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Regulation of *De Novo* Ceramide Synthesis: The Role of Dihydroceramide Desaturase and Transcriptional Factors NFATC and Hand2 in the Hypoxic Mouse Heart

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We have previously shown that ceramide, a proapoptotic molecule decreases in the mouse heart as it adapts to hypoxia. We have also shown that its precursor, dihydroceramide, accumulates with hypoxia. This implicates the enzyme dihydroceramide desaturase (DHC-DS), which converts dihydroceramide to ceramide, in a potential regulatory checkpoint in cardiomyocytes. We hypothesised that the regulation of *de novo* ceramide synthesis plays an important role in the cardiomyocyte adaptation to hypoxia. We used an established mouse model to induce acute and chronic hypoxia. Cardiac tissues were extracted and quantitative real-time polymerase chain reaction (qRT-PCR) was used to evaluate the expression levels of DHC-DS. Electrophoretic Mobility Shift Assays (EMSAs) and qRT-PCR were used to evaluate the activity and expression levels of an array of transcription factors that might regulate *DEGS1* gene expression. We demonstrated that *DEGS1* mRNA levels decrease with time in hypoxic mice concurrent with the decrease in *HAND2* transcripts. Interestingly, the *DEGS1* promoter harbors overlapping sites for Hand2 and Nuclear Factor of Activated T-cells (NFATC) transcription factors. We have demonstrated a physical interaction between NFATC1 and the E-Box proteins with EMSA and coimmunoprecipitation assays. The regulation of *de novo* ceramide synthesis in response to hypoxia and this newly described interaction between E-box and NFATC transcription factors will pave the way to identify new pathways in the adaptation of the cardiomyocyte to stress. The elucidation of these pathways will in the long-term provide insights into potential targets for novel therapeutic regimens.

Introduction

A constant supply of oxygen is indispensable for cardiac viability and function. However, the role of oxygen and oxygen-associated processes in the heart is complex; these processes are either correlated with cardioprotective functions, or contribute to cardiac dysfunction and death (Giordano, 2005). Hypoxia is an extracellular event that can activate a myriad of intracellular pathways in cardiomyocytes. The acclimatization to chronic hypobaric hypoxia was shown to be regulated by a differential transcriptional profile between the right and left ventricles in adult rats. Specifically, there is restoration of normal postnatal gene expression in the left ventricle (LV), whereas chronic hypoxia is associated with persistent fetal metabolic reprogramming in the right ventricle (RV) (Adrogue *et al.,* 2005).

We had previously shown, using a neonatal mouse model, that the total ceramide content in the hypoxic animal increases at day 1 followed by a significant decrease at week 4 in the left ventricle; on the other hand, a significant decrease occurs in the right ventricle at 1, 4, and 8 weeks (Noureddine *et al.,* 2008). We have also shown that Dihydro-N-Palmitoyl-D-erythro-Sphingosine (DHC-16-Cer) levels accumulate with increasing time in hypoxia specifically in the right ventricle. This is accompanied by a parallel decrease in the quantity of N-Palmitoyl-D-erythro-Sphingosine (C16-Cer). DHC-16-Cer being the direct precursor of C16-Cer implicates the final desaturation step of the *de novo* ceramide pathway in the regulation of ceramide synthesis following hypoxia.

Dihydroceramide desaturase (DHC-DS) is the enzyme responsible for the desaturation of dihydroceramide to ceramide (Schulze *et al.,* 2000). We hypothesize that the

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regulation of this enzyme plays a central part in the ceramide response to hypoxia. The role of DHC-DS as a key enzyme in the *de novo* pathway of ceramide generation was previously investigated in human neuroblastoma cells. The partial loss of DEGS1 inhibited cell growth in neuroblastoma cells leading to a cell cycle arrest at G0/G1(Kraveka *et al.*, 2007). The rate-limiting step in the *de novo* synthesis of ceramide is known to be at the level of the enzyme serine palmitoyltransferase (SPT) (Perry *et al.*, 2000). Hypoxia is reported to increase the SPT enzyme activity (Kang *et al.*, 2010), thus shifting the rate-limiting step to the enzyme DHC-DS under hypoxic conditions. This is illustrated in neuronal ceroid lipofuscinosis, specifically the CLN9 type. CLN9-deficient fibroblasts have a distinctive phenotype of rapid growth, increased apoptosis, and diminished levels of ceramide, dihydroceramide, and sphingomyelin (Schulz *et al.*, 2006).

The ability to sense and react to changes in environmental oxygen levels requires a system that links extracellular changes to the intracellular pathways. The overlap or cross talk between the various growth and death pathways in the myocardium exists in a delicate balance where, for example, slight reductions in growth signaling may favor pathways leading to cardiac myocyte apoptosis (Melchert *et al.*, 2002). Several studies indicate that prolonged exposure to hypoxia causes alterations in the expression and activity of several calcium handling pathways in pulmonary arterial smooth muscle cells (Shimoda *et al.*, 2006). These pathways include calcium influx through store-operated calcium release-activated calcium channels (CRAC) required for T cell activation, cytokine synthesis, and proliferation. When CRAC is blocked by CD95 stimulation, the activation of acidic sphingomyelinase (ASM) and ceramide release occurs in parallel with the influx of calcium into the cell. Furthermore, CD95 stimulation or the addition of ceramide prevents store-operated calcium influx, activation of the transcriptional factor Nuclear Factor of Activated T-cells (NFATC), and IL-2 synthesis (Lepple-Wienhues *et al.*, 1999). This provides an essential bridge between ceramide and intracellular mechanisms.

