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Down Syndrome Critical Region-1 Is a Transcriptional Target of Nuclear Factor of Activated T Cells-c1 within the Endocardium during Heart Development*

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Abstract

Patients with Down syndrome have characteristic heart valve lesions resulting from endocardial cushion defects. The Down syndrome critical region 1 (*DSCR1*) gene, identified at the conserved trisomic 21 region in those patients, encodes a calcineurin inhibitor that inactivates nuclear factor of activated T cells (NFATc) activity. Here, we identify a regulatory sequence in the promoter region of human *DSCR1* that dictates specific expression of a reporter gene in the endocardium, defined by the temporal and spatial expression of *Nfatc1* during heart valve development. Activation of this evolutionally conserved *DSCR1* regulatory sequence requires calcineurin and NFATc1 signaling in the endocardium. NFATc1 proteins bind to the regulatory sequence and trigger its enhancer activity. NFATc1 is sufficient to induce the expression of *Dscr1* in cells that normally have undetectable or minimal NFATc1 or *DSCR1*. Pharmacologic inhibition of calcineurin or genetic *Nfatc1* null mutation in mice abolishes the endocardial activity of this *DSCR1* enhancer. Furthermore, in mice lacking endocardial NFATc1, the endogenous *Dscr1* expression is specifically inhibited in the endocardium but not in the myocardium. Thus, our studies indicate that the *DSCR1* gene is a direct transcriptional target of NFATc1 proteins within the endocardium during a critical window of heart valve formation.

Down syndrome (DS),⁵ produced by trisomy of all or part of chromosome 21, occurs at a frequency of 1 in 43 spontaneous abortions and 1 in 750 live births (1). Most DS fetuses (up

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⁵The abbreviations used are: DS, Down syndrome; NFATc, nuclear factor of activated T cells; DSCR, Down syndrome critical region; CnA*, constitutively active calcineurin; IRES, internal ribosomal entry site; wt, wild-type; PECAM, platelet endothelial cell adhesion molecule; RT, reverse transcription; NRE, NFAT-responsive element; GFP, green fluorescent protein; E, embryonic day; MEF, mouse embryonic fibroblast; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

to 50–75%) die *in utero* (2,3). Approximately 40–50% of the surviving DS patients have characteristic congenital heart disease (4). Indeed, DS is a major cause of congenital heart defects in humans (5). Endocardial cushion defect is the predominant cardiac abnormality in DS, leading to characteristic atrioventricular septal or heart valve lesions (4,6). Previous work from our laboratory and others demonstrates that the calcineurin/ nuclear factor of activated T cells (NFATc) signaling pathway is essential for the development of endocardial cushions and heart valves (7–9), and that perturbations of the pathway lead to many features of Down syndrome (7,10,11).

Calcineurin is a Ca^{2+} /calmodulin-dependent phosphatase (12). Activated calcineurin dephosphorylates NFATc proteins, triggering NFATc translocation from the cytoplasm into the nucleus, where NFATc proteins form transcription complexes with cofactors to regulate target gene expression (13,14). Two genes, *DYRK1a* and *DSCR1*, localized within the Down syndrome critical region (DSCR) of human chromosome 21, were shown to synergistically reduce the nuclear occupancy of the NFATc proteins (10,15). *DYRK1a* is a kinase that promotes NFATc nuclear export (10,15), and *DSCR1* (RCAN1, calcipressin1, or MCIP1) encodes an inhibitor of calcineurin (16–19). Apparently a 1.5-fold overexpression of these two proteins leads to the dysregulation of calcineurin/NFAT signaling, resulting in a constellation of DS features that are almost perfectly reproduced in *Nfatc* mutant mice (10).

Although recent work has shown that the expression of *DSCR1* in neurons and the heart is induced by the NFAT pathway (20,21), it is not clear how *DSCR1* interacts with NFATc1 within the endocardium during heart valve development. The role of calcineurin in regulating *DSCR1* expression in the heart remains undefined. Nor is there unambiguous evidence that *DSCR1* is a direct transcriptional target of NFATc1 in the heart. Here, we identify an enhancer of the human *DSCR1* gene that contains all the elements required to direct endocardial *DSCR1* expression that is entirely defined by calcineurin/NFATc1 activity in the heart. We present both *in vitro* and *in vivo* evidence that the *DSCR1* gene is a direct downstream target of the NFATc1 transcription factor in the endocardium of a developing heart.

EXPERIMENTAL PROCEDURES

Mouse Husbandry

All mouse strains were maintained in outbred backgrounds at the Stanford University Research Animal Facility under the National Institutes of Health guidelines. The date of observation of a vaginal plug was set as day 0.5, which was confirmed by ultrasonography prior to sacrificing pregnant mice (22) and examination of embryo morphology and somite number. *Nfatc1-c4* mutant mouse lines were described previously (7).

