes were isolated from the neonatal heart. At this stage, myocytes display significant levels of early cardiac markers such as *Gata4* and *Nkx2.5* but rapidly mature into beating cardiomyocytes in vitro. Isolated cells were transfected with modified antisense oligonucleotides (GapmeRs) targeting mouse *Carmen* (Fig. 8F). *Carmen* was depleted by approximately 50%, with this being accompanied by the significant downregulation of key cardiac TFs and structural proteins (*Gata4*, *Nkx2-5* and *Myh6*). This transcriptional response was indicative of a dedifferentiation of isolated CMs upon *Carmen* silencing, suggesting therefore that *Carmen* plays a critical role in maintaining a differentiated cardiac fate in mature cardiomyocytes.

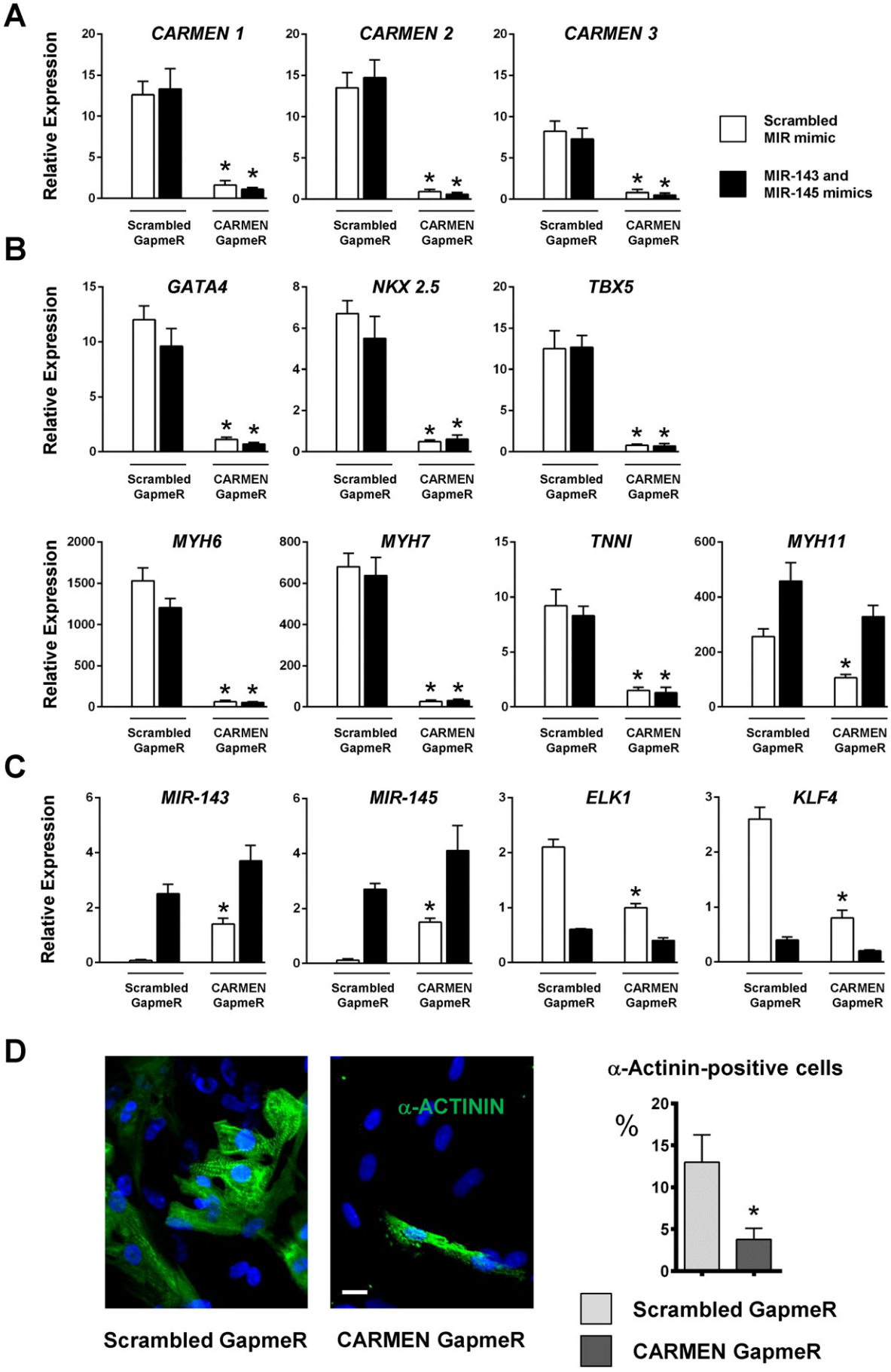
3. Discussion

Dynamic regulation of gene expression is critical for cell fate transitions during cardiac lineage commitment, and faulty regulation can lead to developmental failure and disease. A thorough understanding of these processes can facilitate the targeted derivation of important cardiovascular lineages required for cell replacement therapies. Cardiovascular lineage specification and differentiation is a multistep process regulated by a network of transcription factors [3]. Though many of the genetic factors that control cardiac development and CPC lineage determination and differentiation are known, we suspect that lncRNAs represent an additional layer of regulation [40]. Here, we identify hundreds of differentially modulated human lncRNAs during cardiac differentiation of isolated human fetal CPCs. Interestingly, many of these validated candidates are mapping to bona fide human fetal and adult cardiac enhancers, suggesting they represent the newly identified enhancer-associated lncRNA class [10,11]. We have recently demonstrated that a significant fraction of novel murine cardiac polyadenylated and multi-exonic lncRNAs are derived from developmental cardiac enhancers [11,22]. The human lncRNAs described here are comparable in their chromatin state and likely *cis*-acting function. In support of this, we find that several human lncRNA candidates are highly correlated in their expression with proximal coding genes. We further characterized one of these candidate lncRNAs, which we named *CARMEN*. *CARMEN* is highly conserved in mouse, and appears to be an important regulator of cardiovascular cell specification and differentiation. Furthermore, *CARMEN* is derived from a human SE, which has been shown to be active in the adult heart. Considering the master regulatory roles of SEs in controlling key cell identity genes, we suspect that *CARMEN* represents a novel SE-associated lncRNA critical for cell fate determination and differentiation, akin to other recently described SE-derived lncRNAs such as MyoD enhancer RNA and CCATL-1 [16,17].