The aim of the current study is to explore such cross talks to correlate expression levels of enzymes involved in ceramide biogenesis to the transcriptional regulators enriched in the heart. We hereby identify *DEGS1* as the key gene repressed in the right ventricle and responsible for the decreased levels of C16-Cer. In parallel, the Hand2 transcription factor, a basic-helix-loop-helix (bHLH) protein mainly expressed in the secondary heart field, was also decreased in the right ventricle of hypoxic mice. *DEGS1* promoter analysis led to the identification of several potential binding sites for E-Box proteins among which, a particular site next to a putative NFATC-binding site. Additionally, our data demonstrated a novel interaction between NFATC and the bHLH family of transcriptional factors.

Materials and Methods

In vivo *animal studies*

We made use of the hypoxic mouse model as previously described (Noureddine *et al.*, 2008). The same batch of mice was used. All animals received care in accordance with approved institutional animal care guidelines and according to the Guide for the Care and Use of Laboratory Animals of the National Academy of Science and US National Institutes of Health guidelines.

Quantitative real-time polymerase chain reaction

RNA was extracted from animal tissue using the Tripure reagent (Roche). Complementary DNA was synthesized from the extracted and quantified RNA using M-MLV Reverse Transcriptase (Amersham). Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to quantify the transcripts in hypoxic versus control samples using SYBR Green (BioRad). The primers used are summarized in Table 1. Results were normalized to Tubulin (*TUBA1A1*).

Plasmids

The coding region of *NFATC1* and *HAND2* and Hand2 were amplified by PCR and subcloned into the pCEP4 plasmid harboring an N-terminal Flag epitope (Invitrogen), or the pCGn plasmid harboring an N-terminal HA epitope. The generated plasmids were amplified and sequenced before being used in transfection assays. The CMV-driven plasmid encoding the bHLH protein Pan was a generous gift from Dr. Mona Nemer. The 1.5-kb mouse *DEGS1* promoter was subcloned into pGL3 luciferase reporter (*Kpn*I/*Hin*dIII) by amplifying the corresponding DNA. The plasmid was transformed into XL1 bacteria, isolated, purified, and sequenced.

Cell culture

Modified human embryonic kidney cells (AD293) were used for transfection assays. The Stratagene's AD-293 cell

Table 1. Oligonucleotides (5'.... 3') Used in Quantitative Real Time PCR

Gene	*Sequence*	*Annealing Temp*
LASS1	gttctacttggcctgttgga	Forward:57.8C
LASS1	tgacctccagtcatagaaga	Reverse:53.1C
LASS2	aaggagaaaacccgactgcg	Forward: 63.8C
LASS2	agaaaaggtctacctccacc	Reverse: 54.9C
LASS3	tacacctctagcaaatgcac	Forward: 54.0C
LASS3	tggtttgcttgtggaatgct	Reverse: 61.6C
LASS4	acctccatcctctaccatga	Forward: 56.9C
LASS4	gcagtgaaaggtagaagccc	Reverse: 58.9C
LASS5	acccttgagaaggtgttcgt	Forward: 58.6C
LASS5	tgtcctgattcctccgatgg	Reverse: 63.3C
LASS6	gagattagaagggctctcca	Forward: 56.1C
LASS6	cacatgctctcacagaacct	Reverse: 56.3C
GATA4	atgtcccagacattcagtac	Forward: 52.2C
GATA4	tgacaggagatgcatagcct	Reverse: 58.4C
GATA6	cttctccttctacacaagcg	Forward: 55.4C
GATA6	tgtagaggccgtcttgacct	Reverse: 59.9C
NPPA	agagacggcagtgctctagg	Forward: 59.8C
NPPA	gaagcagctggatcttcgta	Reverse: 58.2C
HIF1A	gagttgatgggttatgagcc	Forward: 57.0C
HIF1A	atcctgtactgtcctgtggt	Reverse: 54.3C
TUBA1A	gcatctccatccatgttggc	Forward:63.8 C
TUBA1A	cctgtctcactgaagaaggt	Reverse:54.3C
DEGS1	atgtctttggcagctgcctt	Forward:62.2C
DEGS1	cattccaaaccagcggttcc	Reverse:64.8C
HAND2	tccaagatcaagacactgcg	Forward:60.0C
HAND2	tcttcttgatctccgccttg	Reverse:60.5C
ACTB	catggagaaaatctggcacc	Forward:60.5C
ACTB	tgatctgggtcatcttctcg	Reverse:58.7C

line, a derivative of the commonly used HEK293 cell line, was maintained in DMEM-enriched media supplemented with 10% FBS (Gibco). Transfections were done using the calcium phosphate precipitation method as previously described (Nemer and Nemer, 2003). The luciferase assays were carried out 36 h after transfections using the luciferase-reporter system from Promega according to the manufacturer's instructions. Results are expressed as fold activation and are the mean of the 3 experiments done in duplicates.

Electrophoretic mobility shift assays

Whole cell extracts of AD293 cells overexpressing NFATC, Hand2, or TCF-3(Pan), were used in the presence of the ^{32}P-labeled probe harboring both the NFATC- and E-Box-binding sites found on the promoter of the *DEGS1* gene (forward primer: 5′ TCTTTAGGAAAGTCATCTGGTCTGC 3′, reverse primer: 5′ TCTTTACCAAAGTCATCTGGTCTGC 3′). The mixture was incubated for 20 min at room temperature in the presence of poly dI-dC (Roche) and a binding buffer as previously described (Nemer and Nemer, 2002). The reactions were resolved on a 4% polyacrylamide gel. Results were visualized by exposing the dried gels to X-Ray films.