Generation of *DSCR1-lacZ* Mice

The plasmid used in transgenic mouse production was constructed by linking the NFAT-dependent human *DSCR1* enhancer upstream of the basal *hsp68* promoter and *lacZ* reporter gene (23). The *DSCR1* enhancer contained 904 base pairs derived from sequences flanking exon4 (–874 to +30) with the first nucleotide of exon 4 designated as 1. After removal of the prokaryotic sequences, transgene fragments were introduced by pronuclei microinjection of fertilized oocytes of C57B6/C3H mice.

Gel Mobility Shift Assay

Binding of nuclear proteins to DNA oligonucleotide probes was done as described previously (24,25). Nuclear proteins were extracted from embryonic hearts at embryonic day (E) 11.5 and E12.5. Anti-NFATc1 (clone 7A6, Santa Cruz Biotechnology) was used for antibody supershift experiments. Oligonucleotides used were: wild-type (wt)-NFAT-responsive element (NRE)-1,

TCAGCTGTTTTTCCATTCTCCCAAGC; mutant-NRE1,
 TCAGCTGTTTGCTAGATTCTCCCAAGC; wt-NRE2,
 AGCATAGGGAAAATGACTAAGG; mutant NRE2, AGCATAGCTAGCATGACTAAGG;
 wt-NRE3, ACGTCACCTCTTCCAGTAGAACTTACAC; mutant NRE3,
 ACGTCACCTCGCTAGAGTAGAACTTACAC (sequences underlined represent the intact
 or mutant NFAT core binding sites).

Plasmids, Cell Culture, and Transfection

Full-length mouse NFATc1 was cloned into pMES vector. The constitutively active calcineurin and *DSCR1* luciferase reporter were described previously (26,27). Nine copies of wt or mutant NRE3 were cloned into pGL3-promoter (Promega). Mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and nonessential amino acids. Cells were transfected with Lipofectamine 2000 (Invitrogen).

lacZ Staining, RNA in Situ Hybridization, and Immunostaining

Whole-mount *lacZ* staining was performed as described previously (7). The mouse *Dscr1* 3'-untranslated region corresponding to exons 5, 6, and 7 was generated by reverse transcription (RT)-PCR and subcloned into pCRII vector. This probe was common to all *DSCR1* isoforms. *In situ* hybridization was performed using a standard protocol. For platelet endothelial cell adhesion molecule (PECAM)-1 staining, paraffin-embedded tissue sections were trypsin-digested and incubated with rat anti-PECAM-1 antibody (Pharmingen). NFATc1 antibody (Santa Cruz Biotechnology) was applied to paraformaldehyde-fixed REN cells. Alexa fluorophor-conjugated secondary antibodies (Molecular Probes) were used to visualize antibody staining.

Adenoviral Infection, cDNA Microarray Analysis, and RT-PCR

The REN cell was infected with an adenoviral expression cassette consisting of a cytomegalovirus promoter, a constitutively activated NFATc1, and downstream green fluorescent protein (GFP) linked by internal ribosomal entry site (IRES). Cells were harvested for RNA isolation 2 days after infection. Human 10K cDNA chip was used for cDNA microarray analyses that were performed at Genomics/Microarray Facility, The Wistar Institute. The arrayed DNA (expressed sequence tag AA629707) was located at the 3' end of *DSCR1* for all four human transcript isoforms. For RT-PCR, total RNAs were isolated from independently infected REN cells. RT-PCR was performed using a standard protocol with the *DSCR1* specific primers.

RESULTS

The *DSCR1* Regulatory Promoter Region Is Evolutionally Conserved and Under Calcineurin/NFATc1 Regulation

A promoter element located upstream of exon 4 of the human *DSCR1* gene was identified previously (27). The promoter showed an over 75% homology among rat, mouse, and human sequences, and the NREs within were perfectly conserved (Fig. 1A). By gel mobility shift analysis, we found that endogenous NFATc proteins present in the nuclear extracts of mouse embryonic hearts bound strongly to oligonucleotides containing the NRE1, -2, or -3 present in the promoter (Fig. 1B, lanes 1, 3, and 5). The presence of NFATc1 proteins in the binding complex was confirmed by disruption of the protein-DNA complexes with anti-NFATc1 antibody (Fig. 1B, lanes 2, 4, and 6). Furthermore, mutations of the canonical NFAT binding sites of NREs from GGAA(A/T)TTCC to CTAG(C/G)CTAG abolished the binding of

endogenous NFATc1 proteins to NRE2 and -3 but not NRE1 (Fig. 1C, lanes 2, 4, and 6), suggesting that NFATc1 binds to canonical sites of NRE2 and -3, and a noncanonical site of NRE1, perhaps through GGAGA/TCTCC that is similar to the canonical site. Overall, these findings indicate that endogenous NFATc1 proteins bind to the NREs of the *DSCR1* enhancer.

