Stem Cell Reports
Article

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CITED2 Cooperates with ISL1 and Promotes Cardiac Differentiation of Mouse Embryonic Stem Cells

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http://dx.doi.org/10.1016/j.stemcr.2016.10.002

SUMMARY

The transcriptional regulator CITED2 is essential for heart development. Here, we investigated the role of CITED2 in the specification of cardiac cell fate from mouse embryonic stem cells (ESC). The overexpression of CITED2 in undifferentiated ESC was sufficient to promote cardiac cell emergence upon differentiation. Conversely, the depletion of *Cited2* at the onset of differentiation resulted in a decline of ESC ability to generate cardiac cells. Moreover, loss of *Cited2* expression impairs the expression of early mesoderm markers and cardiogenic transcription factors (*Isl1*, *Gata4*, *Tbx5*). The cardiogenic defects in *Cited2*-depleted cells were rescued by treatment with recombinant CITED2 protein. We showed that *Cited2* expression is enriched in cardiac progenitors either derived from ESC or mouse embryonic hearts. Finally, we demonstrated that CITED2 and ISL1 proteins interact physically and cooperate to promote ESC differentiation toward cardiomyocytes. Collectively, our results show that *Cited2* plays a pivotal role in cardiac commitment of ESC.

INTRODUCTION

Cardiac morphogenesis results from the specification, differentiation, and migration of spatially and temporally distinct sets of cardiac precursor cells that give rise to the mature cardiac tissue. The first multipotent cardiogenic cells originate from the mesoderm formed at early stages of gastrulation when cells of the epiblast ingress to the primitive streak (Costello et al., 2011; David et al., 2011; Garry and Olson, 2006; Kitajima et al., 2000; Tam et al., 1997). The mesoderm first expresses the markers BRACHYURY and FLK1 and subsequently the cardiogenic marker MESP1 (Bondue et al., 2008; Chan et al., 2013; Ishitobi et al., 2011; Saga et al., 1999). The first (FHF) and second (SHF) heart fields then arise from the cardiogenic mesoderm to ultimately generate the atria, ventricles, and outflow tract of the nascent heart (Cai et al., 2003; Domian et al., 2009; Moretti et al., 2006). Both FHF and SHF progenitors express the transcription factors NKX2.5, TBX5, GATA4, MEF2C, and ISL1, although TBX5 is predominantly present in cells of the FHF, and Isl1 expression is a hallmark of SHF progenitors (Laugwitz et al., 2005; Moretti et al., 2006; Vincent et al., 2010). In mouse, the transcriptional modulator CITED2 is required for normal embryogenesis. Deletion of *Cited2* in the epiblast results in embryonic lethality associated with multiple cardiovascular defects (Bamforth et al., 2001, 2004; MacDonald et al., 2008, 2013; Weninger et al., 2005; Yin et al., 2002). Of important note, however, although *Cited2* is expressed in the early mesoderm (Dunwoodie et al., 1998), conditional deletion of *Cited2* in BRACHYURY-expressing mesoderm cells or MESP1-expressing cardiogenic mesoderm progenitors did not significantly affect cardiac development (MacDonald et al., 2008). In humans, mutations in the gene encoding CITED2 are associated with congenital heart disease (Chen et al., 2012; Sperling et al., 2005).

The specification and differentiation of cardiac progenitor cells (CPC) and mature cardiovascular cells during the in vitro differentiation of pluripotent stem cells recapitulate the cellular and molecular processes of embryonic development (Blin et al., 2010; Bondue et al., 2008, 2011; Christoforou et al., 2008; Gai et al., 2009; Kattman et al., 2011; Kouskoff et al., 2005; Laugwitz et al., 2005; Moretti et al., 2006; Van Vliet et al., 2012; Yang et al., 2008). In mouse, an acute *Cited2* depletion reduces the self-renewal capacity of most embryonic stem cells (ESC), but a small population of *Cited2*-null ESC with apparent characteristics of undifferentiated cells adapt to the loss of *Cited2* (Kranc et al., 2015; Li et al., 2012). Interestingly, *Cited2*-null ESC showed an impairment of differentiation, including cardiac commitment (Li et al., 2012). To better understand the role of *Cited2* at early stages of mouse ESC differentiation, we here employ *Cited2* loss- and gain-of-function approaches to examine the role of *Cited2* during cardiac differentiation. *Cited2* depletion at the onset of differentiation significantly impairs the expression of *Brachyury*, *Mesp1*, *Isl1*, *Gata4*, and *Tbx5*. Conversely, CITED2 overexpression stimulates the expression of these genes in undifferentiated ESC and promotes cardiac lineage commitment and differentiation. We further show that *Cited2* expression is highly associated with CPC populations, particularly cardiac progenitors of the SHF. Finally, we show that CITED2 is recruited to the promoter of the *Isl1* gene, and provide evidence that the human CITED2 and ISL1 proteins physically interact and synergize to promote cardiogenesis from ESC. Collectively our results show that *Cited2* is a key regulator of early cardiac lineage commitment and differentiation of ESC.

RESULTS

CITED2 Overexpression Promotes ESC Differentiation to Cardiac Lineages

The knockout of *Cited2* in mouse ESC has been previously reported to impair cardiomyocyte differentiation (Li et al., 2012). To investigate whether the overexpression of CITED2 promotes ESC differentiation into cardiac lineages, we transfected undifferentiated mouse E14/T ESC with an episomal plasmid expressing a FLAG-tagged human CITED2 (flag-CITED2) or a control vector as previously described (Kranc et al., 2015). Upon differentiation, high levels of flag-CITED2 expression were detected in E14/T ESC (named E14T/flagCITED2 hereafter) at the onset of differentiation (day 0 [D0]) in comparison with the endogenous *Cited2* expression in control cells (named E14T/Control hereafter). However, the ectopic expression of flag-CITED2 in cells derived from E14T/flagCITED2 ESC rapidly declined during the first 3 days of differentiation, and returned to control levels by D6 (Figure 1A). An increase in the number of spontaneous contractile clusters (beating foci), marking the occurrence of terminal cardiomyocyte differentiation, was observed in cell cultures derived from E14T/flagCITED2 ESC in comparison with control cells (Figure 1B). Thus, the overexpression of flag-CITED2 significantly promoted cardiomyocyte differentiation, as confirmed by the elevated expression of cardiac structural genes, such as *α-cardiac myosin heavy chain* (*Myh6*) and *cardiac troponin T* (*cTnT*) transcripts detected in these cells in comparison with control cells (Figure 1C). The *vascular endothelial growth factor receptor 2* (*VegfR2*, a marker of endothelial differentiation) expression was also increased in cells derived from E14T/flagCITED2 ESC, while the expression of the *skeletal troponin I* (*sTnI*, a marker of skeletal muscle differentiation) was unaffected by the overexpression of flag-CITED2 (Figure S1A), suggesting that flag-CITED2 overexpression supports ESC specification to cardiovascular cell lineages. To unravel the mechanism by which flag-CITED2 promoted the cardiac differentiation process, we assessed the expression of transcription factors known to play critical roles in the specification of ESC to cardiac lineages, particularly *Isl1*, *Gata4*, *Nkx2.5*, and *Tbx5*. Surprisingly, the overexpression of flag-CITED2 significantly increased the transcript levels of these factors in undifferentiated ESC (D0), and of *Isl1* and *Gata4* at D6 of differentiation (Figure 1D). The increase in the expression of Isl1 and Gata4 proteins was also detected in undifferentiated E14/T ESC overexpressing flag-CITED2 (Figure S1B). Since CITED2 is a transcriptional modulator, we hypothesized that flag-CITED2 enhanced *Isl1*, *Gata4*, *Nkx2.5*, and *Tbx5* expression by a direct effect on their transcriptional regulatory regions. Therefore, we investigated by chromatin immunoprecipitation (ChIP) assays whether flag-CITED2 was recruited at the promoters of these factors. A significant enrichment of the *Isl1* promoter region was detected in E14T/flagCITED2 ESC extracts immunoprecipitated with an anti-flag antibody in comparison with E14T/Control extracts (Figure 1E). No difference of enrichment was observed for the exon 1 of *Isl1*, or for the *Nkx2.5*, *Gata4*, and *Tbx5* promoter fragments tested in the same conditions (Figure 1E). Interestingly, flag-CITED2 overexpression also significantly enhanced the occupancy of *Isl1* promoter and exon 1 by histones H3 trimethylated on lysine 4 (H3triMeK4), a mark of actively transcribed chromatin (Figure 1F). No enrichment of H3triMeK4 occupancy was detected at the promoters of *Nkx2*.5, *Gata4*, and *Tbx5* in the same extracts. Although these experiments do not enable us to rule out the presence of flag-CITED2 at other regulatory elements of *Nkx2.5*, *Gata4*, and *Tbx5* genes, they exposed its presence at the *Isl1* promoter. Moreover, the presence of flag-CITED2 at *Isl1* regulatory regions was also correlated with an increased recruitment of H3triMeK4 at these regions, suggesting that flag-CITED2 may exert a direct positive effect on the expression of *Isl1*.

Depletion of *Cited2* in ESC Differentiation Affects Cardiac Commitment

To further investigate the role of Cited2 in cardiac differentiation, we used $C2^{fl/fl}$[Cre] ESC, which allow the conditional knockout of *Cited2* by supplementation of 4-hydroxytamoxifen (4HT) to the culture medium (Kranc et al., 2015). First, we analyzed *Cited2* expression kinetics by qPCR during the differentiation of control $C2^{fl/fl}$[Cre] ESC (Figures 2A and 2B, black bars). The pattern of *Cited2* expression was biphasic, with a decrease of the transcript levels from D0 to D2 of differentiation, followed by an elevation from D3 onward (Figure 2B, black bars).