Western blots

Western blots were carried out using either the Flag antibody (Sigma M2), or the HA antibody (sc-805, Santa Cruz biotechnologies) to detect overexpression of NFATC1 or Hand2 overexpressed proteins in AD293 cells, respectively. Briefly, 20 μg of nuclear extracts were resolved on a 10% SDS acrylamide gel, transferred to a PVDF membrane (GE Health Care Life Sciences), and incubated with the corresponding antibody. Revelation was carried out using a horseradish peroxidase coupled secondary antibody and the Western Blot ECL kit from GE (RPN 2232).

Coimmunoprecipitation

Fifty micrograms of whole cell extracts from AD293 cells overexpressing Hand2 was immunoprecipitated with the Hand2 antibody (sc-22818X; Santa Cruz biotechnologies) in the presence of either the same amount of proteins from mock transfected AD293 cells, or cells transfected with NFATC1. The reactions were incubated in the presence of Protein G using the MagnaBead system from Invitrogen. The reactions were resolved on a 12% PAGE and Western blots were carried out using the anti-Flag antibody from Santa Cruz (sc-807).

Bioinformatics

Promoter analysis was performed online at http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home; this Transcriptional Regulatory Element Database retrieves promoters and allows analysis for transcription factor binding sites. To assess for hypothetical interactions between NFATC1 and Hand2 proteins, we docked different associations of the DNA-binding domains of both proteins using Hex 4.5; the structures were manipulated and visualized using the Accelerys DS Visualiser.

Statistics

The mean normalized expression of the different proteins analyzed is the arithmetic mean ± S.E.M. of two independent RNA extractions followed by qPCR done on five samples from each group of mice (normal and hypoxic at 1 day, 1, 4, and 8 weeks). The differences between the normal and hypoxic hearts were considered significant when $p < 0.05$. The unpaired *t*-test was utilized to perform the comparison between the normal and hypoxic states independently at each time (1 day, 1, 4, and 8 weeks). The LV and RV were not compared. All data points were analyzed for outliers using the Grubb's test.

Results

Aberrant regulation of DEGS1 *might account for sustained expression of DHC-16-Cer in the right ventricle*

Our previous study did show a decreased ceramide content in the right ventricle of hypoxic animals starting at week 1 and persisting till week 8. We evaluated the expression of the different *LASS* (longevity-assurance homologue) genes in hypoxic versus control mice to assess the role of these ceramide synthases. qRT-PCR using specific oligonucleotides (Table 1) for the *LASS* genes failed to show a regulation pattern (for 5/6 *LASS* genes) that recapitulates that of the ceramide content over time in hypoxic animals versus controls as seen in Figure 1. However, *LASS5* was upregulated early on in both left and right ventricles at day 1 of hypoxia, paralleling the slight increase in the ceramide content that was detected previously at this particular time. Interestingly, the levels of *LASS5* were subsequently downregulated in the right ventricle as is the case of total ceramide. This diminished expression of *LASS5* does not account, however, for the increased levels DHC-16-Cer in the right ventricle as previously described. This precursor of C16-Cer accumulates in hypoxic right ventricles starting at week 4 reaching a maximum at week 8 concurrent with a decrease in the C16-Cer content at all ages in hypoxic right ventricles. This data suggest that the desaturase enzyme DEGS1, which converts DHC-16-Cer into C16-Cer, might be downregulated between week 4 and 8 in the right ventricles of hypoxic mice and may account for the sustained accumulation of DHC-16-Cer. The expression of *DEGS1* was, in fact, significantly increased by more than 20-fold in the hypoxic left ventricle, while it was significantly decreased by 50% in the hypoxic right ventricle at week 8 concomitant with the accumulation of DHC-16-Cer as seen in Figure 2. The diminished expression of the *DEGS1* transcript prompted us to study the transcriptional profile of the hypoxic right ventricular heart to map its upstream regulators.

Transcriptional profile of hypoxic ventricles

Using real-time PCR, we evaluated the expression of cardiac-enriched transcription factors in the ventricles in an attempt to correlate the observed changes to the expression of DEGS1 and subsequently to ceramide levels. We started with *HIF1A* (hypoxia-inducing factor), which was shown to be involved in an early response to hypoxia in different organs. As seen in Figure 3, a sharp increase in the *HIF1A* transcript was observed at 8 weeks in the left ventricle with minimal changes in the right ventricle at all ages. This expression pattern parallels that of the atrial natriuretic factor (*NPPA*), a marker of cardiac hypertrophy, suggesting that