Interestingly, *CARMEN* is proximal to an important developmental miRNA locus, encoding the MIR-143 and -145. These miRNAs have been previously shown to be crucial post-transcriptional regulators of key pathways implicated in pluripotency and cardiovascular lineage commitment [33,34]. It is important to emphasize that the *CARMEN* isoforms assessed here do not represent precursor transcripts for MIR-143/-145. Indeed, previous analysis of promoter-specific histone modifications and poly(A) signal frequencies demonstrate that *CARMEN* transcript boundaries are well defined [7]. The majority of cDNA and EST fragments overlapping *CARMEN* terminate at the position annotated by GENCODE, i.e. upstream of miR-143. In addition, a non-canonical polyadenylation sequence is located within 20 nucleotides of the termination site. The end of *CARMEN* is also marked by an SVM-predicted polyadenylation site [41]. Furthermore, MIR-143 and -145 expression does not correlate with *CARMEN* expression during cardiac differentiation, and *CARMEN* depletion does not lead to reduced miRNA expression. Most importantly, miRNA overexpression in *CARMEN*-depleted fetal CPCs is not able to rescue the differentiation defect induced by *CARMEN* knockdown, suggesting that *CARMEN*-mediated induction of cardiogenesis is independent of the proximal miRNAs. Altogether, these different pieces of evidence refute that CARMEN represents a precursor of the two miRNAs.

Mouse ES cells with deletions of both miR-143 and miR-145 have been shown in previous studies not to phenocopy *Bvht* depleted cells [18], supporting a miRNA-independent role for *Bvht*. In the mouse, *Bvht* is necessary for the activation of a core cardiac gene network and functions upstream of Mesp1 [18]. It regulates the cardiac gene network during CM differentiation through interacting with SUZ12, a component of PRC2, suggesting that *Bvht* mediates epigenetic regulation of cardiac commitment. *Bvht* is also important for maintaining a cardiac identity in neonatal CMs, as shown for *Carmen*. Our luciferase reporter assay provides support that the *cis*-acting regulatory activity of the enhancer locus is potentiated by the active transcription of *CARMEN*, a finding comparable with recently published findings for other lncRNAs [42]. We also find that *Carmen* directly interacted with PRC2 through both

Fig. 6. Knockdown of *CARMEN* impacts upon cardiac specification and differentiation. Human fetal CPCs were transfected with *CARMEN* GapmeR, with or without MIRNA-143 and -145 mimics, and then differentiated for 10 days. Target gene expression levels of *CARMEN* (A), *GATA4*, *NKX2.5*, *TBX5*, *MYH6* and *MYH7*, *TNNNI*, and *Myh11* (B) and *MIR-143*, *MIR-145*, *ELK1* and *KLF4* (C) were assessed by qRT-PCR. (D) Decreased capacity to produced α-actinin-positive CMs in *CARMEN*-depleted fetal CPCs. *P < 0.05, SEM (n = 3). Scale bars: 20 μm.


A
CARMEN 1
CARMEN 2
CARMEN 3
Relative Expression
Scrambled GapmeR
CARMEN GapmeR
Scrambled MIR mimic
MIR-143 and MIR-145 mimics
B
GATA4
NKX 2.5
TBX5
MYH6
MYH7
TNNI
MYH11
C
MIR-143
MIR-145
ELK1
KLF4
D
α-ACTININ
α-Actinin-positive cells
%
Scrambled GapmeR
CARMEN GapmeR

EZH2 and SUZ12. How *Carmen* targets PRC2 within the genome either in *cis* or *trans* is, however, currently not known. Potentially, *Carmen* may, like other cardiogenic lncRNAs such as *Bvht* and *Fendrr*, either compete for PRC2 binding at specific loci during cardiac differentiation or actively target PRC2 [18,19]. Our work suggests therefore that *Carmen* mediates cardiac specification and differentiation, in part through *cis*- and *trans*-dependent epigenetic regulation, which appears to represent an emerging paradigm for lncRNAs.

The loss-of-function data suggest that *Carmen* is directly implicated in the earliest steps of lineage commitment, able to control the expression of both pluripotency and cardiac mesodermal specifying genes. Since the human *CARMEN* locus does not contain an ortholog of mouse *Bvht*, it suggests that *Bvht* is dispensable for cardiac specification in human CPCs. The loss of *Bvht* in the human lineage represents an interesting evolutionary event in species-specific heart developmental pathways, which requires further investigation. In contrast, the fact that *CARMEN* is conserved and expressed in human supports an evolutionary conserved role in this process. Our work suggests that *CARMEN* functions upstream of and in the same regulatory pathway as EOMES and MESP1 in a miRNA-independent fashion, as a reprogramming factor necessary for cardiac commitment and differentiation of CPCs. Importantly, EOMES and MESP1 can specify all cell types of the cardiovascular lineage [43,44]. MESP1 is able to induce transdifferentiation of dermal fibroblasts into CPCs [45]. This indicates that *CARMEN* could also be a critical factor for inducing somatic cell reprogramming toward a cardiovascular state. A number of recent studies have demonstrated that specific combinations of key cardiac TFs and miRNAs are able to reprogram cardiac fibroblasts into mature CMs in vitro and in vivo [46–49]. This represents a very attractive therapeutic strategy for the promotion of cardiac regeneration. Our data suggest that *CARMEN*, in addition to other lncRNAs identified in the present study, could represent interesting cardiogenic reprogramming factors.