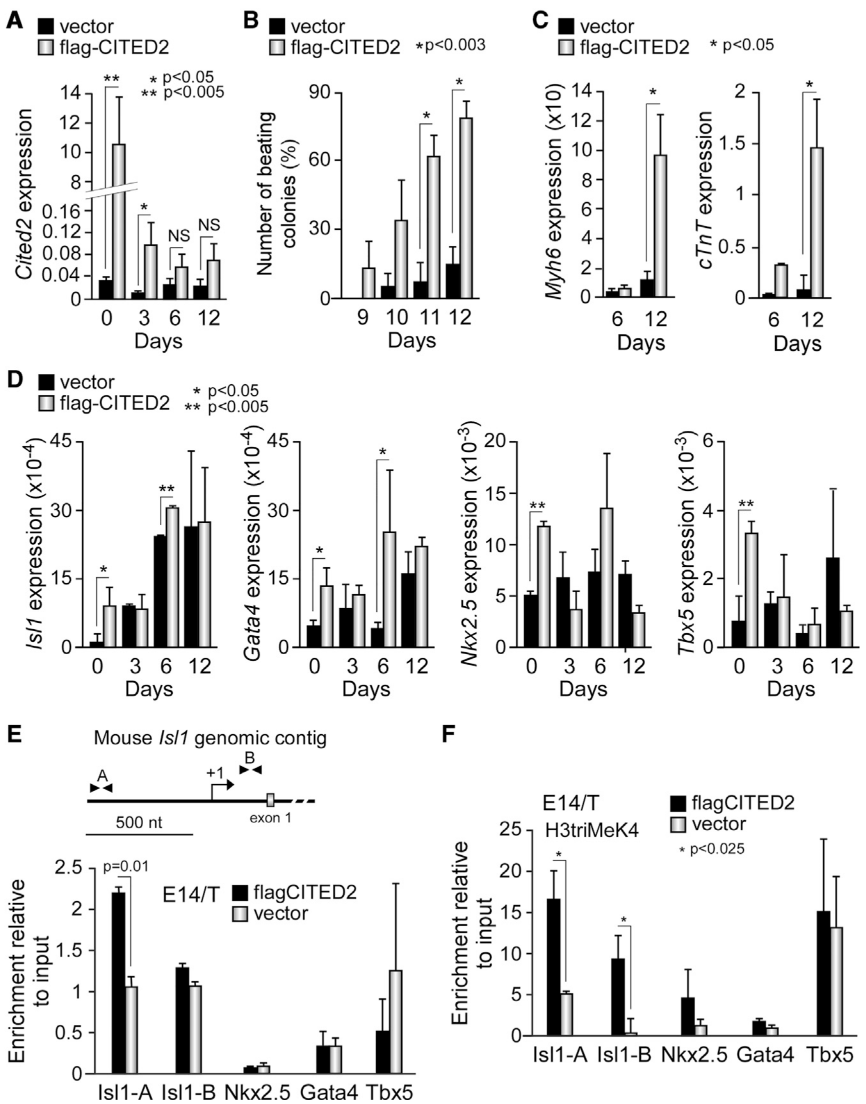

Figure 1. Ectopic Expression of Human CITED2 Promotes Cardiac Differentiation
(A) Relative expression of flag-Cited2 during differentiation, determined by qPCR in E14/T ESC transfected with a plasmid expressing FLAG-tagged CITED2 (flag-CITED2) or the control empty plasmid (vector). NS, not significant.
(B) Number of colonies with beating foci counted at the indicated days of differentiation in cells treated as described in (A).
(C) Expression levels of *Myh6* and *cTnT* determined by qPCR at D6 and D12 of differentiation in cells treated as described in (A).
(D) Expression of *Isl1*, *Gata4*, *Nkx2.5*, and *Tbx5* at D0, D3, D6, and D12 of differentiation in E14/T-derived cell extracts prepared as described in (A).
(E) Top: diagram of the mouse *Isl1* genomic contig showing the transcriptional start site (arrow), exon 1 (gray box), and positions of PCR primers (arrow heads) used in ChIP assays. Bottom: enrichment of *Isl1* promoter (Isl1-A) and exon 1 (Isl1-B), and *Nkx2.5*, *Gata4*, and *Tbx5* promoters in extracts of undifferentiated E14/T ESC overexpressing flag-CITED2 or control cells by ChIP assays with anti-FLAG monoclonal antibody.
(F) Enrichment of the genomic regions of *Isl1*, *Nkx2.5*, *Gata4*, and *Tbx5* in ChIP assays with extracts and primers described in (E), using an anti-histone H3triMeK4-specific antibody.
Results are presented as the mean ± SEM of three independent biological experiments.

A similar expression pattern for *Cited2* was also observed in E14T/Control cells (Figure 1A, black bars). Next, we examined *Cited2* expression in cells derived from $C2^{fl/fl}$[Cre] ESC treated with 4HT at D0 of differentiation during 48 hr, and compared it with control cells derived from $C2^{fl/fl}$[Cre] ESC treated with ethanol used as vehicle (Figures 2A–2C). *Cited2* depletion was incomplete during the time course of differentiation since *Cited2* transcripts remained detectable at D5 and D12 of differentiation in cells treated with 4HT, although these levels were significantly reduced in comparison with control cells (Figure 2B). Noticeably, the number of beating foci was reduced in cell cultures derived from ESC depleted of *Cited2* at D0 compared with control cells (Figure 2D). The decline of *Myh6* and *cTnT* transcripts expression and the reduced number of cells expressing cTNT protein in cultures treated with 4HT supported the requirement of *Cited2* expression for ESC differentiation into cardiac lineages (Figures 2E and 2F). To evaluate the expression and organization of sarcomeric proteins in cardiomyocytes, we performed fluorescent immunodetection of the α-ACTININ and MYOSIN HEAVY CHAIN (MF20) proteins in cells at D10 of differentiation derived from $C2^{fl/fl}$[Cre] ESC treated either with ethanol or 4HT at D0 (Figure 2G). Approximately 25%–30% of the cells derived from control $C2^{fl/fl}$[Cre] ESC were positively stained for α-ACTININ or MF20 and presented some degree of organization of the sarcomeric apparatus (Figures 2G and S1C). The depletion of *Cited2* by treatment of $C2^{fl/fl}$[Cre] ESC with 4HT resulted in the decline of α-ACTININ and MF20 protein detection, and in the diminution of the number of cells stained for α-ACTININ or MF20 in comparison with the control cells (Figures 2G and S1C). Together, these

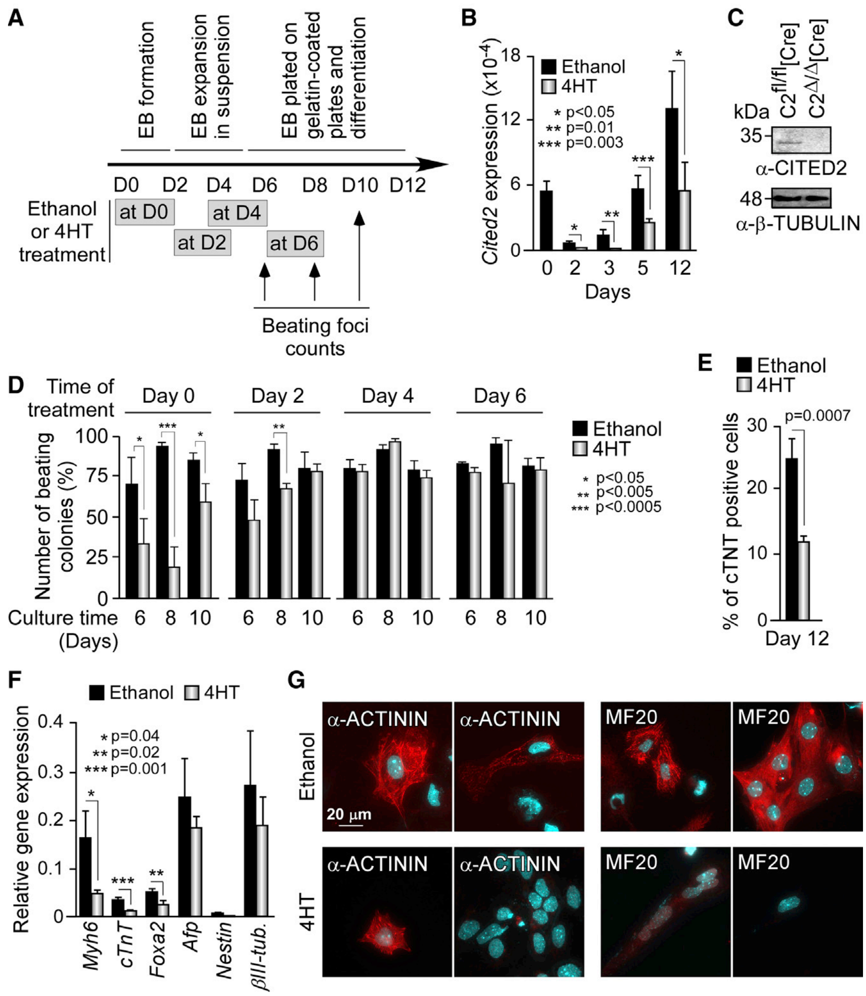

Figure 2. *Cited2* Is Important for Early Steps of Cardiac Differentiation

(A) Timeline depicting the protocol used for differentiation of mouse ESC from D0 onward. The time of ethanol or 4HT treatment and the days of beating activity assessment are indicated.

(B) Expression of *Cited2* determined by qPCR at D0, D2, D3, D5, and D12 of differentiation in cells derived from $C2^{fl/fl}$[Cre] ESC treated at D0 with 4HT or ethanol for 48 hr.

(C) CITED2 protein levels determined by western blotting in extracts from $C2^{fl/fl}$[Cre] ESC, 48 hr after incubation with ethanol or 4HT. Loading in each lane was monitored by detection of β-TUBULIN.

(D) Percentage of colonies with beating foci derived from $C2^{fl/fl}$[Cre] ESC treated with ethanol or 4HT at D0, D2, D4, and D6 of differentiation.

(E) Quantification by flow cytometry of cells expressing cTNT at D12, treated as described in (B). The percentage of cells expressing cTNT over the total number of cells is indicated.

(F) Expression levels determined by qPCR of *Myh6*, *cTnT*, *Foxa2*, α-fetoprotein (*Afp*), *Nestin*, and *βIII-tubulin* (*βIII-tub.*) at D12 of differentiation in cells treated as described in (B).

(G) Immunofluorescent detection of α-ACTININ (left panels, red staining) and MYOSIN HEAVY CHAIN MF20 (right panels, red staining) in $C2^{fl/fl}$[Cre] ESC-derived cells at D10 of differentiation, after treatment for 2 days with ethanol (upper panels) or 4HT (lower panels) at D0. Nuclei were counterstained using DAPI (blue), and cells were examined on at 100× magnification.

Results in (B), (D), (E), and (F) are presented as the mean ± SEM of three independent biological experiments.

observations were consistent with the requirement of *Cited2* for ESC-derived cardiogenesis. In contrast, *Cited2* depletion did not significantly alter the expression of *Nestin* and *βIII-Tubulin* (markers of neural cells, ectoderm origin) and the α-fetoprotein (hepatic marker, endoderm origin). *Cited2* depletion did result in a reduction of *Foxa2* (endoderm and hepatocyte marker) expression at D12 (Figure 2F), consistent with the role played by *Cited2* in liver development (Qu et al., 2007). Interestingly, the treatment of $C2^{fl/fl}$[Cre] ESC with 4HT at D2 of differentiation only mildly affected the emergence of beating clusters, while no alteration was observed after 4HT treatment at D4 or D6 of differentiation in comparison with control cells (Figure 2D). These observations suggest that *Cited2* supports ESC specification to cardiomyocyte lineages during the first 2–3 days of differentiation. In agreement with these results, we showed that *Cited2*-null $C2^{\Delta/\Delta}$[LA11] ESC devoid of *Cited2* expression (Kranc et al., 2015) have a reduced capacity to generate cardiac contractile clusters when compared to control $C2^{fl/fl}$ ESC (Figure 3A). To confirm that the cardiac differentiation defects observed in *Cited2*-depleted cells were due to the loss of *Cited2* expression, $C2^{fl/fl}$[Cre] ESC treated with 4HT at D0 were transduced with a human recombinant CITED2 protein (sharing interchangeable functions with the mouse protein in cardiac development [Chen et al., 2012; Kranc et al., 2015]) fused at its N-terminal domain with a stretch of eight arginines (termed 8R-CITED2). Polyarginine peptides are transduction domains that enable proteins to cross the cellular membrane when applied to the culture medium (Lundberg et al., 2003). The purified 8R-CITED2 added into the culture medium crossed the cellular membrane, translocated into the nucleus of *Cited2*-null ESC, and interfered with the high-affinity binding of overexpressed human CITED2