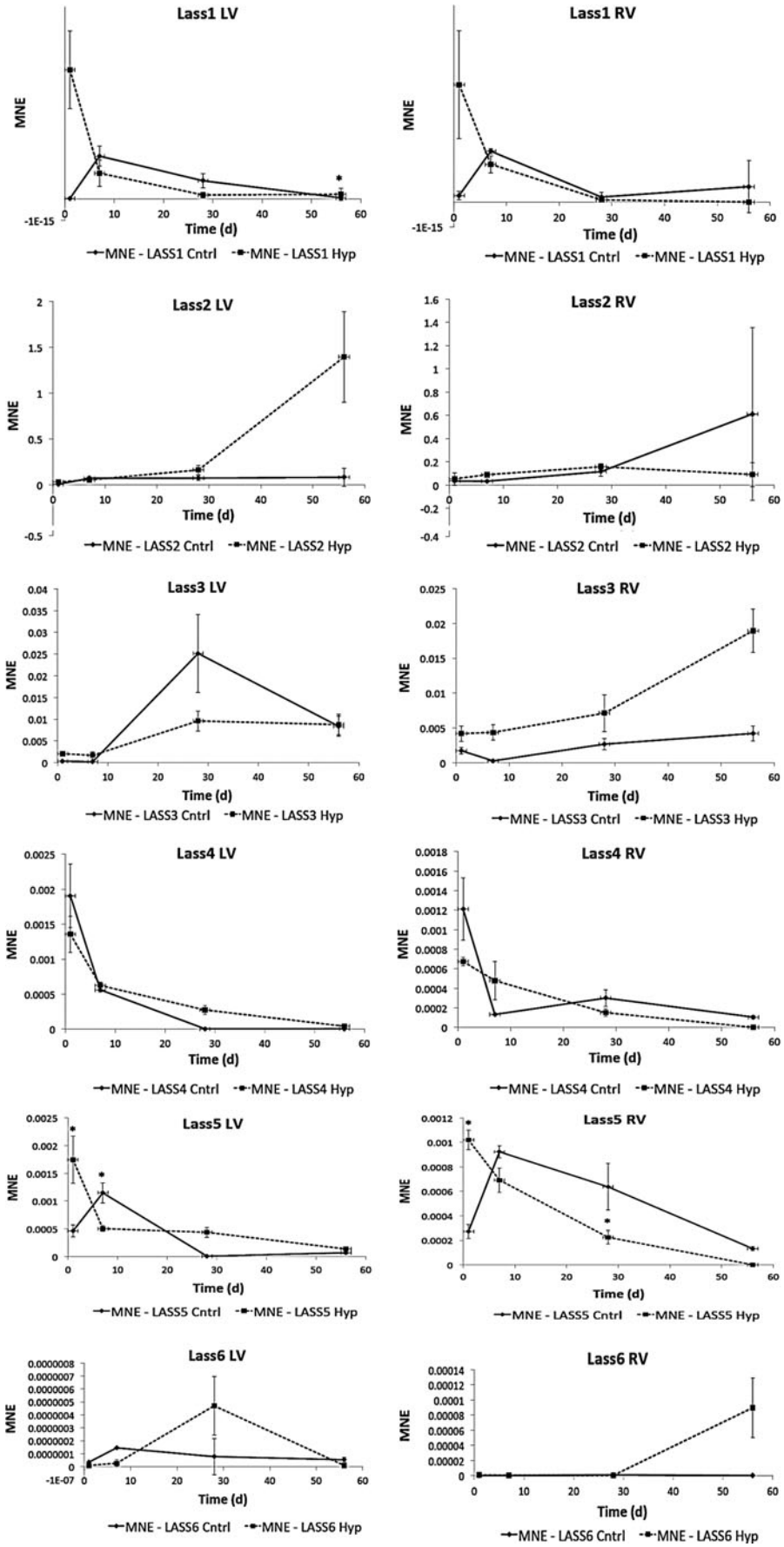

FIG. 1. *LASS* genes regulation of *de novo* ceramide synthesis in the hypoxic heart. Comparison of the mean normalized expression (MNE) of the different ceramide synthetase enzymes (*LASS1* to *LASS6*) between normal and hypoxic heart tissues at different ages (1 day, 1, 4, and 8 weeks). The results are the mean of two independent RNA extractions followed by qPCR done on five samples from each group of mice (normal and hypoxic at 1 day, 1, 4, and 8 weeks). $^{*}p < 0.05$. LV, left ventricle; RV, right ventricle.