Pathological cardiac remodeling and the subsequent transition to heart failure are typically associated with the re-activation of a fetal gene program [1]. We have previously demonstrated that many developmental cardiac enhancer-associated lncRNAs are transcriptionally induced in the remodeling and failing heart [11,22]. Interestingly, *Carmen* is induced in mouse models of cardiac disease, and human *CARMEN* isoforms are induced in AOS and DCM patients. Importantly, *Carmen*-depleted cardiomyocytes demonstrate reduced expression of cell identity genes. *Carmen* expression appears therefore critical for maintaining a differentiated state in cardiomyocytes. An increasing number of studies are beginning to illustrate the roles of lncRNAs in the adult remodeling heart [20–23], supporting the notion that cardiac lncRNAs, including *CARMEN*, could represent unique targets for modulating the cardiac pathological response, and possibly promoting regeneration [40]. Indeed, it is conceivable that increased *CARMEN* expression in the stressed heart reflects upregulation in a subset of cardiac cells other than cardiomyocytes, in particular CPCs. If proved to be true, *CARMEN* could be involved in initiating a cardiogenic program in CPCs that are mobilized in the adult heart upon damage. In a broader context, the manipulation of super enhancer-associated lncRNAs represents a new avenue for controlling the gene regulatory networks that neocardiogenesis in the adult heart. *CARMEN* could potentially represent therefore an attractive therapeutic target for future regenerative and cell-based therapies.

4. Methods

Detailed methods can be found in Supplementary information.

4.1. Human p300 and super enhancer ChIP-Seq data

For ChIP-Seq analysis of human fetal and adult hearts, we utilized previously published data sets [29,50]. Super enhancer annotations in the adult left ventricle were obtained from [14].

4.2. Cardiac injury models

Ligation of the left anterior descending artery — Myocardial infarction in mice was induced as previously described [22].

4.3. Echocardiography

Transthoracic echocardiographies were performed using a 30-MHz probe and the Vevo 770 Ultrasound machine (VisualSonics, Toronto, ON, Canada).

4.4. Primary cell cultures

Human fetal heart chambers and cardiac progenitor cells were isolated as previously described [27].

4.5. Immunohistochemistry on fetal CPCs

Please refer to Supplementary material online.

4.6. RNA isolation, reverse transcription, end-point PCR and quantitative PCR

Primer sequences for qRT-PCR are provided in Suppl. Table S1. For TaqMan probe based qRT-PCR expression was analyzed using fluorescent-labeled TaqMan Probes (ABI), which are described in Suppl. Table S1. Analysis was carried out using an ABI Prism 7500 cycler and relative expression quantified using the ΔΔCt method. For end-point PCR aliquots of PCR mixtures were taken during different cycles for agarose gel analysis to determine linear range of amplification. All reactions were run on a 1.5% agarose gel stained with Ethidium Bromide.

4.7. Cell culture and transfection

Please refer to Supplemental material online.

4.8. Differential expression analysis of lncRNAs

Total RNA (100 ng) was labeled using Low Input Quick Amp Labeling kit (Agilent 5190-2305) following manufacturer instructions. mRNA was reverse transcribed in the presence of T7-oligo-dT primer to produce cDNA. cDNA was then in vitro transcribed with T7 RNA polymerase in the presence of Cy3-CTP to produce labeled cRNA. The labeled cRNA was hybridized to the Agilent SurePrint G3 gene expression 8x60K microarray according to the manufacturer's protocol (Agilent SurePrint 8 × 60 nt technology). The arrays were washed, and scanned on an Agilent G2565CA microarray scanner at 100% PMT and 3um resolution. Intensity data was extracted using the Feature Extraction software (Agilent). Differentially expressed microarray probes were identified using the limma package in R comparing microarray data from 3 proliferating and 3 differentiating cell cultures. Data sets have been deposited in the Gene Expression Omnibus Database under accession number (pending).

4.9. LncRNA analysis

Phastcons data: Base-level mean PhastCons scores were calculated across the indicated genomic features [7].

4.10. Subcellular localization analysis

Nuclear, Cytoplasmic and Chromatin RNAseq data from human cell lines were obtained from ENCODE [51]. Localization of transcripts was estimated by the ratio of RPKM (reads per kilobase per million mapped reads) values, with the condition that both values must have a non-zero