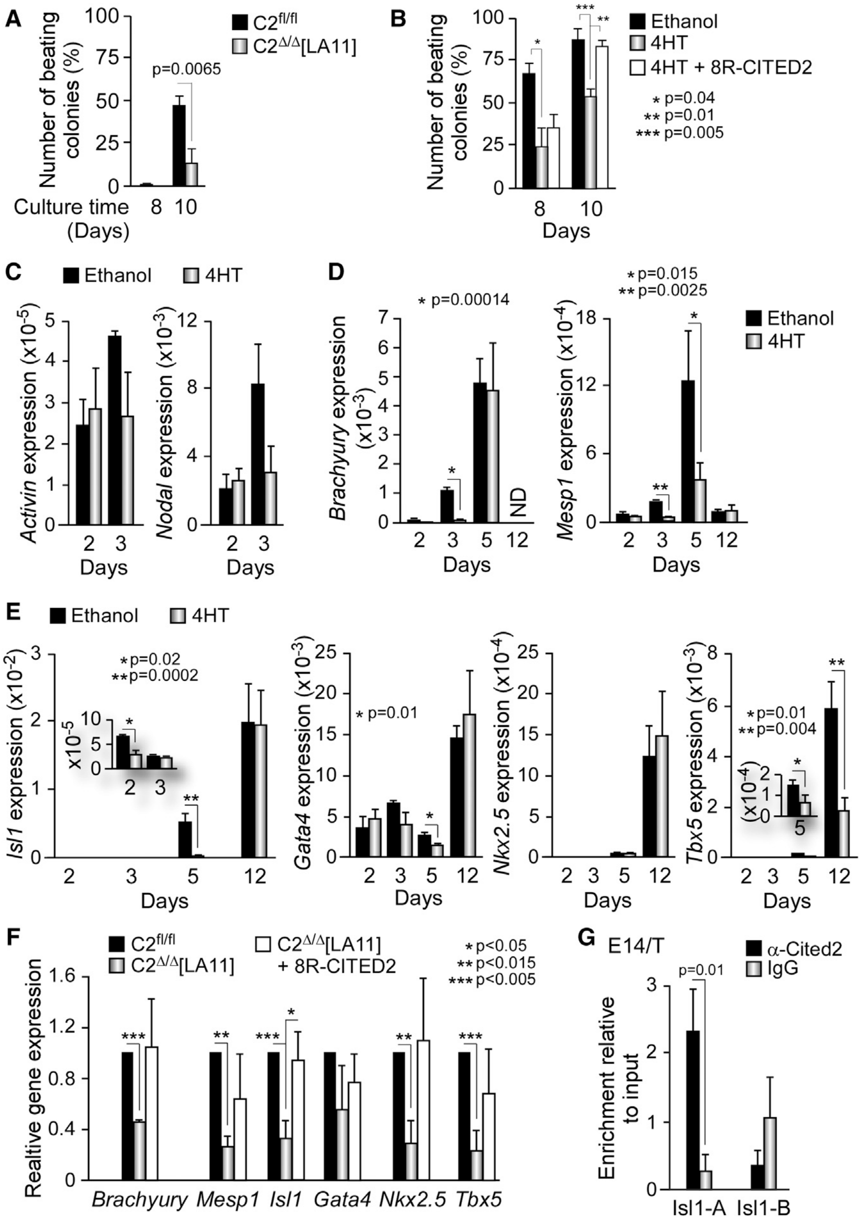

Figure 3. Loss of *Cited2* Impairs Expression of Genes Specifying Cardiac Mesoderm

(A) Percentage of colonies with beating foci derived from $C2^{fl/fl}$ and $C2^{\Delta/\Delta}$[LA11] ESC at D8 and D10 of differentiation.

(B) Percentage of colonies with beating foci counted at 8 and 10 days after the initiation of differentiation in cell cultures derived from $C2^{fl/fl}$[Cre] ESC treated with ethanol or 4HT at D0 of differentiation, and with 4HT at D0 of differentiation and supplemented with recombinant 8R-CITED2 protein at D2 of differentiation (4HT + 8R-CITED2).

(C) Expression of *Activin A* and *Nodal* determined by qPCR at D2 and D3 of differentiation in cultures derived from $C2^{fl/fl}$[Cre] ESC treated with 4HT or ethanol at D0 for 48 hr.

(D) Expression of mesoderm markers (*Brachyury* and *Mesp1*) at D2, D3, D5, and D12 of differentiation in cells generated from $C2^{fl/fl}$[Cre] ESC treated as described in (Figure 2B).

(E) Expression of *Isl1*, *Gata4*, *Nkx2.5*, and *Tbx5* in cell cultures as described in (D). The inserts for *Isl1* and *Tbx5* detail the expression of these genes at D5 and D3, respectively.

(F) Relative expression of *Brachyury*, *Mesp1*, *Isl1*, *Gata4*, *Nkx2.5*, and *Tbx5* determined by qPCR at D5 of differentiation in cultures derived from untreated $C2^{fl/fl}$ and $C2^{\Delta/\Delta}$[LA11] ESC, and $C2^{\Delta/\Delta}$[LA11] ESC supplemented with the recombinant 8R-CITED2 protein ($C2^{\Delta/\Delta}$[LA11] + 8R-CITED2) at D2 of differentiation for 48 hr. Gene expression in $C2^{fl/fl}$ ESC was set to 1.

(G) Enrichment of *Isl1* genomic regions in extracts of E14/T ESC-derived cells at D5 by ChIP assays with anti-CITED2 or control (immunoglobulin G) polyclonal antibodies.

Results are presented as the mean ± SEM of three independent biological experiments.

protein to the CH1 domain of p300 (Freedman et al., 2003), suggesting that 8R-CITED2 is functional (Figure S2). Interestingly, the supplementation of 8R-CITED2 at D2 of differentiation in the culture medium of $C2^{fl/fl}$[Cre] ESC treated with 4HT at D0 restored the emergence of beating foci to control levels (Figure 3B). These results argue that the cardiogenic defects of *Cited2*-depleted ESC are caused by the loss of Cited2 expression. Altogether, these observations suggested that *Cited2* is important for early events of ESC commitment to cardiac cell lineages.

Cited2 Is Necessary for the Expression of Genes Specifying Cardiac Mesoderm and Cardiac Progenitors

During early differentiation, the expression of pluripotency markers *Oct4*, *Sox2*, and *Nanog* was silenced with comparable rates in control and 4HT-treated cells (Figure S1D). Thus, a misregulation of pluripotency factors cannot account for the deficiency in cardiac differentiation of *Cited2*-depleted ESC. Next, we assessed the expression of genes marking early (cardiac) mesoderm induction such as *Activin A*, *Nodal*, *Brachyury*, and *Mesp1* (Figures 3C and 3D). No significant alteration in *Nodal* and *Activin A* expression was observed at D2 and D3 in control and D0 *Cited2*-depleted cell cultures (Figure 3C). In contrast, *Brachyury* expression was significantly reduced at D3 but restored at D5, and *Mesp1* expression was markedly downregulated at D3 and D5 (Figure 3D). The decrease of *Brachyury* and *Mesp1* expression is in agreement with a previous report that had indicated that *Cited2* supports mesoderm differentiation (Li et al., 2012) and with our observations suggesting that CITED2 is important during the first 2–3 days of ESC differentiation (Figure 2). In addition, the expression of *Isl1*, *Gata4*, and *Tbx5* transcripts, as well as the expression of Isl1 and Gata4 proteins, was reduced at D5 of differentiation in *Cited2*-depleted cells (Figures 3E and S1B). *Isl1* expression levels were also lower at D2 while *Tbx5* expression remained decreased at D12 (Figure 3E). The expression of *Nkx2.5* was unchanged in similar conditions (Figure 3E).

Since the treatment of $C2^{fl/fl}$[Cre] ESC with 4HT at D0 only achieved a partial knockout of *Cited2* (Figure 2B), the remaining *Cited2* expression may confound the effects of *Cited2* depletion on gene expression. Therefore, to clarify the consequences of *Cited2* loss on *Brachyury*, *Mesp1*, *Isl1*, *Gata4*, *Nkx2.5*, and *Tbx5* expression, we differentiated control $C2^{fl/fl}$ ESC and *Cited2*-null $C2^{\Delta/\Delta}$[LA11] ESC, which are impaired for cardiac differentiation (Figure 3B). In this context *Gata4* expression was not altered, but a significant downregulation of *Brachyury*, *Mesp1*, *Isl1*, *Nkx2.5*, and *Tbx5* expression was detected in cultures derived from $C2^{\Delta/\Delta}$[LA11] ESC at D5 of differentiation in comparison with control cells (Figure 3F). Interestingly, supplementation of $C2^{\Delta/\Delta}$[LA11] ESC-derived cells at D2 of differentiation with 8R-CITED2, stimulated *Brachyury*, *Mesp1*, *Isl1*, *Nkx2.5*, and *Tbx5* expression at D5 of differentiation with *Isl1* expression being restored to control levels (Figure 3F). This observation further argues for a mechanistic link between *Cited2* and *Isl1* expression levels. To determine whether endogenous CITED2 is recruited to the promoter of *Isl1* at D5 of differentiation, we performed ChIP using anti-CITED2 and control polyclonal antibodies with cellular extracts prepared from cells derived from E14/T ESC (Figure 3G). Endogenous CITED2 was specifically detected at the promoter region of *Isl1* in these cells at D5 of differentiation, suggesting that CITED2 binds to *Isl1* promoter in cells derived from ESC. Together, these observations suggested that *Cited2* not only supports mesoderm induction but also regulates the expression of key genes during CPC specification including *Isl1*.

Cited2 Is Expressed in Cardiac Progenitors

Since *Cited2* expression was associated with the expression of CPC markers, such as *Isl1*, *Nkx2.5*, *Gata4*, and *Tbx5* (Figures 1D, 3E, and 3F), we sought to determine whether *Cited2* was expressed in CPC. For this purpose, we used mouse AD2 ESC harboring the dsRed (R) and eGFP (G) genes under the control of the *Mef2c*/AHF and *Nkx2.5* cardiac-specific enhancers, respectively (Domian et al., 2009). The differentiation of AD2 ESC originates R^+/G^-, R^-/G^+, R^+/G^+, and R^-/G^- cell populations equivalent to CPC of the pharyngeal mesoderm, FHF CPC, committed ventricular CPC of the SHF, and non-cardiac cells, respectively (Domian et al., 2009). *Cited2* expression, assessed by qPCR in AD2-derived cell populations isolated by fluorescence-activated cell sorting at D6 of differentiation, revealed that *Cited2* transcripts were enriched in all CPC populations when compared with R^-/G^- cells, with higher levels of expression observed in R^+/G^+ cells (Figures 4A and 4B). The expression of *Isl1*, *Nkx2.5*, *Gata4*, and *Tbx5* in the distinct CPC populations (Figure S1E) was as previously reported (Domian et al., 2009). To determine whether Cited2 promotes the emergence of CPC, 2 days prior to differentiation we transiently transfected AD2 ESC with either a control vector or the plasmid expressing flag-CITED2 (Figure 4C). The expression of flag-CITED2 stimulated endogenous *Isl1* expression at D0, as observed in E14/T cells (Figure 1D), and significantly increased the number of R^+/G^+ cells derived from AD2 ESC differentiation at D6 (Figure 4D). In addition, the expression profile of *Cited2* in cardiac progenitors in vivo was assessed in CPC populations isolated from embryonic day 9.5 (E9.5) hearts of mouse transgenic embryos harboring the *dsRed* and *eGfp* genes (Domian et al., 2009). Interestingly, *Cited2* expression was enriched in the R^+/G^+ subpopulation simultaneously expressing *Isl1* and *Nkx2.5* (Figure 4E). Collectively, these observations indicated that *Cited2* is expressed in FHF and SHF CPC derived from ESC.