To study whether NFATc1 could transactivate the *DSCR1* promoter, we performed a luciferase reporter assay. The regulatory sequence of *DSCR1* gene was cloned upstream of the luciferase reporter gene (27). In the MEFs, we found that overexpression of NFATc1 and constitutively active calcineurin (CnA*) (7) significantly enhanced the luciferase reporter activity (Fig. 1D). Overexpression of NFATc1 alone, however, had minimal activation of the reporter gene in MEFs because of the fact that NFATc proteins, whether overexpressed or endogenous, were located predominantly in the cytoplasm of MEFs in the absence of CnA* (data not shown). CnA* triggered the nuclear localization of endogenous or overexpressed NFATc proteins, leading to reporter activation (data not shown and Fig. 1D). Thus, the ability of NFATc1 proteins to bind to the enhancer and trigger the enhancer activity depends on their nuclear localization controlled by calcineurin. This indicates that NFATc proteins regulate the expression of *DSCR1* at the transcriptional level. To examine whether NFATc1 could exert its transcriptional activity through the NREs of *DSCR1* enhancer, we cloned the NRE3 element upstream of the luciferase reporter gene. We found that CnA* and NFATc1 strongly activated the luciferase expression of this reporter (Fig. 1E). Mutations of the NFAT binding site on NRE3 significantly reduced the transcriptional activity of NFATc1 on the reporter gene (Fig. 1E). Taken together, these studies indicate that NFATc1 proteins transcriptionally activate the *DSCR1* enhancer through the NREs.

NFATc1 Induces Endogenous *DSCR1* Expression in Cells That Normally Have Undetectable or Minimal Expression of NFATc1 or *DSCR1*

To test whether NFATc proteins were sufficient to trigger endogenous *DSCR1* expression, we chose the human mesothelioma cell line (REN cell) that had no detectable expression of NFATc1 and had only minimal expression of *DSCR1*. These cells were infected with either adenovirus expressing constitutively nuclear NFATc1-IRES-GFP (7) or with GFP and examined for the expression of *DSCR1*. The subcellular localization of the constitutively nuclear NFATc1 proteins was confirmed by immunostaining (Fig. 2A). Western blot analysis showed the expression of constitutive activated NFATc1 proteins in REN cells after infection with adenovirus (Fig. 2B). REN cells infected with control GFP-only virus did not express detectable endogenous NFATc1. Cells expressing NFATc1-IRES-GFP were sorted, cultured, and then subjected to transcript microarray analysis. Transcript analysis revealed that NFATc1 enhanced the expression of *DSCR1* mRNA in REN cells by approximately 10-fold (Fig. 2C). The induction of *DSCR1* mRNA expression was confirmed by semi-quantitative RT-PCR in independently infected cells (Fig. 2D). These results indicate that NFATc1 is sufficient to activate endogenous *DSCR1* expression.

The *DSCR1* Enhancer Activity in the Endocardium Requires Both Calcineurin and NFATc1

Both the *in vitro* binding and cellular transactivation assays suggested that NFATc transcriptionally regulated the expression of *DSCR1*. To investigate whether the enhancer activity of *DSCR1* gene depends on NFATc proteins *in vivo*, we generated a transgenic mouse reporter line (*DSCR1-lacZ*), where a β -galactosidase reporter gene was driven by the human *DSCR1* enhancer. By whole-mount *lacZ* staining, we found that the *DSCR1-lacZ* activity was expressed in the heart and neural tube during a specific time window of development from E9.5 to E12.5 (Fig. 3A). This expression pattern partially recapitulated the endogenous *DSCR1* expression in the heart (28) and corresponded to the time window of NFATc1 expression in the endocardium (7–9). Detailed histological analysis of the heart revealed a specific expression of this transgene in endocardial cells (Fig. 3B). At E9.5, the transgene was expressed

throughout the endocardium. By E11.5, the expression became limited to the cushion endocardium of the cardiac outflow tract and atrioventricular canal. This unique expression pattern was identical to that of NFATc1 as the latter was expressed in all endocardial cells at E9.5, and its expression became restricted to the cushion endocardial cells by E11.5 (7–9).