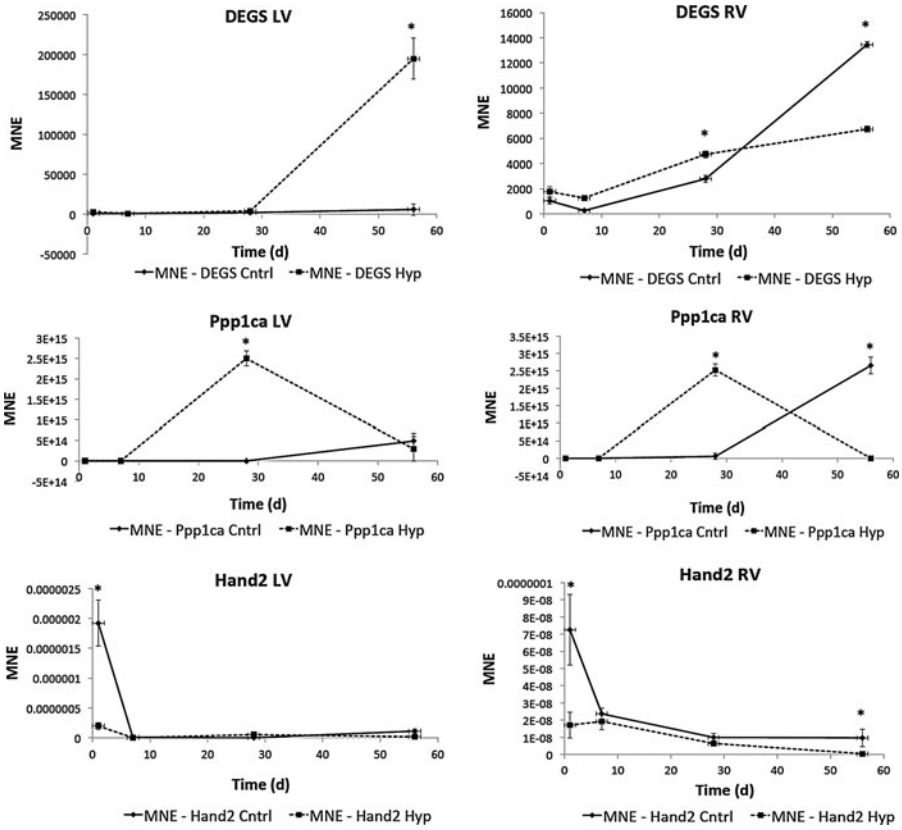

FIG. 2. *DEGS1*, *Hand2*, and activated calcineurin (*PPP3CA*) expression in the hypoxic heart. Comparison of the MNE of the following different transcripts between control and hypoxic left and right ventricles: *DEGS1*, *PPP3CA*, *Hand2*. The results are the mean of two independent RNA extractions followed by qPCR done on five samples from each group of mice (normal and hypoxic at 1 day, 1, 4, and 8 weeks). $*p < 0.05$. LV, left ventricle; RV, right ventricle.

HIF-1α might be an upstream regulator of *NPPA* expression in hypoxic hearts and that cardiac hypertrophy in this model occurs at a late stage. The cardiac GATA proteins were assessed because of the previous link between GATA2 and hypoxia in endothelial cells (Yamashita *et al.*, 2001). Our results demonstrate that both *GATA4* and *GATA6* are upregulated at day 1 in the left ventricles, while only *GATA4* is upregulated in the right ventricle starting week 1 through week 8 (Fig. 3). *GATA4* expression, however, does not seem to be correlated to that of *DEGS1*, which was upregulated at later stages in hypoxia (Fig. 2). This prompted us to look at right ventricle-enriched transcripts. *HAND2* encodes a bHLH protein highly restricted to the right ventricle and the outflow tract of the mammalian hearts. *HAND2* transcript levels were assessed in both ventricles of hypoxic versus control mice. At day 1, *HAND2* mRNA was sharply decreased in both ventricles suggesting that it is an early response gene implicated in hypoxia. At week 8, however, only *HAND2* transcripts were significantly reduced in the right ventricles of hypoxic mice (Fig. 2). This suggested that Hand2 might be a regulator of *DEGS1* expression in the right ventricle.

Hand2 *and NFATC physically interact on the* DEGS1 *promoter*

We analyzed 1.5 Kbp of the mouse *DEGS1* promoter and identified several putative E-box and GATA elements. Interestingly, an NFATC putative-binding site was identified juxtaposed with an E-box-binding sequence (Fig. 4A). This pattern of overlapping binding sites for transcription factors is very rare and in most cases suggests a combinatorial interaction between the two classes of proteins in regulating gene expression (Bruneau *et al.*, 2001). Since nuclear localization of the NFAT proteins is modulated by dephosphorylation via calcineurin, we performed qRT-PCR on the catalytic subunit of calcineurin (*PPP3CA*) and found that they were only upregulated after 1 week of hypoxia in both ventricles, and then downregulated at 8 weeks especially in the right ventricles (Fig. 2). This downregulation at 8 weeks in the right ventricles is concurrent with the decreased expression of *DEGS1*.

Docking studies were carried out with Hex 4.5. The Rel domain of NFATC1 and the bHLH domain of Pan were uploaded from the protein data bank (www.rcsb.org, PDBID: 1NFA and 1HLH), whereas the Hand2 bHLH structure was constructed by homology-based modeling using the Insight II Grid software (Accelrys). The simulated interaction between NFATC1 and Hand2 depicted a favorable exothermic association with a docking energy of −500 KJ/mol (Fig. 4B). The interaction simulated between the NFATC1 and Pan proceeded with an exothermic energy of −546 KJ/mol; suggesting that the Rel domain of NFATC1 can interact with both bHLH domains of Hand2 and Pan.

To assess the potential binding of both the NFATC and Hand2 protein to the overlapping site on the DEGS1 promoter, gel shift assays were performed using NFATC1 and Hand2 overexpressed proteins in AD293 cells. As shown in Figure 4D, both proteins can bind the overlapping site, while Hand2 is able to bind only as a heterodimer with its ubiquitously expressed partner Pan/E47. Interestingly, when both NFATC1 and Pan or NFATC1 and Hand2/Pan proteins are expressed, a stable ternary complex is formed showing that these proteins can physically interact (Fig. 4D).