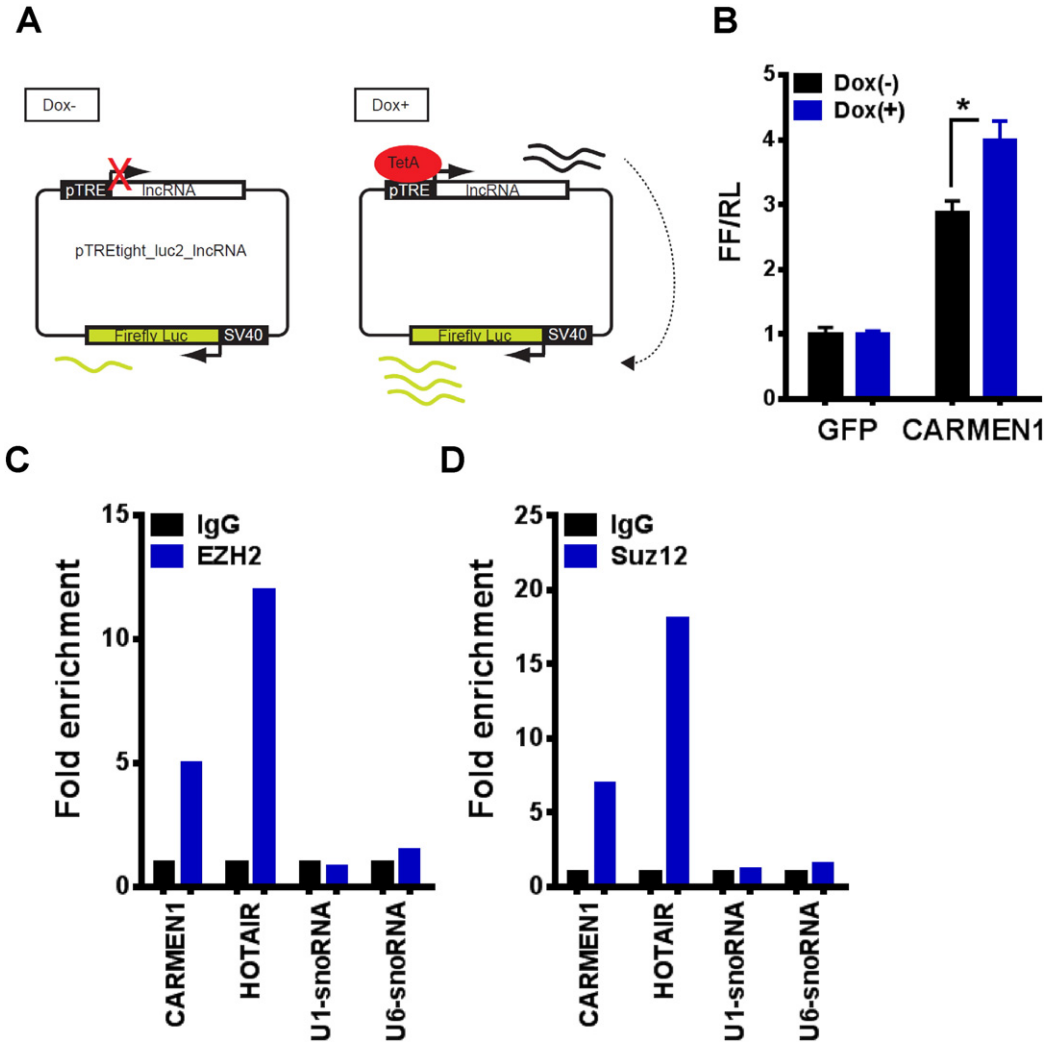


Fig. 7. *CARMEN* is a *cis*-acting regulatory factor and interacts with PRC2. (A) Schematic representation of luciferase-lncRNA reporter constructs used to probe *cis*-acting function of *CARMEN*. (B) Relative luciferase activity of reporter plasmids with and without Doxycycline supplementation. (C and D) RNA Immunoprecipitation (RIP) of lncRNAs using anti-Suz12 and anti-EZH2 antibodies. Black bar represents control IgG and blue bar represents the indicated specific antibody. The indicated immunoprecipitated transcripts were quantified by qRT-PCR *P < 0.05, SEM (n = 3–5).

value and must meet the confidence threshold represented by an IDR value (Irreproducible Discovery Rate) of 0.1 [7].

4.11. Protein-coding potential

We used a series of widely used tools to evaluate protein-coding potential of *CARMEN* isoforms (ENST00000505254, ENST00000518014) using default settings, with the indicated cutoffs. CNCI [52]: >0, CPAT (PMID:23335781): >0.43, CPC [53]: >0.54, iSeeRNA [54]: <0.71, PhyloCSF [30]: >28.34 and RNACode [55]: >37.55. All methods indicate non-protein coding status for both transcript isoforms.

4.12. Cross-species screening pipeline

Please refer to Supplemental material online.

4.13. CARMEN expression in Gtex samples

Tissue RNAseq quantifications were obtained from the Broad Institute in the form of gene-level RPKM [39].