Because the expression of *DSCR1-lacZ* colocalized with NFATc1 expression both temporally and spatially during heart development, we investigated whether the *DSCR1* promoter activity was indeed regulated by NFATc1 *in vivo*. In *Nfatc1* null embryos harboring the *DSCR1-lacZ* reporter gene, we found that the expression of *lacZ* in the endocardium was abrogated (Fig. 3C), indicating that NFATc1 was required *in vivo* for *DSCR1-lacZ* expression. However, the expression of *DSCR1-lacZ* in the neural tube was not affected in the absence of NFATc1, consistent with the observation that NFATc1 was expressed in the endocardium (7–9,29) but not in the neural tube at that developmental stage. Furthermore, we found that the dependence of *DSCR1* expression on NFATc1 required calcineurin activity in the endocardium. Using cyclosporin A to inhibit calcineurin activity *in utero* (7), we showed that cyclosporin-treated embryos failed to express *DSCR1* enhancer activity in the heart (Fig. 3D). NFATc1 proteins were localized to the cytoplasm in cyclosporin-treated embryos (Fig. 3E) and in embryos lacking endocardial calcineurin (data not shown), indicating that nuclear localization of NFATc1 controlled by calcineurin is crucial for *DSCR1* activation in the endocardium. To investigate whether NFATc2, -c3, and -c4 were required for endocardial *DSCR1* expression, we examined the expression of *DSCR1-lacZ* activity in *Nfatc2/3/4* triple null embryos (7) and found that the *DSCR1* enhancer activity was expressed normally in the heart in the absence of *Nfatc2/3/4* (Fig. 3F). Of note, the *DSCR1* enhancer activity in the neural tube required NFATc2/3/4 but not NFATc1 (Fig. 3, C and F). These results demonstrated a specific and unique *in vivo* requirement of calcineurin/NFATc1 for the *DSCR1-lacZ* expression in the endocardium.

Endogenous *DSCR1* Gene Expression in the Endocardium Requires NFATc1

To address whether endogenous *DSCR1* expression required NFATc1 in the endocardium, we performed a RNA *in situ* hybridization in *Nfatc1* null embryos with a probe specific for *DSCR1*. This probe was generated from the 3'-untranslated region corresponding to exons 5, 6, and 7 of mouse *Dscr1* and detected all the common isoforms of *Dscr1*. We found that *DSCR1* was expressed in both the endocardium and myocardium of wild-type mice at E12.5 (Fig. 4A). In mice lacking NFATc1, the expression of *DSCR1* in the cushion endocardium was significantly reduced or abolished (Fig. 4, B and D). In contrast, its expression was preserved in the myocardium that did not normally express NFATc1 (Fig. 4B). To rule out a global change of gene expression in the endocardium of *Nfatc1*^{-/-} mice, we examined the expression of endocardial PECAM-1 in the mutant mice. By immunostaining, we found no significant changes of endocardial PECAM-1 expression in *Nfatc1*^{-/-} embryos (Fig. 4F). These observations indicate that NFATc1 proteins specifically regulate the expression of endogenous *DSCR1* in the endocardium and that the expression of *DSCR1* in the myocardium is NFATc1-independent. Overall, our studies unequivocally demonstrate that the regulation of endocardial *DSCR1* expression depends on calcineurin/NFATc1 signaling and that *DSCR1* is a direct downstream target of NFATc1 proteins within the endocardium.

DISCUSSION

We have presented multiple lines of evidence indicating that the calcineurin inhibitor *DSCR1* is a direct transcriptional target of NFATc1 during critical windows of heart valve development. Because the activity of endocardial NFATc1 itself depends on calcineurin (Fig. 3E) (7), the regulation of *DSCR1* by NFATc1 may provide a negative feedback control mechanism to maintain endocardial NFATc1 homeostasis during heart development (Fig. 4G). This working

model suggests that reduced NFATc1 activity attenuates DSCR1 expression, boosting calcineurin activity and promoting further NFATc1 nuclear entry to restore NFATc1 level. Excessive NFATc1 activity, in contrast, may enhance the expression of DSCR1, which inhibits calcineurin and blocks the entry of additional NFATc1 molecules into the nuclei, and thus normalizes NFATc1 activity. This working model is further supported by previous observations that enhanced DSCR1 expression inhibits NFATc1 nuclear localization and transcriptional activity in the heart (10,30). The negative feedback loop may serve to maintain endocardial NFATc1 protein function at a stable level during heart valve morphogenesis. However, mice lacking DSCR1 and/or DSCR1-like 1 survive to adulthood with no apparent severe defects in cardiac morphogenesis (31). These findings may suggest the presence of redundant pathways that compensate for the loss of function of DSCR1.