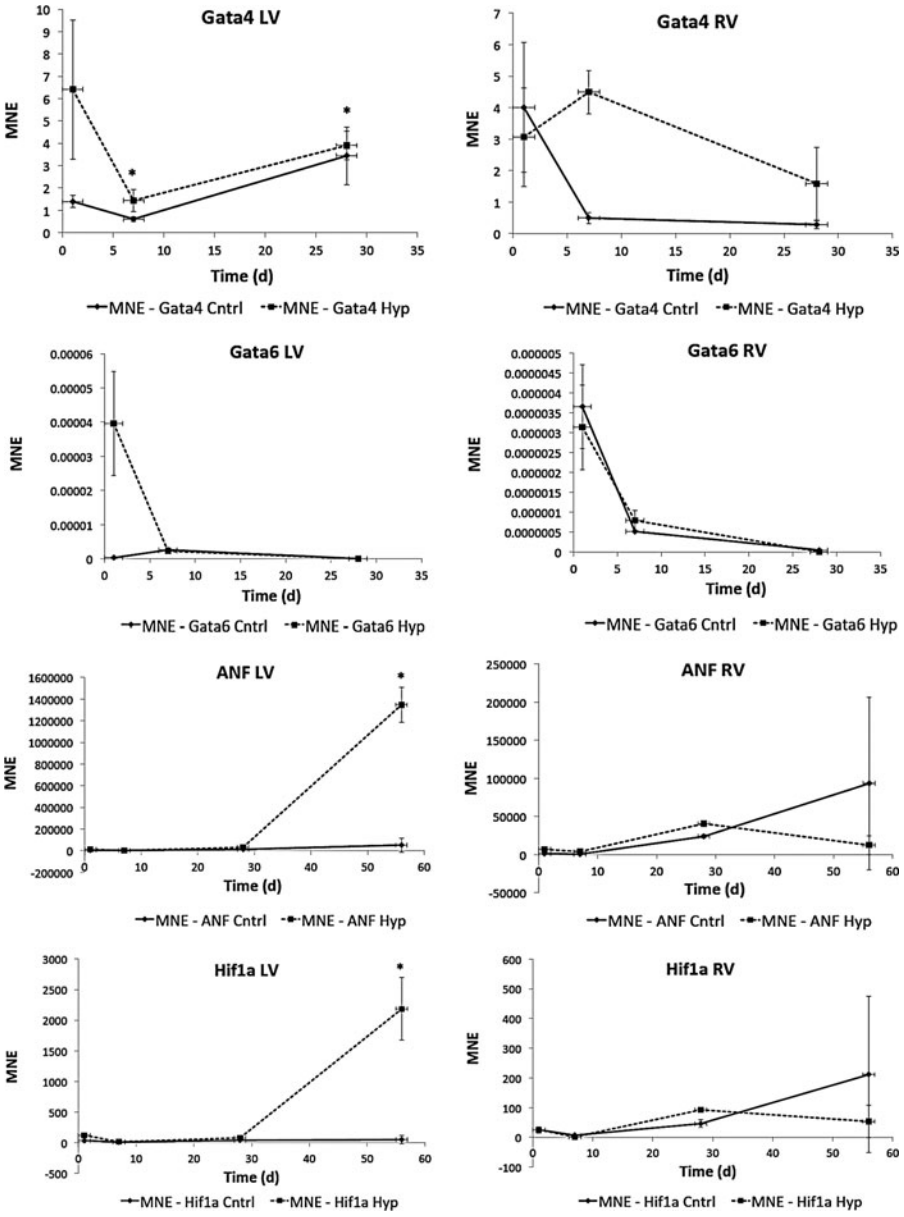

FIG. 3. Cardiac-enriched transcription factors and *NPPA* (Atrial natriuretic factor, ANF) expression. Comparison of the MNE of *GATA4, GATA5, HIF-1a* and ANF (*NPPA*) between normal and hypoxic heart tissues at different ages (1 day, 1 week, 4 weeks, and 8 weeks). The results are the mean of two independent RNA extractions followed by qPCR done on 5 samples from each group of mice (normal and hypoxic at 1 day, 1, 4, and 8 weeks). $^*p < 0.05$. LV, left ventricle; RV, right ventricle.

The physical interaction over the DNA-binding sites was tested also in the absence of DNA by carrying co-immunoprecipitation studies. Using AD293 cells over-expressing NFATC1 and Hand2, we demonstrated a strong physical interaction between the two proteins confirming the simulation studies (Fig. 4C). Moreover, to prove that this physical interaction is functional *in vivo*, we cloned the *DEGS1* 1.5-kbp promoter upstream of the luciferase reporter. Our results show that either NFATC1 or Hand2 alone can slightly activate the promoter. When expressed together, the two proteins produced a synergistic effect on promoter activation as seen in Figure 4E. This synergy was also seen between Pan and NFATC1 (data not shown), suggesting that both bHLH proteins can interact with NFATC1. Furthermore, transient overexpression of NFATC1 or Hand2 in AD293 cells (Fig. 5A) alone only slightly upregulated *DEGS1* mRNA expression (Fig. 5B); however, maximum induction of *DEGS1* was observed with the coexpression of these proteins suggesting that both factors are required to upregulate the *DEGS1* transcript.

Discussion

In the current study, we used two parameters to assess the acclimatization of the right ventricle to chronic hypoxia. The first involves sphingolipid metabolism, specifically, *de novo* ceramide synthesis. The second deals with the transcriptional profile of the hypoxic cardiac tissue. Pulmonary hypertension is the first response to the hypoxic insult resulting in pressure overload on the right side of the heart leading to hypertrophy and a myriad of regulatory mechanisms to prolong cardiomyocyte adaptation and survival. Cardiac hypertrophy is underscored by the interplay of several transcriptional modulators. These emerge as a response to the genetic reprogramming that results in reinduction of fetal

genes characterizing the hypertrophied myocytes (Nemer and Nemer, 2001). The reactivation of the fetal metabolic program involves *NPPA* upregulation via cooperative interactions between different classes of proteins, mainly GATA, MADS (SRF and MEF2), and NFATC (Georges *et al.*, 2008; Molkentin *et al.*, 1998; Morin *et al.*, 2000).