CITED2 and ISL1 Proteins Interact and Synergize to Enhance Cardiac Differentiation

ISL1 is a protein with two LIM domains located at its N-terminal domain, both important for SHF expansion and morphogenetic control of cardiogenesis (Witzel et al., 2012). Since CITED2 binds to the LIM domain of the transcription factor LHX2 (Glenn and Maurer, 1999), we hypothesized that ISL1 and CITED2 proteins might physically interact. We therefore synthesized a ^{35}S-labeled myc-tagged full-length human ISL1 protein (myc-ISL1) by coupled in vitro transcription-translation, and tested for its ability to bind to a glutathione S-transferase (GST)-CITED2 fusion protein (Figure 5A). Myc-ISL1 interacted specifically with GST-CITED2 full-length protein and the C-terminal residues of CITED2 (amino acids 66–270). It also interacted weakly with CITED2 N-terminal residues (amino acids 2–214). To confirm the interaction between CITED2 and ISL1 in cells, we used the anti-FLAG antibody to immunoprecipitate protein extracts from HEK293T cells expressing either myc-ISL1 alone or myc-ISL1 and flag-CITED2. Immunopurified proteins were then analyzed by

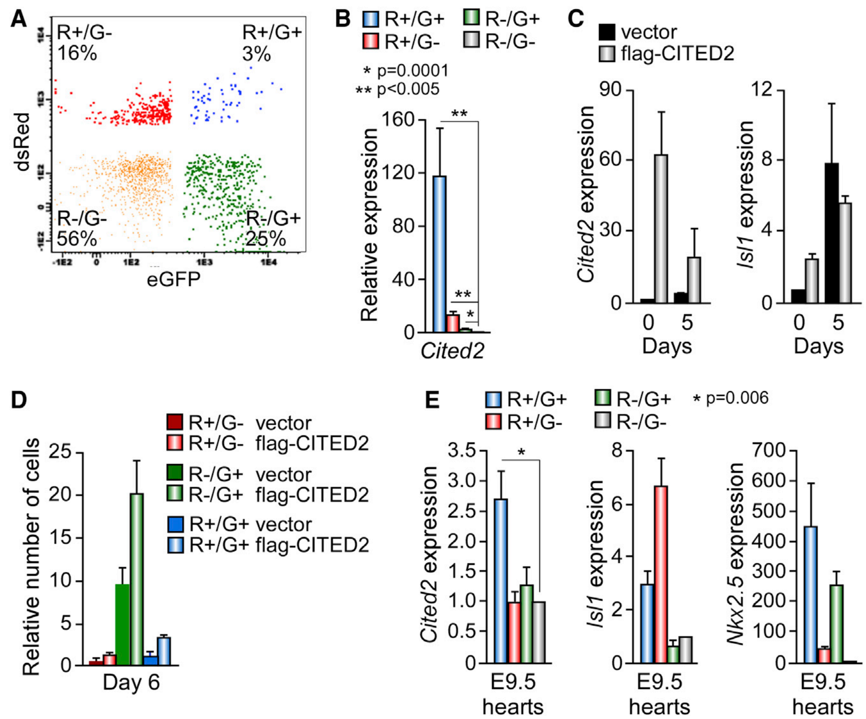

Figure 4. CITED2 Is Expressed in Cardiac Progenitors and its Overexpression Promotes Cardiac Progenitor Specification

(A) Representative flow cytometry plots of the R+/G$^-$, R$^-$/G+, R+/G+, and R$^-$/G$^-$ cell populations derived from AD2 ESC at D6 of differentiation. Numbers indicate percentage of cells within each gate.

(B) Relative expression of *Cited2* determined by qPCR in cell populations derived from AD2 at D7 differentiation. Expression of the indicated genes is reported relative to their expression in non-cardiac cells R$^-$/G$^-$ set at 1.

(C) Relative expression of ectopic CITED2 and endogenous *Isl1* determined by qPCR at D0 and D5 of differentiation, in AD2 ESC transiently transfected with a plasmid expressing flag-CITED2 or a control vector. flag-*Cited2* and *Isl1* expressions are relative to their expression in cells transfected with the control vector set at 1.

(D) Quantification, at D6 of differentiation by flow cytometry, of R+/G$^-$, R$^-$/G+, R+/G+, and R$^-$/G$^-$ populations derived from AD2 ESC transfected with a plasmid expressing flag-CITED2 or a control vector. Cell numbers are reported relative to background determined at the onset of differentiation, which was set at 1.

(E) Relative expression of *Cited2*, *Isl1*, and *Nkx2.5* determined by qPCR in embryonic cardiac progenitor populations at E9.5.

Results in (B) and (E) are presented as the mean ± SEM of three independent biological experiments, while results in (C) and (D) are presented as the mean ± SEM of two independent biological experiments each performed in technical triplicates.

western blot with anti-ISL1 and anti-FLAG antibodies (Figure 5B). Myc-ISL1 was co-immunoprecipitated with flag-CITED2, implying that ISL1 and CITED2 were in protein complexes in HEK293T cells. We also visualized ISL1-CITED2 interaction in living cells using vectors expressing either ISL1 in fusion with the N-terminal domain of the fluorescent protein VENUS (VEN-ISL1), CITED2 in fusion with the C-terminal domain of VENUS (VEC-CITED2), or control vectors to perform bifluorescence complementation (BiFC) assays (Machado-Oliveira et al., 2015). The co-transfection of VEN-ISL1 and VEC-CITED2 in E14/T cells resulted in a specific detection of fluorescence, confirming that ISL1 and CITED2 associate in these cells (Figures 5C and S3).

To test the functional significance of CITED2-ISL1 interaction, we co-transfected Hep3B cells with vectors expressing myc-ISL1 and flag-CITED2 together with a reporter construct harboring *luciferase* under the control of a composite regulatory region containing the *Mef2c* cardiac-specific enhancer, which is a direct target of Isl1 (Witzel et al., 2012). The transfection of myc-ISL1 vector increased the reporter activity, and this was further enhanced by co-transfection of flag-CITED2 vector (Figure 5D). These observations provide evidence that myc-ISL1 and flag-CITED2 synergize to stimulate the activity of an ISL1-responsive reporter.

To determine whether CITED2 and ISL1 cooperate to promote ESC differentiation toward cardiac cell fate, the number of beating foci originating from undifferentiated E14/T ESC expressing VEC-CITED2 and VEN-ISL1 individually or in combination was evaluated at D8, D10, and D12 of differentiation (Figure 5E). The transfection of VEC-CITED2 or VEN-ISL1 individually led to the emergence of beating foci at similar levels, while the co-transfection of VEC-CITED2 and VEN-ISL1 significantly increased the number of contractile foci at D12. Altogether, these observations provide evidence for a physical and functional interaction between ISL1 and CITED2 that promotes ESC differentiation toward cardiac cell lineages.

DISCUSSION

In this study, we show that *Cited2* is required for early cardiac commitment of mouse ESC, in agreement with a previous study (Li et al., 2012). We and others have

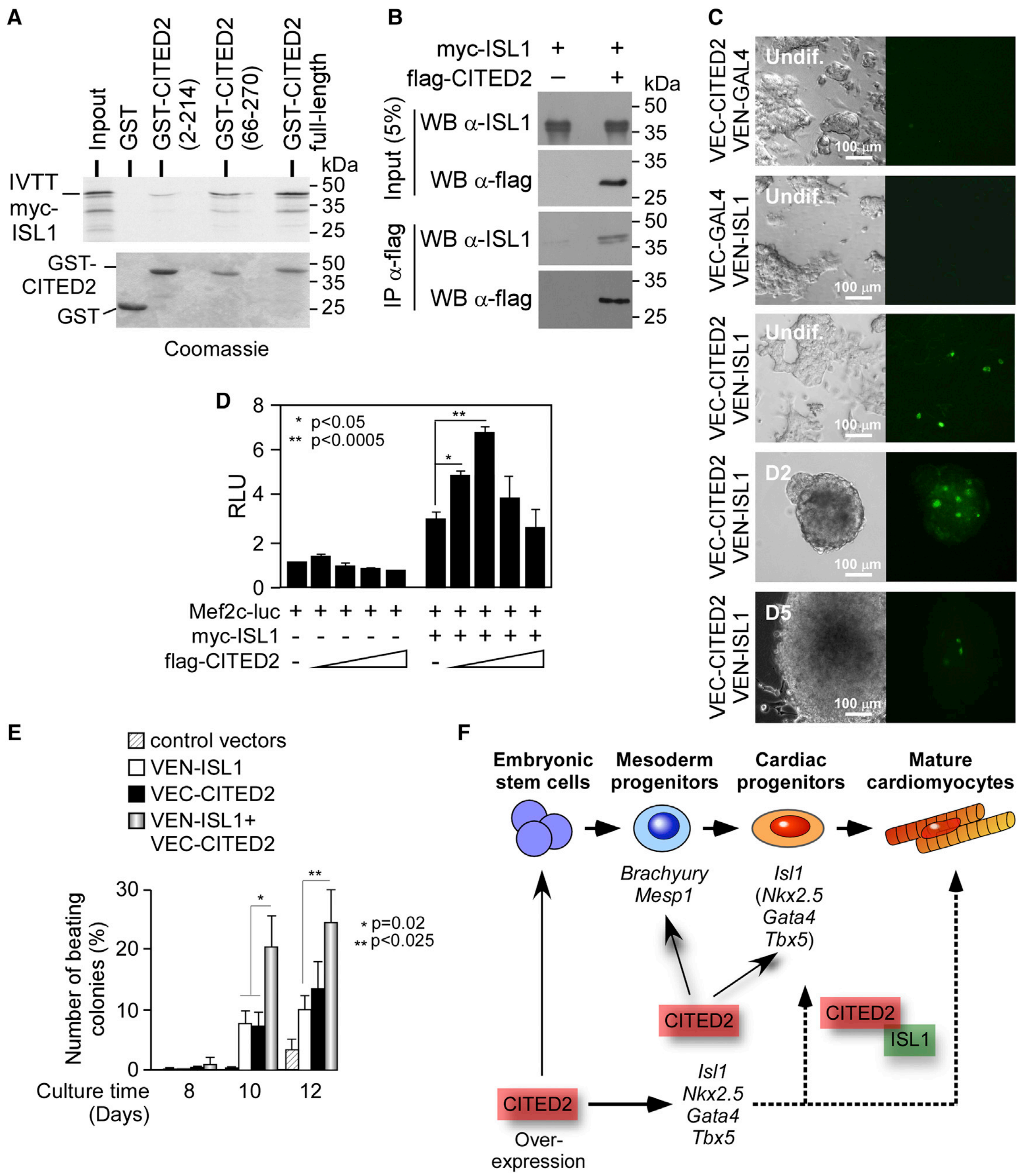

Figure 5. CITED2 Binds to ISL1 and Both Synergize to Enhance Cardiac Differentiation

(A) Binding of ^{35}S-labeled myc-ISL1 protein either to GST alone or GST fused to full-length CITED2 (GST-CITED2, amino acid residues 2–270), the N-terminal fragment GST-CITED2(2–214), or the C-terminal fragment GST-CITED2(66–270). Ten percent of labeled myc-ISL1 used for the binding assay was loaded as the input. Top panel: autoradiogram showing the binding of ^{35}S-labeled ISL1 to GST and GST-CITED2 proteins. Bottom panel: Coomassie blue stain of the gels showing relative amounts of GST and GST-CITED2 proteins.

(B) Whole-cell extracts from HEK293T cells expressing myc-ISL1 alone or in combination flag-CITED2 immunoprecipitated (IP) with an anti-FLAG antibody, and proteins detected by western blot (WB) with anti-ISL1 and anti-FLAG antibodies. Five percent of the input was also loaded.

(C) Interaction between CITED2 and ISL1 visualized by BiFC assays in undifferentiated E14/T ESC. Fluorescence emission (right panels) and morphological aspect of E14/T cells as observed in bright field (left panels) was examined 1 day after transfection and in embryoid bodies at D2 and D5 of differentiation.