A mouse *Dscr1* enhancer (~700 bp) was used previously to drive lacZ transgene expression in an embryonic heart. Weak transgene expression was detected in the endocardial cushion of the outflow tract at E9.5–E12.5 and in a small subset of NFATc1-expressing cells of the atrioventricular canal (21), suggesting that the enhancer did not contain all elements required to recapitulate endogenous DSCR1 expression in the endocardium defined by NFATc1 activity. In this study, the lacZ transgene is controlled by the homologous human enhancer (~900 bp). We have observed broader panendocardial expression of the transgene at E9.5. By E11.5, the expression is restricted to the cushion endocardium of the cardiac outflow tract and atrioventricular canal (Fig. 3). This unique spatial-temporal expression profile is identical to that of NFATc1 (7–9) and demonstrates a functional conservation of the *DSCR1* enhancer between the human and mouse species. Indeed, the transgene expression is abolished in *Nfatc1* null embryos (Fig. 3), indicating that endocardial DSCR1 expression is both spatially and temporally defined by NFATc1. Thus, the longer human enhancer used in our study contains a complete set of elements required to direct endocardial DSCR1 expression in a NFATc1-dependent manner. Additionally, we show that endogenous NFATc1 proteins physically bind to the enhancer through the NREs and that NFATc1 is both necessary and sufficient for the activation of DSCR1 expression (Figs. 1 and 2). Among NFATc signaling, NFATc1, but not NFATc2/3/4, is uniquely required for activating endocardial DSCR1 expression (Fig. 3). We further show that the DSCR1 expression requires calcineurin to trigger NFATc1 nuclear localization and activation (Figs. 1 and 3).

These observations provide direct evidence that the endogenous DSCR1 expression in the developing endocardium is controlled by calcineurin/ NFATc1 signaling. NFATc1 transcriptionally activates DSCR1 expression through a conserved enhancer region. The regulation of DSCR1 and NFATc1 may provide a feedback mechanism to ensure proper maintenance of NFAT signaling strength during heart development.

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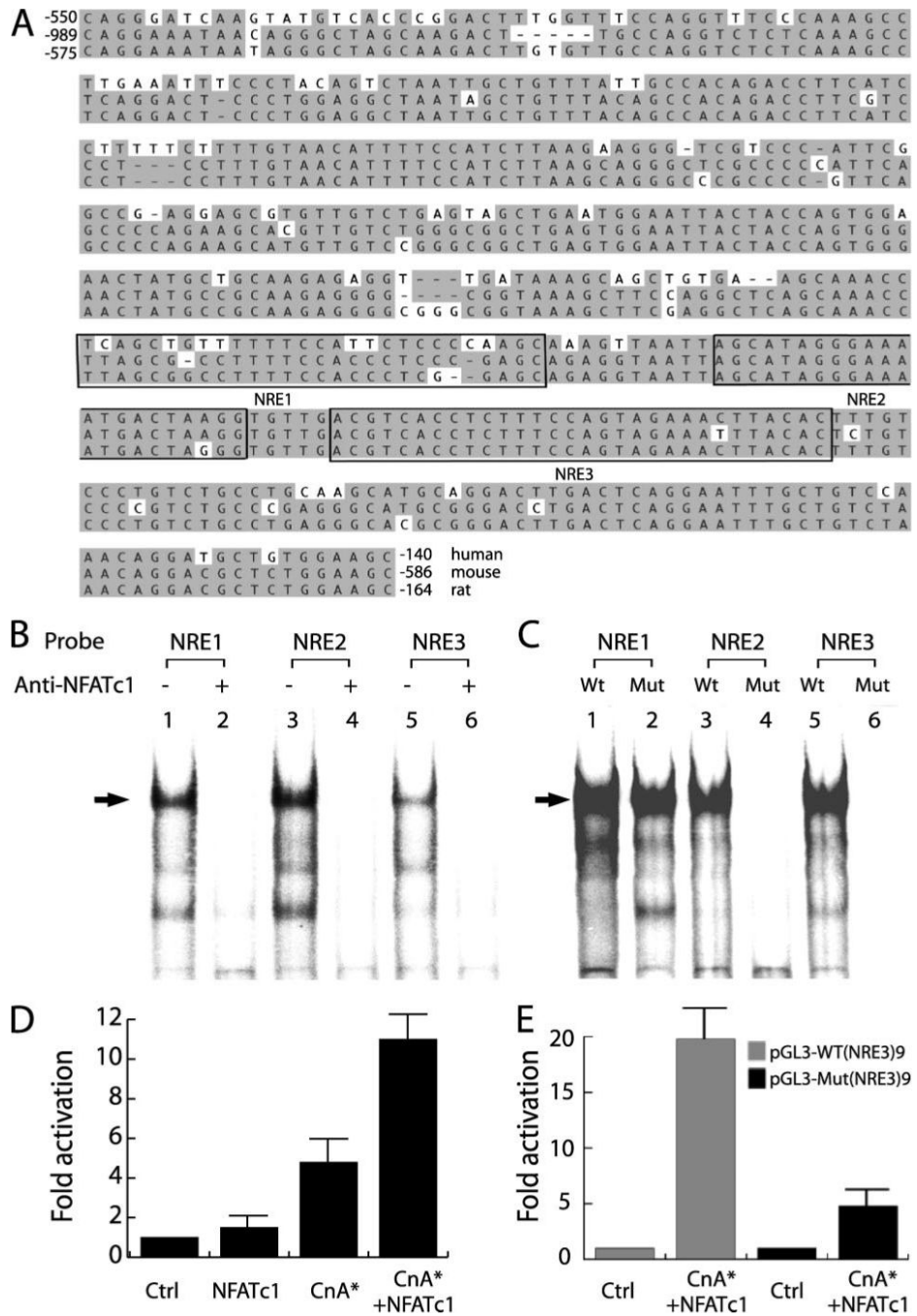