4.14. Chromatin states

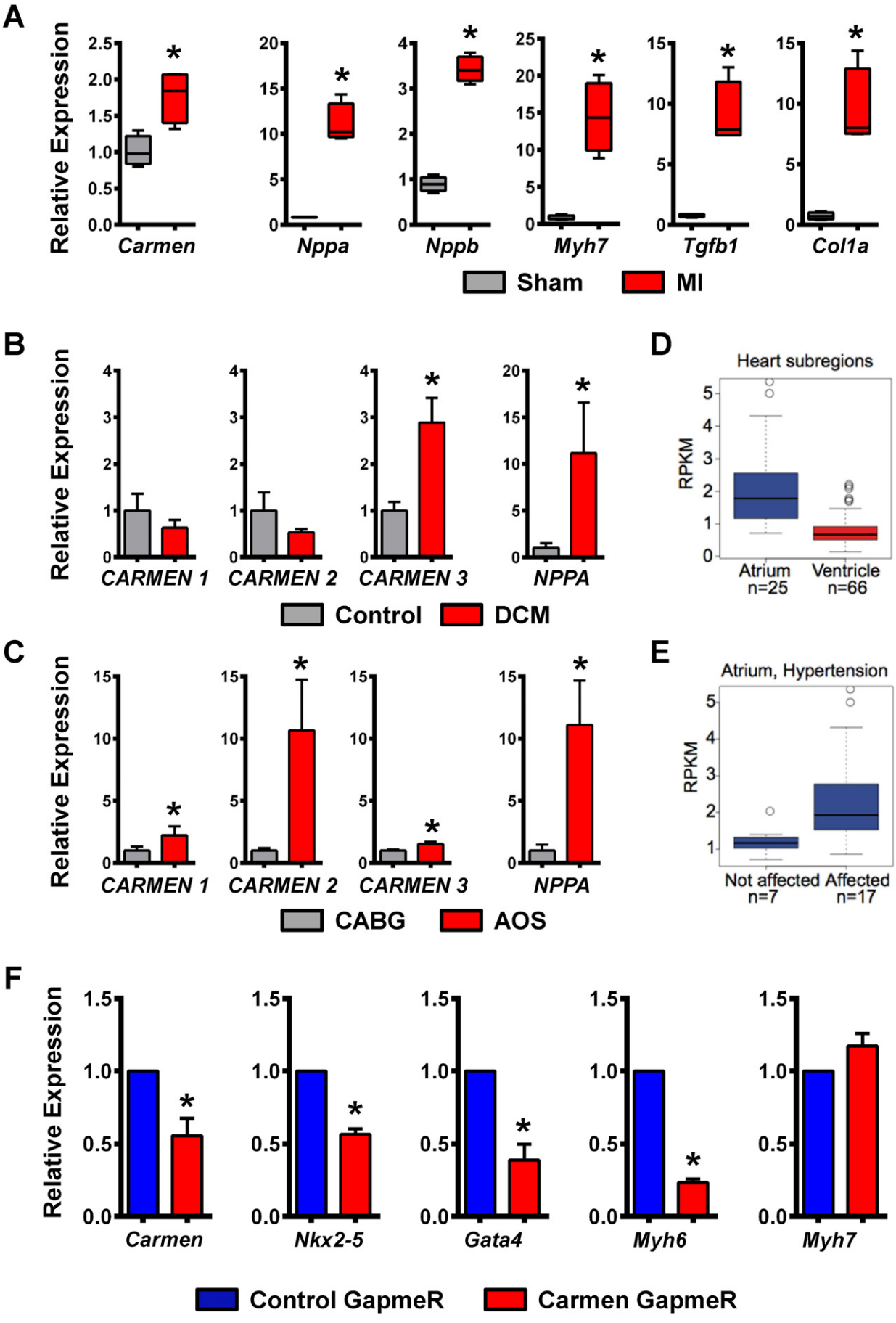
To analyze the presence of chromatin marker peaks at promoters during cardiac differentiation of mouse embryonic stem cells, we used publicly available data published by Wamstad et al. [38].

4.15. Embryonic stem cell culture and differentiation

Mouse embryonic stem (ES) cells were differentiated into CMs as previously described [36].

4.16. P19CL6 cell culture and transfection

P19CL6 cells (RCB2318, RIKEN Cell Bank, Japan) were cultured in DMEM with 10% FCS and antibiotics. Transfection of P19CL6 cells with pLKO.1-puro-UbC-Tag635™ (containing shRNAi, Sigma Aldrich) was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. P19CL6 was a kind gift of Dr. Elizabeth Robertson, University of Oxford, UK.


A
Relative Expression
Carmen
Nppa
Nppb
Myh7
Tgfb1
Col1a
Sham
MI
B
Relative Expression
CARMEN 1
CARMEN 2
CARMEN 3
NPPA
Control
DCM
C
Relative Expression
CARMEN 1
CARMEN 2
CARMEN 3
NPPA
CABG
AOS
D
Heart subregions
RPKM
Atrium n=25
Ventricle n=66
E
Atrium, Hypertension
RPKM
Not affected n=7
Affected n=17
F
Relative Expression
Carmen
Nkx2-5
Gata4
Myh6
Myh7
Control GapmeR
Carmen GapmeR

4.17. Methods related to data obtained in humans

All human material was obtained during routine sampling used for clinical purposes or after obtaining informed consent, stored in a coded way and available for research purposes in accordance with the Declaration of Helsinki and the ethical committee at Maastricht University Medical Center. For further details please refer to Supplemental material online.

4.18. CARMEN LncRNA cloning

Human CARMEN1 was amplified from the FBI-1 cDNA template using primers C222F (TACCGAGCTCGGATCCtcagtgccagctgcttaaaa) and C222R (CTGGACTAGTGGATCCaggcacagtgttagagtttgct) with Expand polymerase (Roche) (PCR conditions: annealing 60 °C, extension 3 min, 1.5 mM Mg, 40 cycles amplification) and cloned into BamHI digested plasmid pcDNA as described [56]. Human CARMEN1 insert was subcloned into inducible plasmid pTretight-SV40-luciferase (*pTre-tight vector from Clontech in which we added a SV40-luciferase ORF). All the clones were checked by Sanger sequencing. (*pTre-tight vector was gift from Susanna de la Luna lab).

4.19. Luciferase assay

We performed luciferase assay using dual-luciferase reporter kit (Promega) following standard protocols.

4.20. RNA immunoprecipitation (RIP)

Cells were detached with Accutase (Millipore), crosslinked in 1% formaldehyde for 15 min and quenched with 2.5 M glycine for 5 min. The cell pellet was resuspended in modified RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) supplemented with RNase inhibitor Superase.In (Ambion) and Complete protease inhibitor (Roche). The cell suspension was briefly sonicated at low amplitude for 5 × 30 s cycles using a Bioruptor sonicator to lyse nuclei. Cell debris was removed by centrifugation at 4 °C, precleared with Protein G dynal beads (Invitrogen) before adding the respective antibodies pre-bound with Protein G dynal beads for 3 h at RT. 25 μg of rabbit anti-SUZ12 antibody (Abcam ab12073) or control rabbit IgG antibody was used for each RIP. Beads were then washed 3 times in modified RIPA buffer, and twice in high salt RIPA buffer (1 M NaCl, 50 mM Tris, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40). Crosslinks were reversed and proteins were digested with Proteinase K (Invitrogen) at 65 °C for 2 h. RNA was extracted in Trizol (Invitrogen) following the manufacturer's instructions. RNA was then reverse transcribed using High Capacity cDNA kit (Ambion) and used for quantitative PCR (RIP-qPCR).