Early response to hypoxia is linked to increased ceramide de novo *synthesis via* LASS5

We have previously shown that acute hypoxia for 1 day causes an increase in ceramide levels in the right cardiac ventricle when compared to normoxic controls (Noureddine *et al.*, 2008). However, this rapid increase was subsequently followed by a gradual decrease that was maximal in week 8. Careful examination of the expression of the six *LASS* genes implicated in ceramide synthesis showed that both *LASS1* and *LASS5* were upregulated at day 1 in the right ventricles of hypoxic mice. The subsequent decrease in ceramide levels was correlated with a similar decrease in the expression of *LASS5*. Interestingly, *LASS5* is the only *LASS* gene highly expressed in the mammalian heart and is mainly involved in the synthesis of short chained ceramide (<C20) (Mizutani *et al.*, 2005). The early increase in the Lass5 activity and the persistent predominance of C16-Cer as the major ceramide species present in the right ventricle under acute hypoxic conditions could suggest a protective effect of this ceramide species. Recent studies in *Caenorhabditis elegans* accentuated the importance of ceramide production to the ability of the organism to survive under hypoxia or anoxia. In fact, mutations in the gene encoding one of the three Lass genes present in *Caenorhabditis elegans* resulted in animals with increased sensitivity to anoxia suggesting that the production of ceramide is not linked to apoptosis, but to survival of some organisms under harsh conditions like anoxia (Lepple-Wienhues *et al.*, 1999). Interestingly, the results also point out to the particular

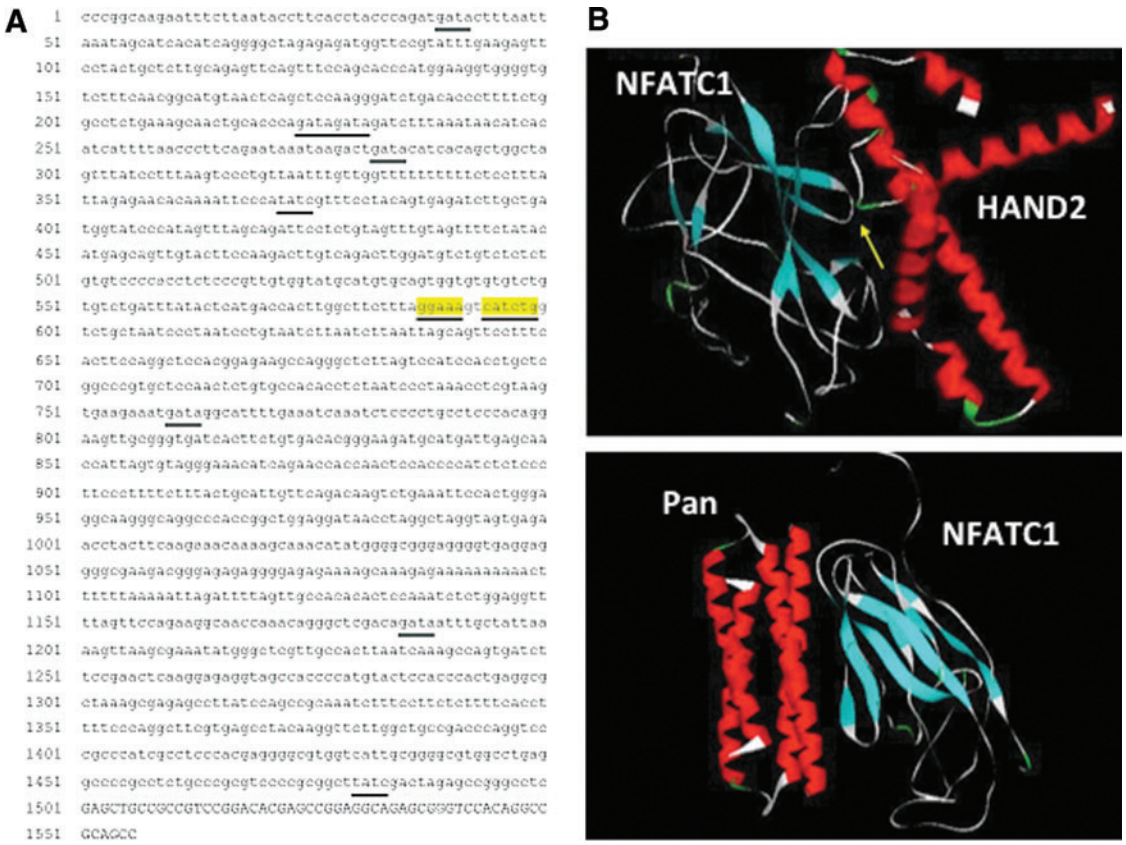

FIG. 4. NFATC and Hand2 regulate the *DEGS1* promoter. **(A)** The mouse *DEGS1* promoter (1500 bp upstream of the transcription initiation site) harbors multiple binding sites for GATA (underlined) and bHLH proteins in addition to two juxtaposed binding sites for bHLH and NFAT (bold). Capital letters are for the transcript and small letters for the 5′ upstream region. **(B)** NFAT and bHLH protein hypothetical interaction using Hex4.5. The Rel domain of NFATC1 (blue) and the bHLH domain of Pan and Hand2 (red) were used and results showed a favorable exothermic association with a docking energy of −500 KJ/mol for HAND2 and −546 KJ/mol for Pan. **(C)** Co-immunoprecipitation of Flag-tagged Hand2 and NFATC1 proteins showing a strong interaction between both transcription factors. Western blots with the Flag antibody (left panel) showing 15% input of protein extracts used for the co-IP. Immunoprecipitation was done using an anti-Hand2 antibody and immunoblotted using the anti-Flag antibody. **(D)** Gel shift analysis with whole cell protein extracts from AD293 cells overexpressing Hand2, NFATC1, and Pan showed that all Pan, NFATC1, and Pan/Hand2 can bind specifically the E-box/NFAT-binding site on the *DEGS1* promoter. They can also form a stable ternary complex (*). Control refers to mock transfected cells. **(E)** A functional interaction between NFATC1 and Hand2. Luciferase assays shows that both proteins interact together to synergistically activate the *DEGS1* promoter (+ and ++ refers to increasing doses of the plasmid being used). *$p < 0.05$. bHLH, basic-helix-loop-helix; NFATC, Nuclear Factor of Activated T-cells. Color images available online at www.liebertpub.com/dna