(legend continued on next page)

demonstrated that *Cited2* expression promotes mouse ESC self-renewal (Chen et al., 2012; Kranc et al., 2015; Pritsker et al., 2006). Interestingly, we show that the deletion of *Cited2* in ESC or depletion of *Cited2* at the onset of differentiation impairs cardiac lineage commitment. Remarkably, we determine that endogenous expression of *Cited2* transcripts is biphasic during ESC differentiation, starting with a decrease from D0 to D2. This decline of *Cited2* expression upon differentiation might be necessary for ESC to switch from a non-permissive to a permissive differentiation state. In a second phase, *Cited2* expression increases from D3 of differentiation onward, implying that *Cited2* might be required for subsequent differentiation processes. This is corroborated by the rescue of the cardiac differentiation defects in *Cited2*-depleted ESC with the supplementation of the recombinant CITED2 protein to the cells at D2 of differentiation, a day before the levels of endogenous *Cited2* start to increase during differentiation.

The impairment of cardiac differentiation resulting from *Cited2* depletion at the onset of ESC differentiation may be due to a consequent downregulation of *Brachyury* and *Mesp1* expression. The decrease of *Isl1*, *Nkx2.5*, *Gata4*, and *Tbx5* expression that we observed in *Cited2*-depleted cells at D5 of differentiation, which corresponds to the time when CPC emerge, might be a consequence of *Mesp1* downregulation, since these genes are activated by MESP1 to promote cardiogenesis and CPC specification during cardiac development and ESC differentiation (Bondue et al., 2008; David et al., 2011). On the other hand, we show that *Cited2* expression is enriched in CPC, and CITED2 protein is recruited to *Isl1* promoter at D5 of differentiation during CPC specification. This suggests that CITED2 may play a role in CPC specification, proliferation, and/or differentiation. In agreement with this hypothesis, flag-CITED2 overexpression promoted both the emergence of CPC and terminal cardiomyocyte differentiation from ESC, and increased *Isl1*, *Nkx2.5*, *Gata4*, and *Tbx5* expression in undifferentiated ESC. Fluctuations in the expression of genes encoding transcription factors with capacities to instruct lineage specification have been evidenced in undifferentiated pluripotent ESC and, in accordance with the transcripts expressed, subsets of ESC might be prone to undergo particular differentiation programs (Lanner and Rossant, 2010). The overexpression of CITED2 in undifferentiated ESC might specify and promote CPC and cardiac differentiation by raising the expression of the pro-cardiogenic factors *Isl1*, *Nkx2.5*, *Gata4*, and *Tbx5*. Interestingly, the transdifferentiation of human dermal fibroblasts into cardiac progenitors by expression of MESP1 and ETS2 has been shown to stimulate the expression of CITED2 (Islas et al., 2012), suggesting that CITED2 may play a role in cardiac progenitor specification and/or functions. Therefore, CITED2 overexpression might be instrumental for specification of CPC and cardiomyocyte differentiation from pluripotent stem cells.

We also provided evidence for a privileged regulatory interaction between *Cited2* and *Isl1*. Indeed, our ChIP assays show that CITED2 is specifically recruited at the *Isl1* promoter. Furthermore, supplementation of *Cited2*-null ESC with CITED2 recombinant protein restores the expression of *Isl1* to normal levels at D5 of ESC differentiation. In addition, ESC overexpressing flag-CITED2 are enriched for H3triMeK4 at the *Isl1* regulatory elements, which marks transcriptionally active chromatin. The mechanisms by which flag-CITED2 overexpression contribute to the enrichment of H3triMeK4 at the *Isl1* loci and increase *Gata4* and *Nkx2.5* expression in undifferentiated ESC remain to be elucidated. Interestingly, at the protein level we demonstrated that ISL1 and CITED2 interact, delineated the amino acids 66–214 of CITED2 as part of the ISL1 interacting domain, and by transient transfection assays established that CITED2 increased ISL1-mediated *Mef2c*-enhancer activity and that ISL1 and CITED2 have a synergistic effect on cardiac cells derived from ESC. The exact molecular mechanism by which CITED2 and ISL1 cooperate in cardiac specification remains to be clarified, but it has been recently demonstrated that in mouse

(D) Hep3B cells were transiently co-transfected with 40 ng of Mef2c-luc reporter, together with 100 ng of either myc-ISL1 expressing or empty control vector, and increasing amounts of a flag-CITED2-expressing plasmid (0, 50, and 100 ng) combined with the empty plasmid to achieve a total of 400 ng in each condition. The luciferase activity was normalized for the β-GALACTOSIDASE activity conferred by CMV-*lacZ* (100 ng). Relative luminescence units (RLU) are presented relative to values of the *Mef2c*-luc transfected with the control vectors set at 1.

(E) Percentage of colonies with beating foci at D8, D10, and D12 in cultures derived from differentiation of E14/T ESC transfected individually or in combination with vectors expressing VEN-ISL1 and VEC-CITED2, or control vectors at D0.

(F) Model for the role of CITED2 during cardiogenesis of ESC, as supported by the data from our study. CITED2 is required for the normal expression of mesoderm progenitor markers such as *Brachyury* and *Mesp1*. In addition, at this stage CITED2 contributes to the expression of *Isl1*, *Nkx2.5*, *Gata4*, and *Tbx5*, which are CPC markers. The overexpression of CITED2 triggers an increase of *Isl1*, *Nkx2.5*, *Gata4*, and *Tbx5* expression in undifferentiated ESC, which may favor cardiac differentiation. Finally, CITED2 and ISL1 proteins physically interact and cooperatively promote cardiac differentiation.

Results in (D) and (E) are presented as the mean ± SEM of three independent biological experiments.

embryonic hearts and cardiac progenitors ISL1 interacts with p300 to selectively promote H3 acetylation at the *Mef2c* promoter (Yu et al., 2013). Since CITED2 interacts strongly with p300, it would be of interest to determine whether CITED2 cooperates with p300 to promote its functional interaction with ISL1.

Altogether, our results indicated that *Cited2* contributes to the expression of a subset of pivotal cardiopoietic genes involved in mesoderm and cardiac progenitor specification (Figure 5F). During mouse embryonic development, *Cited2* expression was detected in the early mesoderm- and cardiac-derived structures (Dunwoodie et al., 1998), but rather surprisingly a *Brachyury/T-Cre* or *Mesp1-Cre* conditional *Cited2* knockout only resulted in infrequent and minor heart developmental defects of mouse embryos, while *Cited2* knockout in the epiblast consistently caused heart malformations (Bamforth et al., 2001; MacDonald et al., 2008). In the present report, we show that *Cited2* depletion at the onset of differentiation causes the most severe impact on cardiac differentiation, and results also in the impairment of *Brachyury* and *Mesp1* expression at D3. Interestingly, the cardiogenic defects due to the loss of *Cited2* expression at D0 were reversed by supplementation of the 8R-CITED2 at D2 of differentiation. On the other hand, *Cited2* depletion at later time points (D2, D4, and D6) had little or no effect on cardiogenesis. Together, these observations indicate that Cited2 function is important for early commitment of ESC to mesoderm and/or cardiac specification, or at least contribute to the correct expression of *Brachyury* and *Mesp1*, or other genes crucial for these processes. Therefore, the lack of a strong phenotype in *Brachyury/T-Cre* and *Mesp1-CreCited2* conditional knockout might be because the depletion of *Cited2* in these embryos was triggered after *Brachyury* and *Mesp1* were activated and after the requirement for *Cited2*. Of particular interest, we show that CITED2 stimulates the expression of *Isl1*, a marker of SHF cardiac progenitors, and binds to its promoter at the time of CPC specification. We also show that *Cited2* expression is enriched in SHF cardiac progenitors derived either from ESC or mouse E9.5 embryonic hearts. Moreover, *Cited2*-null embryos display a variety of cardiac developmental defects such as ventricular septal defects with overriding aorta or double-outlet right ventricle, outflow tract defects, and transposition of the great arteries, which may result from anomalies of SHF and cardiac neural crest cell progenitors known to express ISL1 (Bamforth et al., 2001; Bruneau, 2013). Therefore, CITED2 and ISL1 may also interact during heart development and play a critical role for the fulfillment of CPC functions in this process. It would be of interest to investigate the contribution of the CITED2-ISL1 interaction in CPC functions both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

All mice were cared for within the Animal Care facilities of the Massachusetts General Hospital under the supervision of an active and functioning Subcommittee on Research Animal Care (SRAC), which serves as the Institutional Animal Care and Use Committee (IACUC) as required by the Public Health Service (PHS) Policy on Humane Welfare Regulations.

Embryonic Stem Cells, Culture Conditions, and Isolation of Cardiac Progenitor Populations

Apple D2 (AD2), $C2^{fl/fl}$, $C2^{\Delta/\Delta}$[LA11], $C2^{fl/fl}$[Cre], and E14/T mouse ESC lines were described previously, and were cultured on gelatin-coated plates in undifferentiating medium supplemented with LIF (Chambers et al., 2003; Domian et al., 2009; Kranc et al., 2015). All ESC lines were differentiated using the hanging-drop method in medium containing 20% fetal bovine serum without LIF supplementation (differentiation medium).

Flow Cytometry Analysis

For detection of cardiac troponin I type 3 (cTNT), cells were fixed with 0.5% paraformaldehyde for 20 min at room temperature, blocked, and permeabilized with PBS containing 0.5% BSA and 0.1% saponin for 5 min at 4°C, washed with blocking solution, and incubated for 1 hr at 4°C with a monoclonal anti-mouse cTNT antibody (NB110-2546, Novus Biologicals) at a 1:800 dilution followed by 1 hr of incubation at 4°C with a secondary goat anti-mouse immunoglobulin G conjugated with Alexa 488 (A21202, Life Technologies) used at 1:2,000 dilution. Flow cytometry analyses were performed on a FACSCalibur (BD Biosciences) operating at 488 nm excitation with standard emission filters. Baseline of noise fluorescence was established with cells incubated only with the secondary antibody.

Real-Time qPCR

Total RNA isolation, cDNA synthesis, and qPCR assays were carried out as previously described (Kranc et al., 2015) with the primers listed in Table S1. The primer set designated Cited2 detects both mouse endogenous *Cited2* and human exogenous *flag-CITED2*. For the selection of reliable reference genes, the expression of three reference genes, *hprt*, *Gapdh*, and *18S* was tested in samples prepared from $C2^{fl/fl}$[Cre] and E14/T ESC at different time points of the differentiation (Figure S3E). *Gapdh* and *18S* showed a very consistent expression across the differentiation process of both cell types. We opted for the normalization of gene expression levels to *Gapdh*, as previously performed (Kranc et al., 2015).

Immunochemistry

Immunocytochemistry was performed with $C2^{fl/fl}$[Cre] ESC treated with ethanol or 4HT at D0 and differentiated for 10 days. Western blotting assays were performed using 20 μg of whole-cell lysates prepared from the indicated mouse ESC as previously described (Kranc et al., 2015).

Chromatin Immunoprecipitation Assays

ChIP experiments and enrichment of target genomic elements by qPCR were performed as previously described (Kranc et al., 2015) using the primers listed in Table S1.

Production and Transduction of the Recombinant 8R-CITED2 Protein

Full-length human CITED2 cDNA and an oligonucleotide encoding eight arginines (8R) were cloned into the pGEX6P1 vector (GE Healthcare Life Sciences) to express a chimeric protein consisting of the GST in fusion with the 8R domain and CITED2 (termed GST-8R-CITED2, Figure S2). Newly constructed plasmids were validated by sequencing and the expression of fusion proteins tested by western blot (Figure S2). Details of plasmid construction and protein purification are available upon request.