4.21. Statistical analysis

Data throughout the paper are expressed as mean ± SEM. One way ANOVA was used to test significance of data comparisons between experimental groups, with P values < 0.05 were considered significant.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2015.09.016.

Sources of funding

This work is in part funded by a grant from the Swiss National Science Foundation within the frame of the National Research Program 63 on “Stem cells and Regenerative Medicine” (TP; grant no. 406340-128129).

Author contribution

SO, RJ and TP designed the research; SO, RJ, RM, CA, IP, DC, BS, FR, MA, CG, SYN, GB and IP performed the experiments; SO, GB, CN, SH, RG, RJ and TP performed the data analysis; SO and RJ wrote the paper; and SO, RJ and TP edited the manuscript.

Disclosure statement

None to disclose.

Acknowledgments

We thank Benoit Bruneau, Gladstone Institute, San Francisco, CA, for providing access to custom ChIP-Seq tracks. In the process of this analysis, we took advantage of CvDC data produced as part of the Bench-to-Bassinet program. We are grateful to the personnel of the Cardiovascular Assessment Facility, University of Lausanne, Lausanne, Switzerland, for microsurgery and evaluation of cardiac function in the mouse.

References

[1] J.A. Hill, E.N. Olson, Cardiac plasticity, N. Engl. J. Med. 358 (13) (2008 Mar 27) 1370–1380.
[2] J.C. Garbern, R.T. Lee, Cardiac stem cell therapy and the promise of heart regeneration, Cell Stem Cell 12 (6) (2013 Jun 6) 689–698.
[3] B.G. Bruneau, Signaling and transcriptional networks in heart development and regeneration, Cold Spring Harb. Perspect. Biol. 5 (3) (2013 Mar) a008292.
[4] T.R. Mercer, J.S. Mattick, Structure and function of long noncoding RNAs in epigenetic regulation, Nat. Struct. Mol. Biol. 20 (3) (2013 Mar) 300–307.
[5] S. Ounzain, S. Crippa, T. Pedrazzini, Small and long non-coding RNAs in cardiac homeostasis and regeneration, Biochim. Biophys. Acta 1833 (4) (2013 Apr) 923–933.
[6] J.L. Rinn, H.Y. Chang, Genome regulation by long noncoding RNAs, Annu. Rev. Biochem. 81 (2012) 145–166.
[7] T. Derrien, R. Johnson, G. Bussotti, A. Tanzer, S. Djebali, H. Tilgner, et al., The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression, Genome Res. 22 (9) (2012 Sep) 1775–1789.
[8] M.N. Cabili, C. Trapnell, L. Goff, M. Koziol, B. Tazon-Vega, A. Regev, et al., Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses, Genes Dev. 25 (18) (2011 Sep 15) 1915–1927.
[9] X.C. Quek, D.W. Thomson, J.L. Maag, N. Bartonicek, B. Signal, M.B. Clark, et al., lncRNAdb v2.0: expanding the reference database for functional long noncoding RNAs, Nucleic Acids Res. 43 (Database issue) (2015 Jan) D168–D173.
[10] A.C. Marques, J. Hughes, B. Graham, M.S. Kowalczyk, D.R. Higgs, C.P. Ponting, Chromatin signatures at transcriptional start sites separate two equally populated yet distinct classes of intergenic long noncoding RNAs, Genome Biol. 14 (11) (2013 Nov 29) R131.
[11] S. Ounzain, I. Pezzuto, R. Micheletti, F. Burdet, R. Sheta, M. Nemir, et al., Functional importance of cardiac enhancer-associated noncoding RNAs in heart development and disease, J. Mol. Cell. Cardiol. 76C (2014 Aug 19) 55–70.
[12] C.T. Ong, V.G. Corces, Enhancers: emerging roles in cell fate specification, EMBO Rep. 13 (5) (2012 May) 423–430.
[13] M.T. Lam, W. Li, M.G. Rosenfeld, C.K. Glass, Enhancer RNAs and regulated transcriptional programs, Trends Biochem. Sci. 39 (4) (2014 Apr) 170–182.
[14] D. Hnisz, B.J. Abraham, T.I. Lee, A. Lau, V. Saint-Andre, A.A. Sigova, et al., Super-enhancers in the control of cell identity and disease, Cell 155 (4) (2013 Nov 7) 934–947.
[15] W.A. Whyte, D.A. Orlando, D. Hnisz, B.J. Abraham, C.Y. Lin, M.H. Kagey, et al., Master transcription factors and mediator establish super-enhancers at key cell identity genes, Cell 153 (2) (2013 Apr 11) 307–319.