FIGURE 1. NFATc proteins bind and transactivate a conserved enhancer derived from the *DSCR1* gene

A, alignment of part of the enhancer from human, mouse, and rat. The nucleotide immediately upstream of the start codon ATG of exon 4 is defined as position -1. Oligonucleotides (NREs) used in the gel shift assay are boxed. B, binding of NFATc proteins to the enhancer. [γ^{32} P] ATP-labeled NRE oligonucleotides were incubated with nuclear extracts from mouse embryonic hearts. DNA-protein complex (arrow) was resolved by gel electrophoresis. For the NFAT, DNA complexes were disrupted after incubating nuclear protein extracts with anti-NFATc1 antibody. C, binding of NFATc proteins to intact and mutant NREs. The consensus NFAT binding sites (GGAAA) were substituted with CTAGC in the mutant NREs. For the

formation of NFAT, DNA complexes (*arrow*) were disrupted for mutant NRE2 and -3. *D*, transactivation of the *DSCR1* enhancer by calcineurin and NFATc1. CnA* and NFATc1 activated the enhancer in MEFs. *E*, NRE3 functions as an NFAT-dependent enhancer. Nine copies of intact or mutant NRE3 were cloned upstream of luciferase reporter. Transactivation of the mutant NRE3 (pGL3-Mut(NRE3)₉) by calcineurin and NFATc1 is greatly reduced compared with intact NRE3 (pGL3-wt(NRE3)₉).

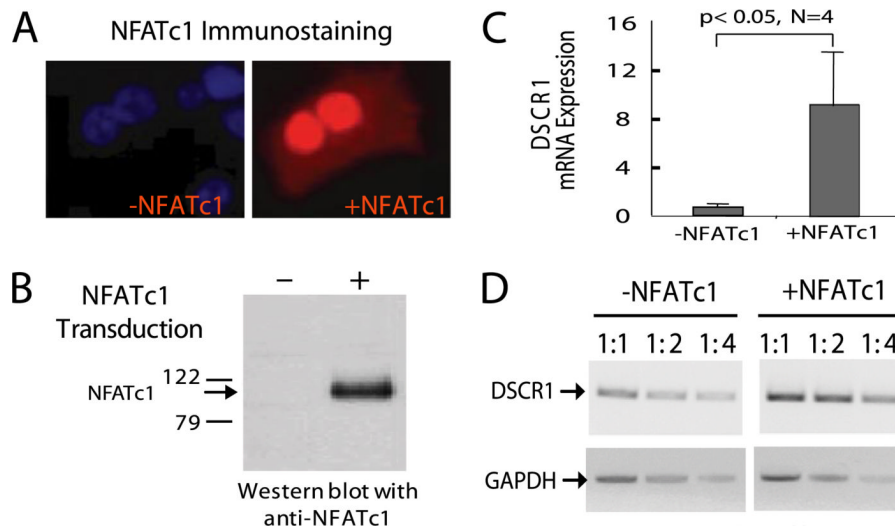


FIGURE 2. The cDNA microarray analysis shows that NFATc1 activates endogenous DSCR1 expression

A, immunofluorescent staining shows nuclear localization NFATc1 proteins (*red*) in cells transfected with the constitutively nuclear NFATc1. *B*, Western blot analysis shows the expression of constitutively activated NFATc1 in REN cells after infection with adeno-NFATc1-IRES-GFP virus. REN cells infected with control GFP-only virus do not express detectable endogenous NFATc1 even with 10-fold protein loading. *C*, a graph showing that the ectopic expression of NFATc1 in REN cells markedly up-regulates *DSCR1* mRNA expression (up to 11-fold, $n = 4$, $p < 0.05$). *D*, semiquantitative RT-PCR showing up-regulation of *DSCR1* mRNA level in virally infected REN cells. Approximately 4-fold induction of *DSCR1* mRNA by NFATc1 was observed.