Protein Interactions and Plasmids

For in vitro binding assays, GST-CITED2 fusion proteins and in vitro translated myc-ISL1 were prepared as previously described (Machado-Oliveira et al., 2015). Myc-ISL1 expression plasmid was constructed in pcDNA3 (Invitrogen) with an amino-terminal myc epitope tag. Plasmids expressing flag-CITED2 and pPyCAGIP were previously described (Chen et al., 2012). For BiFC assays, plasmids expressing VEC-CITED2, VEN-p300CH1, VEC-GAL4, VEN-GAL4, and the VEN vector were described elsewhere (Machado-Oliveira et al., 2015). The plasmid VEN-ISL1 expressing VEN (amino acid residues 1–155 of the VENUS fluorescent protein) in fusion with ISL1 was obtained by subcloning ISL1 cDNA fragment of the Myc-ISL1 expression plasmid into the VEN vector in frame with the VEN domain. All newly constructed plasmids were validated by sequencing and the expression of fusion proteins tested by western blot (Figure S3). Details of plasmid construction are available upon request. For co-immunoprecipitation assays, ~0.4 mg of whole-cell extracts from HEK293T cells transfected with flag-CITED2 expression vector alone or together with myc-ISL1 vector were used.

Reporter Assays

Hep3B cells, a human hepatocellular carcinoma cell line with low levels of endogenous CITED2, were plated in 24-well plates at 2.5×10^4 cells per well and transfected the following day using Lipofectamine 2000 (Invitrogen) with Mef2c-luc reporter (Witzel et al., 2012) and expression vectors. CMV-*lacZ* plasmid was co-transfected in all experiments, and both LUCIFERASE and β-GALACTOSIDASE activities measured as previously described (Kranc et al., 2015).

Statistical Analysis

Statistical significance was determined by two-tailed Student's t tests assuming unequal variance. p Values of <0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.10.002.

AUTHOR CONTRIBUTIONS

I.P.-L., conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; A.C.M., D.V.O., J.M.A.S., R.N., E.G., A.M., A.M.v.D.V., G.M.O., collection and/or assembly of data; G.F., conception and design, provision of equipment and reagents; I.B., conception and design, collection and/or assembly of data, financial support; J.B., conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. A.C.M., D.V.O., and J.M.A.S. contributed equally.

ACKNOWLEDGMENTS

This work is supported by national Portuguese funding through FCT, Fundação para a Ciência e a Tecnologia, project UID/BIM/04773/2013 CBMR, projects PEst-OE/EQB/LA0023/2013 and PTDC/SAU-ENB/111702/2009 to J.B., U01HL100408, NIH/NHLBI to I.D., and SFRH/BPD/74807/2010 to G.M.O. I.P.L. thanks the Biomedical Sciences PhD program and FCT for funding (SFRH/BD/62054/2009). J.M.A.S. is a PhD student of the ProRegeM - PhD Program in Mechanisms of Disease and Regenerative Medicine of the University of Algarve and New University of Lisbon financed by the FCT. We thank Shoumo Bhattacharya (University of Oxford) for CITED2 expressing vectors, Austin Smith (University of Cambridge) for E14/T cells and pPyCAGIP vector, Gergana Dobreva (Max Planck Institute for Heart and Lung Research) for Mef2c-luc reporter, Claudia Florindo, head of the Microscopy Facility (University of Algarve), and José A. Belo (New University of Lisbon) for reagents.

Received: April 6, 2016
Revised: October 5, 2016
Accepted: October 6, 2016
Published: November 3, 2016

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Supplemental Information

CITED2 Cooperates with ISL1 and Promotes Cardiac Differentiation of Mouse Embryonic Stem Cells

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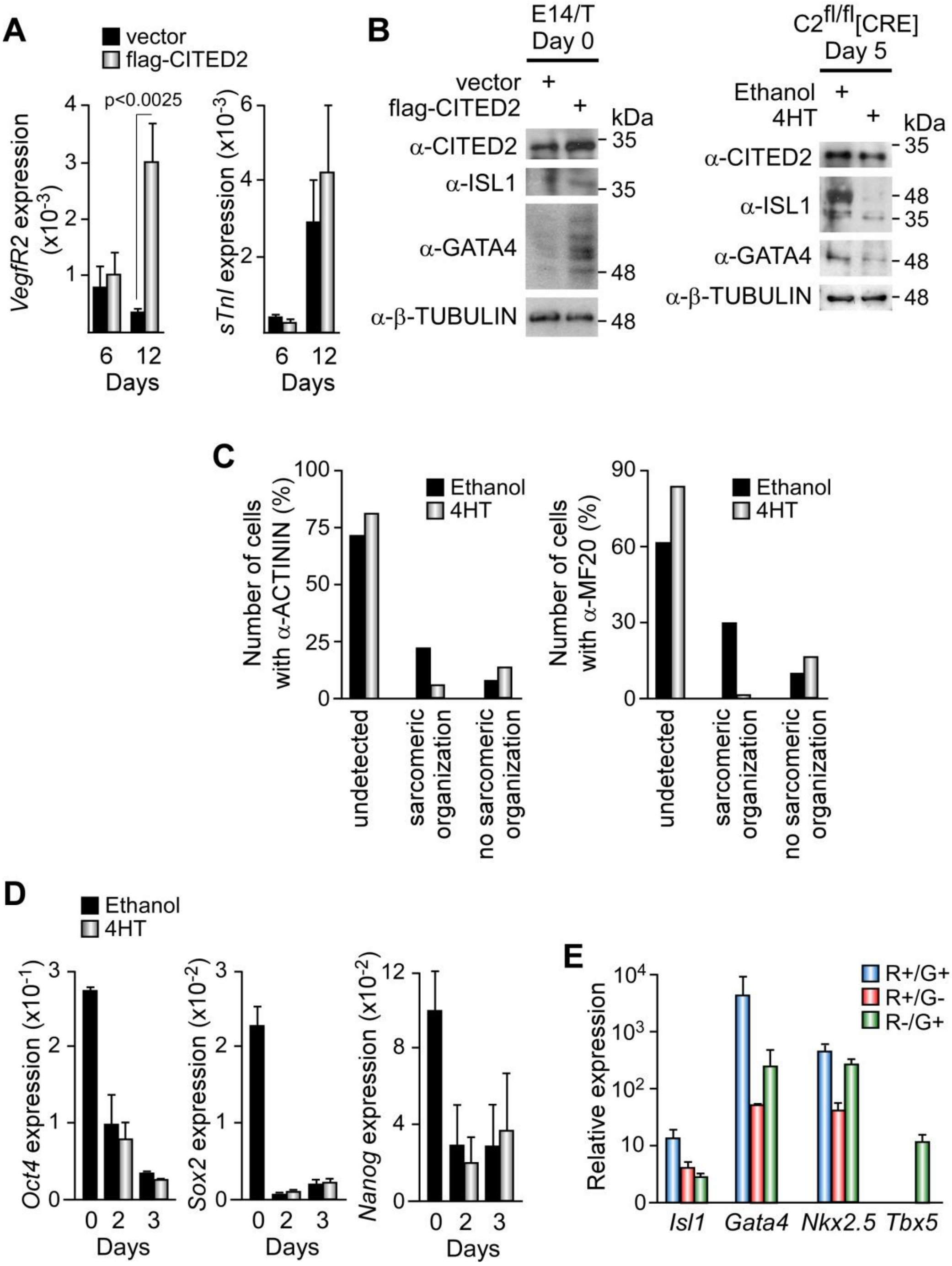
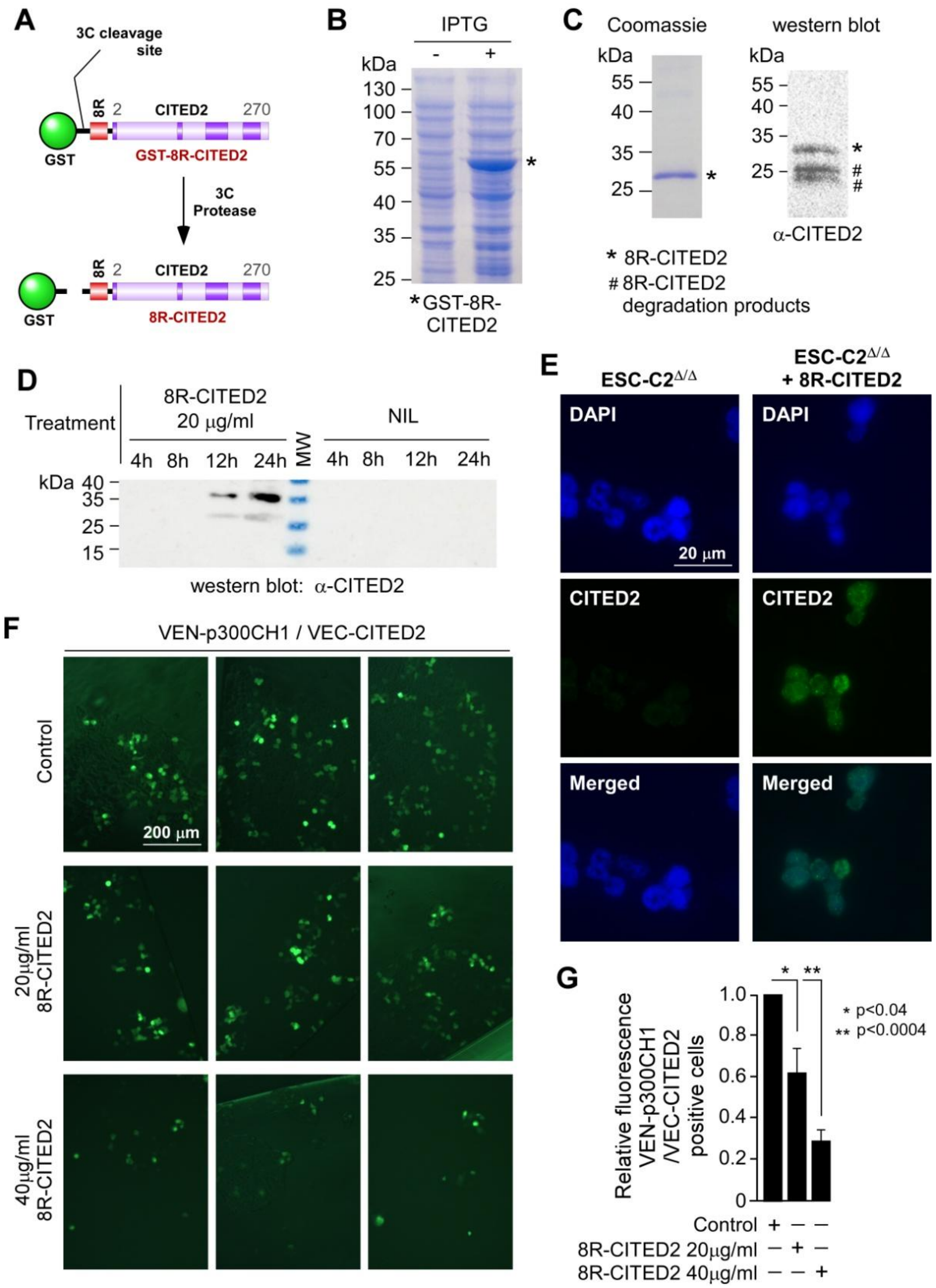
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