Fig. 8. Dysregulation of *CARMEN* in cardiac pathology. (A) qRT-PCR analysis of *Carmen* and classical stress marker genes, *Nppa*, *Nppb*, *Myh6*, *Myh7*, *Tgfb1* and *Col1a*, in sham-operated and MI hearts. (B) qRT-PCR analysis of *CARMEN1*, *2 and 3*, and *NPPA* in cardiac biopsies from patients suffering from DCM. (C) qRT-PCR analysis of *CARMEN1*, *2 and 3*, and *NPPA* in cardiac biopsies from patients suffering from AOS. (D) Gtex-derived RNA-Seq-based *CARMEN3* expression in human atrium and ventricle. (E) Gtex-derived RNA-Seq-based *CARMEN3* expression in atria from patients suffering from hypertension. (F) Mouse neonatal CMs were transfected with GapmeRs targeting *Carmen* or random scrambled sequence. Cells were harvested 48 h post transfection and assayed for *Carmen*, *Nkx2-5*, *Gata4*, *Myh6* and *Myh7* expression. *P < 0.05, SEM (n = 3–12).

[16] K. Mousavi, H. Zare, S. Dell'orso, L. Grontved, G. Gutierrez-Cruz, A. Derfoul, et al., eRNAs promote transcription by establishing chromatin accessibility at defined genomic loci, Mol. Cell 51 (5) (2013 Sep 12) 606–617.
[17] J.F. Xiang, Q.F. Yin, T. Chen, Y. Zhang, X.O. Zhang, Z. Wu, et al., Human colorectal cancer-specific CCAT1-L lncRNA regulates long-range chromatin interactions at the MYC locus, Cell Res. 24 (9) (2014 Sep) 1150.
[18] C.A. Klattenhoff, J.C. Scheuermann, L.E. Surface, R.K. Bradley, P.A. Fields, M.L. Steinhauser, et al., Braveheart, a long noncoding RNA required for cardiovascular lineage commitment, Cell 152 (3) (2013 Jan 31) 570–583.
[19] P. Grote, L. Wittler, D. Hendrix, F. Koch, S. Wahrisch, A. Beisaw, et al., The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse, Dev. Cell 24 (2) (2013 Jan 28) 206–214.
[20] K. Wang, F. Liu, L.Y. Zhou, B. Long, S.M. Yuan, Y. Wang, et al., The long noncoding RNA CHRF regulates cardiac hypertrophy by targeting miR-489, Circ. Res. 114 (9) (2014 Apr 25) 1377–1388.
[21] K.C. Yang, K.A. Yamada, A.Y. Patel, V.K. Topkara, I. George, F.H. Cheema, et al., Deep RNA sequencing reveals dynamic regulation of myocardial noncoding RNAs in failing human heart and remodeling with mechanical circulatory support, Circulation 129 (9) (2014 Mar 4) 1009–1021.
[22] S. Ounzain, R. Micheletti, T. Beckmann, B. Schroen, M. Alexanian, I. Pezzuto, et al., Genome-wide profiling of the cardiac transcriptome after myocardial infarction identifies novel heart-specific long non-coding RNAs, Eur. Heart J. 36 (6) (2015 Feb 7) 353–368.
[23] P. Han, W. Li, C.H. Lin, J. Yang, C. Shang, S.T. Nurnberg, et al., A long noncoding RNA protects the heart from pathological hypertrophy, Nature 514 (7520) (2014 Oct 2) 102–106.
[24] S.J. Matkovich, J.R. Edwards, T.C. Grossenheider, S.C. de Guzman, G.W. Dorn 2nd., Epigenetic coordination of embryonic heart transcription by dynamically regulated long noncoding RNAs, Proc. Natl. Acad. Sci. U. S. A. 111 (33) (2014 Aug 19) 12264–12269.
[25] Y. Devaux, J. Zangrando, B. Schroen, E.E. Creemers, T. Pedrazzini, C.P. Chang, et al., Long noncoding RNAs in cardiac development and ageing, Nat. Rev. Cardiol. 12 (7) (2015 Jul) 415–425.
[26] R. Kumarswamy, C. Bauters, I. Volkmann, F. Maury, J. Fetisch, A. Holzmann, et al., Circulating long noncoding RNA, LIPCAR, predicts survival in patients with heart failure, Circ. Res. 114 (10) (2014 May 9) 1569–1575.
[27] C. Gonzales, N.D. Ullrich, S. Gerber, C. Berthonneche, E. Niggli, T. Pedrazzini, Isolation of cardiovascular precursor cells from the human fetal heart, Tissue Eng. A 18 (1–2) (2012 Jan) 198–207.
[28] M.E. Ritchie, B. Phipson, D. Wu, Y. Hu, C.W. Law, W. Shi, et al., limma powers differential expression analyses for RNA-sequencing and microarray studies, Nucleic Acids Res. 43 (7) (2015 Apr 20) e47.
[29] D. May, M.J. Blow, T. Kaplan, D.J. McCulley, B.C. Jensen, J.A. Akiyama, et al., Large-scale discovery of enhancers from human heart tissue, Nat. Genet. 44 (1) (2012 Jan) 89–93.
[30] M.F. Lin, I. Jungreis, M. Kellis, PhyloCSF: a comparative genomics method to distinguish protein coding and non-coding regions, Bioinformatics 27 (13) (2011 Jul 1) i275–i282.
[31] M. Wilhelm, J. Schlegl, H. Hahne, A. Moghaddas Gholami, M. Lieberenz, M.M. Savitski, et al., Mass-spectrometry-based draft of the human proteome, Nature 509 (7502) (2014 May 29) 582–587.
[32] J. Ma, C.C. Ward, I. Jungreis, S.A. Slavoff, A.G. Schwaid, J. Neveu, et al., Discovery of human sORF-encoded polypeptides (SEPs) in cell lines and tissue, J. Proteome Res. 13 (3) (2014 Mar 7) 1757–1765.