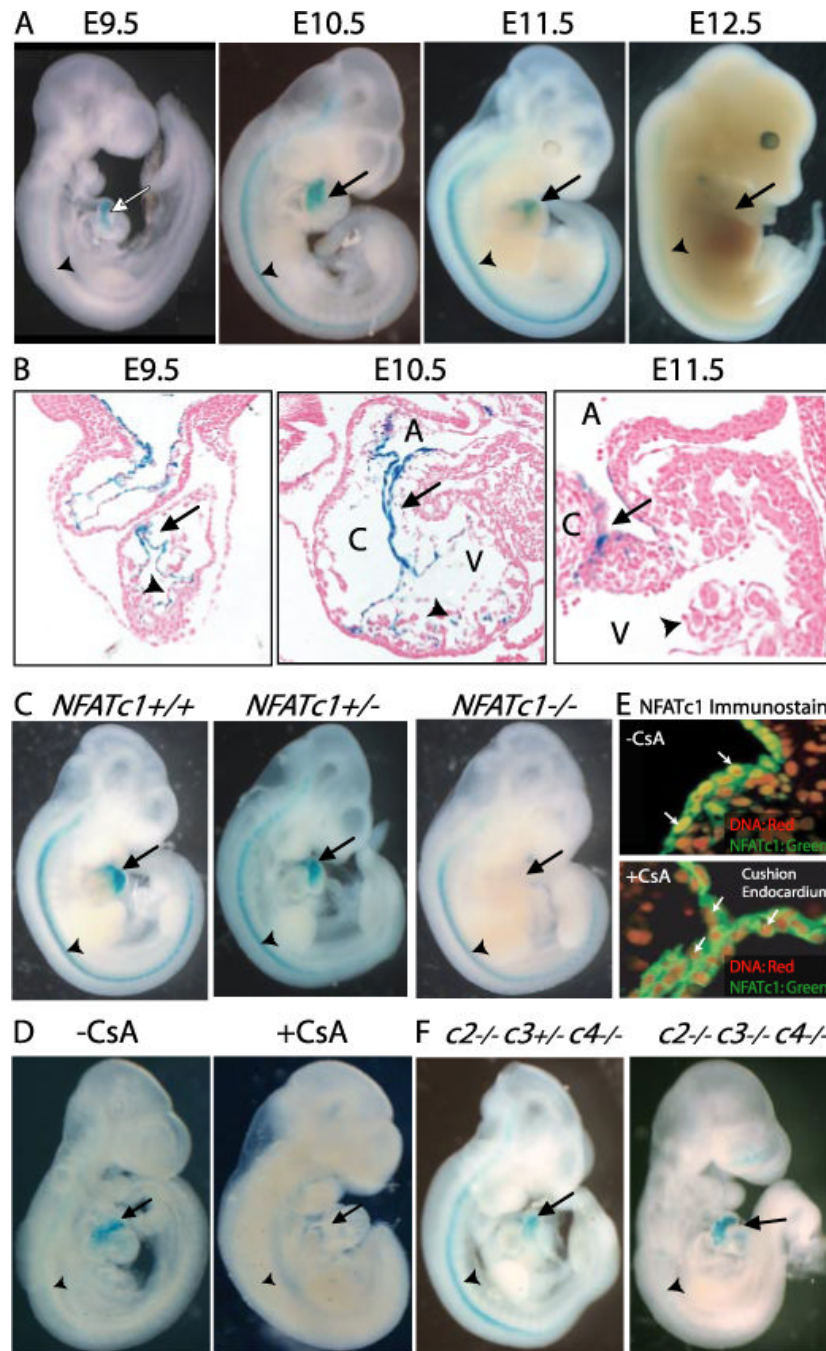


FIGURE 3. The expression of *DSCR1-lacZ* transgene in the endocardium requires calcineurin and NFATc1

A, transgenic embryos harboring the *lacZ* reporter driven by the *DSCR1* enhancer. Images of whole-mount 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) staining of embryos at different developmental stages (E9.5 to E12.5) are shown. *lacZ* is expressed in both the heart (arrows) and neural tube (arrowheads). B, sections of *DSCR1-lacZ* embryonic hearts at E9.5, E10.5, and E11.5 are counterstained with nuclear fast red. Note that the *lacZ* is specifically expressed in endocardium of the cushion (arrows) and ventricle (arrowheads) at E9.5 and E10.5. Its expression is limited to the cushion endocardium at E11.5. A, atrium; C, cushion; V, ventricle. C, images of whole-mount X-gal staining of embryos at E10.5. Breeding of *lacZ*