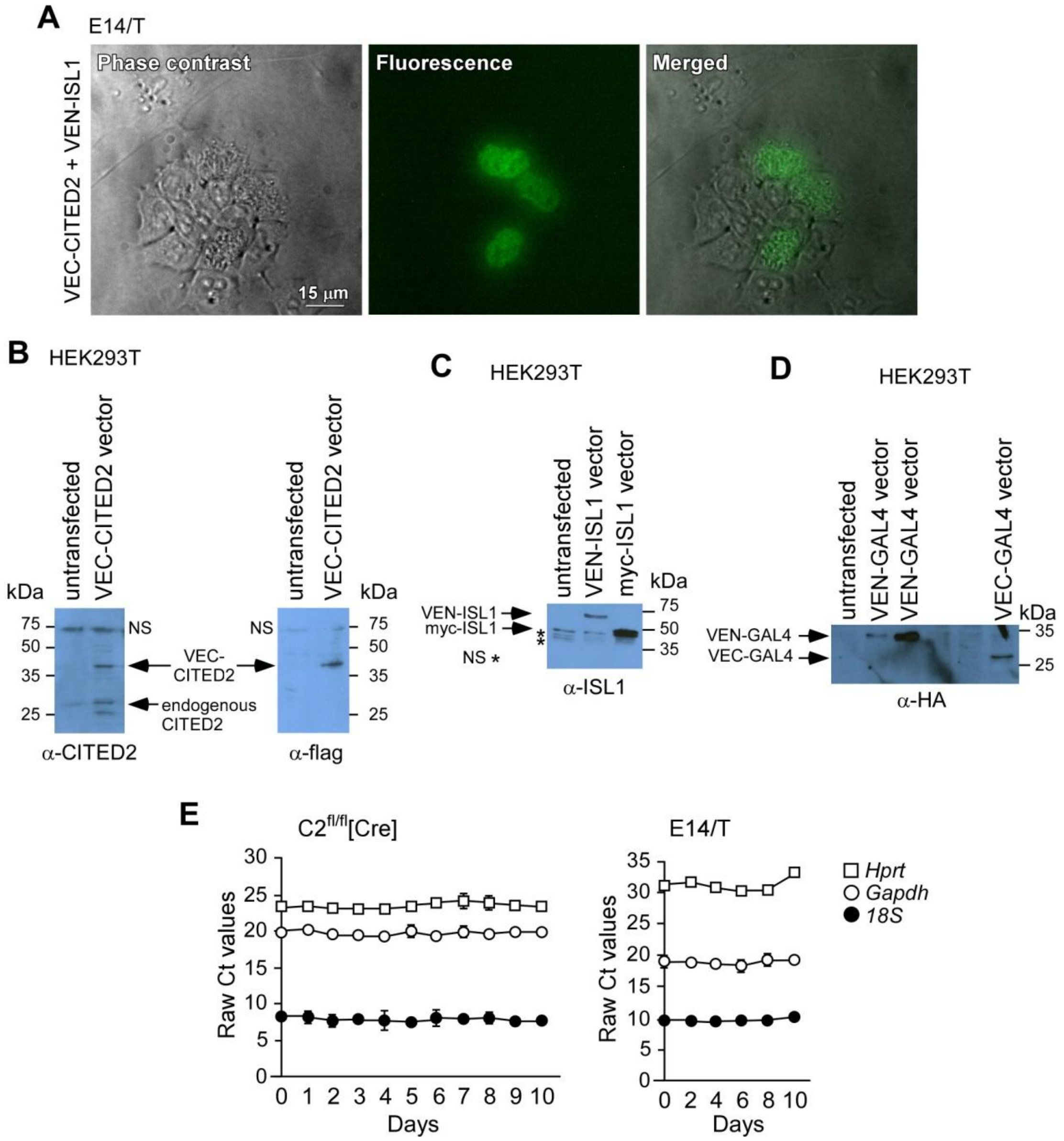


Table S1 – Primers used for qPCR and ChIP qPCR

qPCR	Forward primer	Reverse primer	Reference
18S	CGTCTGCCCTATCAACTTTCG	CCTTGGATGTGGTAGCCGTT	(Chen et al. 2007)
Activin A	CTCCACGATCATGTTCTGAAT	GATGATGTTTTGACCATCATC	(Orimo et al. 1996)
Afp	GCCACCGAGGAGGAAGTG	AGTCTTCTTGCGTGCCAGC	(Gouon-Evans et al. 2006)
Brachyury	CTCTAATGTCCTCCCTTGTTGCC	TGCAGATTGTCTTTGGCTACTTTG	(Ivanova et al. 2006)
Cited2	CTCTAATGTCCTCCCTTGTTGCC	CGCTCGTGGCATTCATGTTG	(Chen et al. 2007)
cTnT	GAGGAGGTGGTGGAGGAGTA	GGCTTCTTCATCAGGACCAA	(Sachinidis and Schwengberg 2006)
Foxa2	GGCCCAGTCACGAACAAAGC	CCCAAAGTCTCCACTCAGCCTC	(Ivanova et al. 2006)
Gapdh	TCCCACTCTTCCACCTTCGATGC	GGGTCTGGGATGGAAATTGTGAGG	(Ivanova et al. 2006)
Gata4	TTCCTGCTCGGACTTGGGAC	TTCCCAGGCAGGTGGAGAATAAG	(Ivanova et al. 2006)
Isl1	CTTAAGCATGCCCTGTAGCTGG	CAGACAGGAGTCAAACACAATCCC	(Ivanova et al. 2006)
Hprt	GTTGGATACAGGCCAGACTTTGTTG	GAGGGTAGGCTGGCCTATAGGCT	(Chen et al. 2007)
Mesp1	TGTACGCAGAAACAGCATCC	TTGTCCCCTCCACTCTTCAG	(Zhong and Jin 2009)
Myh6	GATGGCACAGAAGATGCTGA	CTGCCCCTTGGTGACATACT	(Sachinidis and Schwengberg S 2006)
Nanog	CTCATCAATGCCTGCAGTTTTTCA	CTCCTCAGGGCCCTTGTCAGC	(Zhong and Jin 2009)
Nestin	GGTGCTGAGTATGTCGTGGA	CGGAGATGATGACCCTTTTG	(Sachinidis and Schwengbreg 2006)
Nkx2.5	CCACTCTCTGCTACCCACCT	CCAGGTTCAGGATGTCTTTGA	(Sachinidis and Schwengberg S 2006)
Nodal	TGGCGTACATGTTGAGCCTCT	TGAAAGTCCAGTTCTGTCCGG	(Ogawa et al. 2007)
Oct4	GCAGGAGCACGAGTGGAAAGCAAC	CCAGGCCTCGAAGCGACAGATG	(Ivanova et al. 2006)
Sox2	CGAGATAAACATGGCAATCAAATG	AACGTTTGCCTTAAACAAGACCAC	(Ivanova et al. 2006)
sTnI	GGAAATCCAAGATCACTGCCTCC	GGGCACTGAGGGACAGACCA	(Martínez-Fernandez et al. 2006)
Tbx5	GGACCCAGTCCCTTGAATGG	TCCAGGCTGAGGAGTTCTAGGC	(Ivanova et al. 2006)
VegfR2	ACTGCAGTGATTGCCATGTTCT	TCATTGGCCCGCTTAACG	(Fujimori et al. 2008)
βIII-tubulin	G TATTCAGGCCCGACAACTTT	GGGTGTCAACCAGAGGAAGT	(Suter et al. 2009)

ChIP qPCR	Forward primer	Reverse primer	Reference
Gata4	ACTCCCTTAGGCCAGTCAGC	GGAAAAGAGCAGGGACTCG	(Snyder et al. 2010)
Gapdh	CAAGGCTGTGGGCAAGGT	TCACCACCTTCTTGATGTCATCA	(Chen et al. 2012)
Isl1 A	TTTTGGGTCTAACCGTCCTACTC	CCGCTTTCCTTCACTGACTC	(Nimura et al. 2009)
Isl1 B	ACTATTTGCCACCTAGCCACAG	AGAGGGAGTAATGTCCACAGTG	(Nimura et al. 2009)
Nkx2.5	AGGCAAAGAAATCACTCCACA	TGTTACAATGGCTGGGAAGG	(Snyder et al. 2010)
Tbx5	GAAGCATTTTCTATACTTTGTGAGA	TCAGCCAGCTGTTTTCAGAG	(Snyder et al. 2010)

Supplemental Figure Legends

Figure S1. Related to Figures 1, 2 and 3. The modulation of the expression of CITED2 at the onset of differentiation affects the expression of *VegfR2*, ISL1 and GATA4, α–ACTININ and MYOSIN HEAVY CHAIN (MF20), but does not impair the expression of *sTnI* and of genes involved in the maintenance of pluripotency. (A) *VegfR2* and *sTnI* expression detected by qPCR from extracts isolated from E14/T at D6 and D12 of differentiation in cells expressing normal levels of CITED2 (vector) or overexpressing flag-CITED2. (B) Detection of CITED2, ISL1 and GATA4 protein levels by western blotting in extracts from E14/T ESC transiently transfected with a control- or a flag-CITED2 expression vector (left), and in in extracts from C2fl/fl[Cre] cells differentiated for 5 days and treated with Ethanol or 4HT at D0 for 2 days (right). Loading in each lane was monitored by detection of β-TUBULIN. (C) Percentage of cells either unstained, stained for sarcomeric α–ACTININ (left) or MYOSIN HEAVY CHAIN (right - MF20) and presenting some sarcomeric organization, or presenting a poor expression of α–ACTININ or MF20 and no sarcomeric organization in cells described in Figure 2G. (D) Expression of pluripotency markers (*Oct4*, *Sox2* and *Nanog*) determined by qPCR at D0, D2 and D3 of differentiation in cultures derived from $C2^{fl/fl}$[Cre] ESC treated with 1μM 4HT or ethanol at D0 for 48 hrs, and normalized for *Gapdh*. (E) Relative expression of *Isl1*, *Gata4*, *Nkx2.5* and *Tbx5* determined by qPCR and normalized for *Gapdh* in cell populations derived from AD2 at D6 differentiation as described in Figure 4B. Expression of the indicated genes is reported relative to their expression in non-cardiac progenitor cells R-/G- set at 1. Note the logarithmic axis scale. Results in panels A, D, E are presented as the mean ± s.e.m. of three independent biological experiments, while results in panel C are presented as the mean ± s.e.m. of two independent biological experiments.

Figure S2. Related to Figure 3. Production and characterization of 8R-CITED2 recombinant protein. (A) Schematic representation of the chimeric GST-8R-CITED2 protein produced in bacteria. Oligonucleotides with a sequence corresponding to the 8 arginines (8R) cDNA and a fragment encoding the human CITED2 were cloned in the pGEX6P1 vector (GE Healthcare Life Sciences) to express a chimeric protein consisting of the Glutathione S-transferase (GST) linked to 8R, which are themselves fused to CITED2 (GST-8R-CITED2). The 3C cleavage site encoded at the C-terminal part of the GST by the pGEX6P1 vector is indicated. (B) Detection by SDS-PAGE separation and Coomassie staining of total proteins from BL21 *E.coli* transformed with the GST-8R-CITED2 expression vector and either stimulated (+) or unstimulated (-) by IPTG. The position of GST-8R-CITED2 protein is indicated by an asterisk (*). (C) Detection by SDS-PAGE separation and either Coomassie staining (left panel) or western blotting (right panel) of 8R-CITED2 purified recombinant protein after purification by affinity column and cleavage on column by the Rhonivirus 3C protease coupled to GST. The recombinant 8R-CITED2 (*) and degradation products (#) are indicated. The western blotting was performed with the anti-CITED2 antibody as described in the main text. Cells not supplemented with 8R-CITED2 (NIL) were loaded as control (D) Transduction of 8R-CITED2 into $C2^{\Delta/\Delta}$[MG5] *Cited2*-null ESC described elsewhere (Kranc et al., 2015). Purified 8R-CITED2 was added in the culture medium at a final concentration of 20μg/ml. Detection by western blotting of intracellular 8R-

CITED2 in whole cellular extracts prepared at the indicated times after addition of 8R-CITED2 or in control cells using the anti-CITED2 antibody as described in the main text. (E) Accumulation of 8R-CITED2 in the cellular nuclei of $C2^{\Delta/\Delta}$[MG5] *Cited2*-null ESC detected by immunohistochemical reaction against CITED2 24 hrs after supplementation of 8R-CITED2 as described in D. (F) Fluorescence detection in HEK293T cells co-transfected with plasmids expressing VEN-p300CH1 and VEC-CITED2 (25 ng each), and supplemented in the culture medium 24 hrs post-transfection with 8R-CITED2 at the final concentration of 0 (Control), 20 and 40μg/ml. The morphology of transfected cells (not shown) and the fluorescence signal obtained by BiFC (green panels) were visualized 48 hrs after transfection. (G) Quantification of BiFC detected in HEK293T cells treated as indicated in F. The number of fluorescent cells is presented relative to the fluorescence detected in the control condition (VEN-p300CH1/VEC-CITED2) set to 1. Results are presented as the mean ± s.e.m. of three independent biological experiments.