[33] N. Xu, T. Papagiannakopoulos, G. Pan, J.A. Thomson, K.S. Kosik, MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells, Cell 137 (4) (2009 May 15) 647–658.
[34] K.R. Cordes, N.T. Sheehy, M.P. White, E.C. Berry, S.U. Morton, A.N. Muth, et al., miR-145 and miR-143 regulate smooth muscle cell fate and plasticity, Nature 460 (7256) (2009 Aug 6) 705–710.
[35] J.M. Boucher, S.M. Peterson, S. Urs, C. Zhang, L. Liaw, The miR-143/145 cluster is a novel transcriptional target of Jagged-1/Notch signaling in vascular smooth muscle cells, J. Biol. Chem. 286 (32) (2011 Aug 12) 28312–28321.
[36] A.M. Wobus, G. Kaomei, J. Shan, M.C. Wellner, J. Rohwedel, G. Ji, et al., Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes, J. Mol. Cell. Cardiol. 29 (6) (1997 Jun) 1525–1539.
[37] K. Monzen, I. Shiojima, Y. Hiroi, S. Kudoh, T. Oka, E. Takimoto, et al., Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase kinase kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4, Mol. Cell. Biol. 19 (10) (1999 Oct) 7096–7105.
[38] J.A. Wamstad, J.M. Alexander, R.M. Truty, A. Shrikumar, F. Li, K.E. Eilertson, et al., Dynamic and coordinated epigenetic regulation of developmental transitions in the cardiac lineage, Cell 151 (1) (2012 Sep 28) 206–220.
[39] G.T. Consortium, The Genotype-Tissue Expression (GTEx) project, Nat. Genet. 45 (6) (2013 Jun) 580–585.
[40] S. Ounzain, T. Pedrazzini, The promise of enhancer-associated long noncoding RNAs in cardiac regeneration, TCM 25 (7) (Oct 2015) 592–602.
[41] H. Zhang, J. Hu, M. Recce, B. Tian, PolyA_DB: a database for mammalian mRNA polyadenylation, Nucleic Acids Res. 33 (Database issue) (2005 Jan 1) D116–D120.
[42] K.W. Vance, S.N. Sansom, S. Lee, V. Chalei, L. Kong, S.E. Cooper, et al., The long non-coding RNA Paupar regulates the expression of both local and distal genes, EMBO J 33 (4) (2014 Feb 18) 296–311.
[43] I. Costello, I.M. Pimeisl, S. Drager, E.K. Bikoff, E.J. Robertson, S.J. Arnold, The T-box transcription factor Eomesodermin acts upstream of Mesp1 to specify cardiac mesoderm during mouse gastrulation, Nat. Cell Biol. 13 (9) (2011 Sep) 1084–1091.
[44] J. van den Ameele, L. Tiberi, A. Bondue, C. Paulissen, A. Herpoel, M. Iacovino, et al., Eomesodermin induces Mesp1 expression and cardiac differentiation from embryonic stem cells in the absence of activin, EMBO Rep. 13 (4) (2012 Apr) 355–362.
[45] J.F. Islas, Y. Liu, K.C. Weng, M.J. Robertson, S. Zhang, A. Prejusa, et al., Transcription factors ETS2 and MESP1 transdifferentiate human dermal fibroblasts into cardiac progenitors, Proc. Natl. Acad. Sci. U. S. A. 109 (32) (2012 Aug 7) 13016–13021.
[46] M. Ieda, J.D. Fu, P. Delgado-Olguin, V. Vedantham, Y. Hayashi, B.G. Bruneau, et al., Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors, Cell 142 (3) (2010 Aug 6) 375–386.
[47] L. Qian, Y. Huang, C.I. Spencer, A. Foley, V. Vedantham, L. Liu, et al., In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes, Nature 485 (7400) (2012 May 31) 593–598.
[48] K. Song, Y.J. Nam, X. Luo, X. Qi, W. Tan, G.N. Huang, et al., Heart repair by reprogramming non-myocytes with cardiac transcription factors, Nature 485 (7400) (2012 May 31) 599–604.
[49] T.M. Jayawardena, B. Egemnazarov, E.A. Finch, L. Zhang, J.A. Payne, K. Pandya, et al., MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes, Circ. Res. 110 (11) (2012 May 25) 1465–1473.
[50] M.J. Blow, D.J. McCulley, Z. Li, T. Zhang, J.A. Akiyama, A. Holt, et al., ChIP-Seq identification of weakly conserved heart enhancers, Nat. Genet. 42 (9) (2010 Sep) 806–810.
[51] S. Djebali, C.A. Davis, A. Merkel, A. Dobin, T. Lassmann, A. Mortazavi, et al., Landscape of transcription in human cells, Nature 489 (7414) (2012 Sep 6) 101–108.
[52] H. Luo, D. Bu, L. Sun, R. Chen, Y. Zhao, De novo approach to classify protein-coding and noncoding transcripts based on sequence composition, Methods Mol. Biol. 1182 (2014) 203–207.
[53] L. Kong, Y. Zhang, Z.Q. Ye, X.Q. Liu, S.Q. Zhao, L. Wei, et al., CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine, Nucleic Acids Res. 35 (Web Server issue) (2007 Jul) W345–W349.
[54] K. Sun, X. Chen, P. Jiang, X. Song, H. Wang, H. Sun, iSeeRNA: identification of long intergenic non-coding RNA transcripts from transcriptome sequencing data, BMC Genomics 14 (Suppl. 2) (2013) S7.
[55] S. Washietl, S. Findeiss, S.A. Muller, S. Kalkhof, M. von Bergen, I.L. Hofacker, et al., RNAcode: robust discrimination of coding and noncoding regions in comparative sequence data, RNA 17 (4) (2011 Apr) 578–594.
[56] Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods. 2009 May; 6(5): 343–5.