transgene into *Nfatc1* null mutant background removes lacZ expression in the heart (*arrows*) but not in the neural tube (*arrowheads*). *Nfatc1* heterozygous mutant retains lacZ expression. *D*, images of whole-mount X-gal staining of embryos at E9.5. Staged pregnant transgenic mice (E8) were subcutaneously injected with cyclosporin A (*CsA*) at 50 mg/kg body weight. Embryos were harvested and analyzed 36 h later. *CsA* treatment prevents the transgene expression in the heart (*arrows*) and neural tube (*arrowheads*). *E*, *CsA*-treated embryos showed cytoplasmic translocation of NFATc1 proteins in the cushion endocardium. Fluorescent immunostaining of NFATc1 proteins by anti-NFATc1 antibody (*green*) and nuclei by 7-amino-actinomycin D (*red*) is shown. *F*, NFATc2, c3, or c4 is not required to maintain *DSCR1-lacZ* expression in the endocardium (*arrows*). Breeding of lacZ transgene into *Nfatc2/c3/c4* triple null mutant background removes lacZ expression in the neural tube (*arrowheads*) but not in the heart (*arrows*).

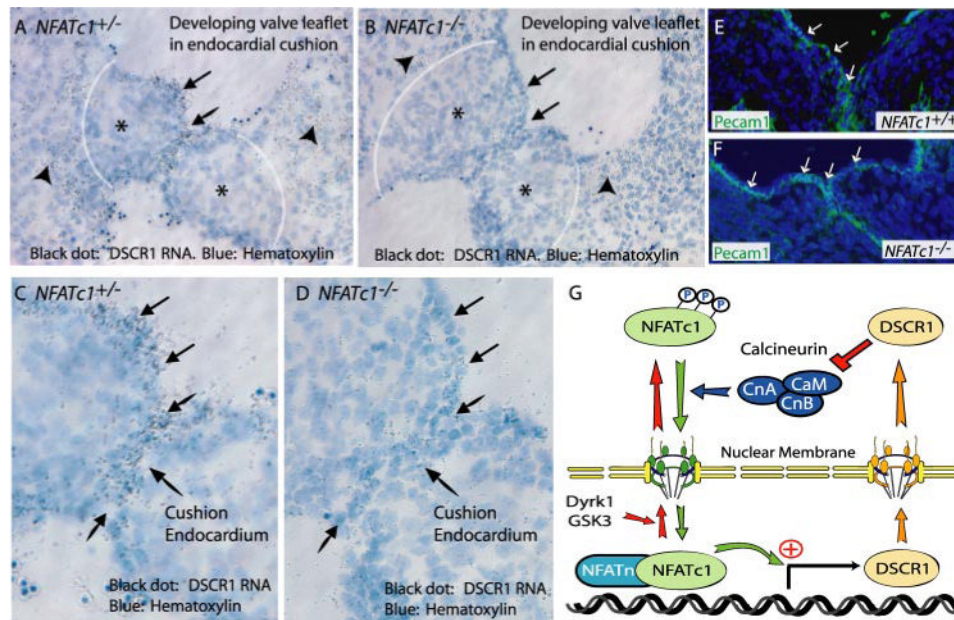


FIGURE 4. NFATc1 activates the expression of endogenous DSCR1 in the endocardium
 A, the expression pattern of DSCR1 mRNA by *in situ* hybridization at E12.5. *Dscr1* mRNA is expressed in both the myocardium (*arrowheads*) and cushion endocardium (*arrows*). *Black dots*, *Dscr1* mRNA; *blue*, hematoxylin counterstain; *, valve leaflet. B, the expression of DSCR1 in the cushion endocardium (*arrow*) is abolished in the absence of NFATc1. DSCR1 expression in the myocardium (*arrowhead*) is preserved. *, valve leaflet. C, higher magnification of *panel A* showing abundant *Dscr1* mRNA (*black dots*) expression in the cushion endocardium (*arrows*). D, higher magnification of *panel B*, showing minimal *Dscr1* mRNA expression in the cushion endocardium of *Nfata1^{-/-}* embryos (*arrows*). E and F, PECAM-1 immunostaining of cushion endocardium of wild-type (E) and *Nfata1^{-/-}* embryos (F) at E12.5. *Green*, PECAM-1 proteins; *blue*, 4',6-diamidino-2-phenylindole nuclear stain. G, a working model illustrating a negative feedback loop that controls NFATc1 activity and DSCR1 expression within the endocardium. DSCR1 inhibits calcineurin and NFATc1 signaling required for its own expression. NFATc1 proteins activate the expression of DSCR1, a calcineurin inhibitor, to regulate its nuclear localization and activity.