Figure S3. Related to Figure 5. CITED2 and ISL1 interaction visualized in living cells by BiFC assays and test expression of fusion proteins. (A) Undifferentiated E14/T ESC transfected with VEN-ISL1 and VEC-CITED2 expression vectors examined one day after transfection for morphological aspect (Phase contrast), and fluorescence emission (Fluorescence). The merged picture is also presented. (B-D) Whole cell extracts from untransfectedHEK293T cells or transiently expressing the indicated proteins were analysed by western blotting. (B) Expression of VEC-CITED2 detected with anti-CITED2 (left panel) and anti-flag (right panel) antibodies in HEK293T tranfected cells. The position of VEC-CITED2 fusion protein (~40kDa), endogenous CITED2 (~30 kDa) and non-specific protein (NS) are indicated. (C) Expression of VEN-ISL1 (~56 kDa) and myc-ISL1 (~40 kDa) detected with anti-ISL1 antibody. The positions of fusion proteins and non-specific proteins (*, NS) are indicated. (D) Expression of VEN-GAL4 (~34 kDa) and VEC-GAL4 (~27 kDa) analysed with anti-HA antibody. The positions of the fusion proteins are indicated. (E) The reference genes *Hprt*, *Gapdh* and *18S* were assayed across the cDNA samples prepared from undifferentiated (D0) $C2^{fl/fl}$[Cre] (left) and E14/T ESC, or differentiated cells up to the time points indicated. The mean of raw threshold cycle (Ct) values obtained from three biological replicates are plotted.

Table S1. Related to all figures. – Primers used for qPCR and ChIP qPCR

Supplemental Experimental Procedures

Embryonic stem cells, culture conditions and isolation of cardiac progenitor populations

Apple D2 (AD2), $C2^{fl/fl}$, $C2^{\Delta/\Delta}$[LA11], $C2^{fl/fl}$[Cre] and E14/T mouse ESC lines were described previously, and were cultured on gelatine-coated plates in undifferentiating medium supplemented with LIF. $C2^{fl/fl}$ ESC harbour both *Cited2* alleles functional and $C2^{\Delta/\Delta}$[LA11] ESC are *Cited2*-knockout cells originated from $C2^{fl/fl}$ ESC. $C2^{fl/fl}$[Cre] ESC which have the exon2 of *Cited2* flanked by LoxP sites and constitutively express a tamoxifen-inducible Cre recombinase were treated with 1μM of 4-hydroxytamoxifen (4HT) during 48 hrs at the indicated time points to delete *Cited2* gene or with Ethanol used as a 4HT vehicle control. All ESC lines were differentiated using the hanging-drop method in medium containing 20% FBS without LIF supplementation (differentiation medium). Briefly, 500 (for E14/T or AD2) or 1000 (for $C2^{fl/fl}$, $C2^{\Delta/\Delta}$[LA11] or $C2^{fl/fl}$[Cre]) cells were cultured in 20μl hanging drops of differentiation medium for 48 hrs to initiate EB formation. Next, EB were grown in differentiation medium in suspension for 3 days in a bacterial petri dish before transfer to 0.1% gelatine coated plates. The puromycin added to the culture medium of undifferentiated E14/T cells to sustain the presence of high levels of flag-CITED2 expressing plasmid (pPyCAGIP-flagCITED2) or the control vector (pPyCAGIP) in these cells as previously described (Kranc et al., 2015), was omitted in the differentiation medium. Progression of differentiation was monitored with inverted microscopes. R+G+, R-G+, R+G- and R-G- labelled cell populations were isolated by FACS from E9.5 *Nkx2.5-eGFP*/SHF-*dsRed* double transgenic heart embryos or AD2-derived cells as previously described (Domian et al., 2009). All mice were cared for within the Animal Care facilities of the Massachusetts General Hospital under the supervision of an active and functioning Subcommittee on Research Animal Care (SRAC), which serves as the Institutional Animal Care and Use Committee

(IACUC) as required by the Public Health Service (PHS) Policy on Humane Welfare Regulations.

Immunochemistry

Immunocytochemistry was performed with $C2^{fl/fl}$[Cre] ESC treated with ethanol or 4HT at D0 and differentiated for 10 days. At D10, cells were dissociated by trypsinization and grown for 24 hours on coverslips coated with 0.1% GELATIN, before being washed with phosphate buffered saline (PBS; Sigma), fixed in 4% formaldehyde (Sigma) for 15 minutes, permeabilized in 0.1% Triton X-100 diluted in PBS (Sigma) for 20 minutes, and blocked at room temperature with a 2% BOVINE SERUM ALBUMIN (BSA; Nzytech) in PBS for at 30 minutes. Samples were then incubated either with an anti-α-ACTININ (sarcomeric) antibody (A7811, Sigma; at 1:500 dilution) or with an anti-sarcomeric MYOSIN (DSHB, MF20; at 1:300 dilution) monoclonal primary antibodies diluted in blocking solution for 2 hours at room temperature. The coverslips were then washed three times in PBS and incubated for 1 hour at room temperature with the AlexaFluor-594 donkey anti-mouse antibodies (Invitrogen) used at 1:500 dilution in blocking solution. The coverslips were then washed three times with PBS and placed onto slides using mounting medium containing DAPI (Mowiol-DAPI). Fluorescence microscopy was performed using an Axio Imager Z2 Fluorescence microscope (Carl Zeiss) at a 100x magnification. Negative controls were used to set up exposure conditions for detection of a specific signal. Western blotting assays were performed using 20μg of whole cell lysates prepared from the indicated mouse ESC as previously described (Kranc et al., 2015). Mouse monoclonal JA22 against CITED2 (AB5155, Abcam), anti-ISL1 (AB109517, Abcam), and anti-flagM2 (F1804, Sigma) mouse monoclonal antibodies were used at 1:2000 dilution. Mouse monoclonal anti-GATA4 (sc-25310, Santa Cruz) was used at 1:200 dilution. Loading was monitored by probing the membrane with a mouse monoclonal anti-β-TUBULIN antibody (T5293, Sigma) used at 1:5000 dilution.

Chromatin immunoprecipitation (ChIP) assays

$1x10^8$ of E14/T cells transfected with a plasmid expressing flag-CITED2 (pPyCAGIP-flagCITED2) or control vector (pPyCAGIP), or untransfected E14/T ESC-derived cells at D5 of differentiation dissociated to a single cells by 0.05% TRYPSIN (SIGMA) at 37°C for 15 min, were fixed with 1% formaldehyde and quenched by glycine to final concentration of 0.125 M (SIGMA). Nuclei were extracted and submitted to 75U of Microccocal Nuclease-MNase (Fermentas) for DNA fragmentation. CITED2 and flag-CITED2 immunoprecipitations were performed with rabbit polyclonal anti-CITED2 (H-220, Santa Cruz Biotechnology) and monoclonal anti-flagM2 (F1804, Sigma) antibodies, respectively. A rabbit IgG-ChIP grade (AB46540, Abcam) was used for control immunoprecipitations of endogenous CITED2. The co-immunoprecipitated DNA was purified by phenol:chloroform:isoamyl extraction and precipitation. ChIP experiments to determine the presence of H3triMek4 were performed using mouse monoclonal anti-H3triMek4 (AB10812, Abcam) and anti-flagM2 (F1804, Sigma) as previously described (Kranc et al., 2015). The enrichment of target genomic elements was determined by qPCR as previously described (Kranc et al., 2015), using primers listed in Supplemental Table S1.

Production and transduction of the recombinant 8R-CITED2 protein

Full-length human CITED2 cDNA and an oligonucleotide encoding 8 arginines (8R) were cloned into the pGEX6P1 vector (GE Healthcare Life Sciences) to express a chimeric protein consisting of the Glutathione S-transferase (GST) in fusion with the 8R domain and CITED2 (termed GST-8R-CITED2, Figure S2). The construct harbours also a 3C cleavage site encoded at the C-terminal part of the GST. GST-8R-CITED2 protein was expressed in BL21 *E. coli* cells, purified by Gluthatione Sepharose Fast Flow (GE Healthcare) resin affinity chromatography using a fast protein liquid chromatography (FPLC) ÄKTA™ (Amersham Biosciences). GST-8R-CITED2 was cleaved on the affinity column by the Rhonivirus 3C protease coupled to GST for 16 hrs at 4ºC. Subsequently, the recombinant 8R-CITED2 was eluted from the column and stored at -80ºC. For cellular transduction, the protein solutions were thawed and diluted with the culture medium 24 hrs and stored at 4ºC prior to supplementation of cell cultures at final concentration of 5-10μg/ml. Newly constructed plasmids were validated by sequencing and the expression of fusion proteins tested by western blot (Figure S2). Details of plasmid construction and protein purification are available upon request.

Protein interactions and plasmids

For *in vitro* binding assays, GST-CITED2 fusion proteins and *in vitro* translated myc-ISL1 were prepared as previously described (Machado-Oliveira et al., 2015). Myc-ISL1 expression plasmid was constructed in pcDNA3 (Invitrogen) with an amino-terminal myc epitope tag. Plasmids expressing flag-CITED2 and pPyCAGIP were previously described (Chen et al., 2012). For BiFC assays, plasmids expressing VEC-CITED2, VEN-p300CH1, VEC-GAL4, VEN-GAL4 and the VEN-vector were described elsewhere (Machado-Oliveira et al., 2015). The plasmid VEN-ISL1 expressing VEN in fusion with ISL1 was obtained by subcloning ISL1 cDNA fragment of the Myc-ISL1 expression plasmid into the VEN-vector in frame with the VEN domain. All newly constructed plasmids were validated by sequencing and the expression of fusion proteins tested by western blot (Figure S3). Details of plasmid construction are available upon request. For co-immunoprecipitation assays, ~0.4mg of whole cell extracts from HEK293T cells transfected with flag-CITED2 expression vector alone or together with myc-ISL1 vector, were prepared in a buffer containing 50 mM Tris pH7.5, 100 mM NaCl, 15 mM EGTA, 0.1% Triton-X100, and Complete protease inhibitors (Roche), and incubated overnight at 4°C with monoclonal anti-flagM2 covalently coupled to agarose beads (Sigma). Immunoprecipitates were washed five times, and eluted by competition with flag peptide (Sigma) added at 200 μg/ml for 30 minutes at 4ºC. Eluted samples were subjected to western blot analysis. For BiFC, $2x10^5$ E14/T ESC were transfected with 250 ng of the indicated vectors and examined for fluorescence emission two days after transfection in undifferentiated cells, or at D2 and D5 of differentiation by EB formation.

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