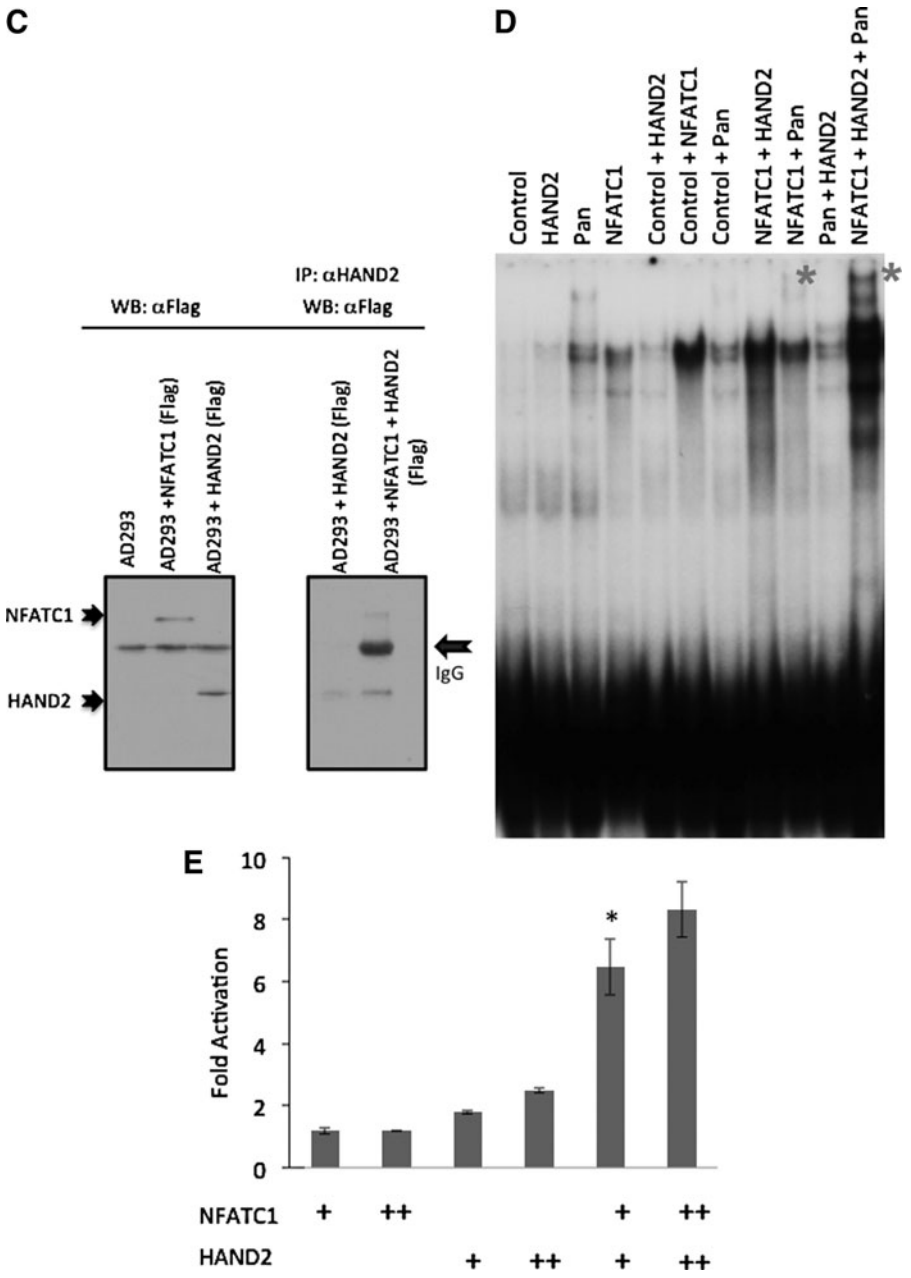

FIG. 4. Continued.

effect of the length of the chain within long-chained ceramide. The relatively shorter chained ceramide species (C16–C20) confer adaptation to anoxic conditions since rescue experiments with longer chained ceramide species (C20–C26) were not as efficient as with the shorter species (Schiffmann *et al.*, 2012). Taken together, one would expect to see mice lacking *LASS5* in the heart to be less resistant to acute hypoxic conditions compared to normal mice or to mice lacking other *LASS* genes. Thus some ceramide species act to protect cells against cell death under harsh conditions.

The acute rise in ceramide levels represents an important signal to downstream regulators in the adaptation of the cardiomyocyte to hypoxia. One essential target is the L-type calcium release-activated calcium channels, which are blocked by the release of ceramide through the activation of ASM (Lepple-Wienhues *et al.*, 1999). This results in the decrease of calcium influx into the hypoxic cell, and thus, repression of the calcium- and calmodulin- dependent phosphatase calcineurin. We have shown that in contrast to the left ventricle, the hypoxic right ventricle compared to control at day 1 did not experience any significant change in transcript levels of calcineurin. This was followed by a 12% increase at week 1 reaching a maximum at week 4 before dipping to a minimum with increasing time in hypoxia. This timely decrease suggests a regulatory mechanism that downregulates the expression of the phosphatase calcineurin in the event of a stressful insult like hypoxia only in the right ventricle. Further studies, including a cell culture model of hypoxia will help confirm these findings.

Post-translational processes have been described to specifically control the level of DHC-DS via ubiquitination and

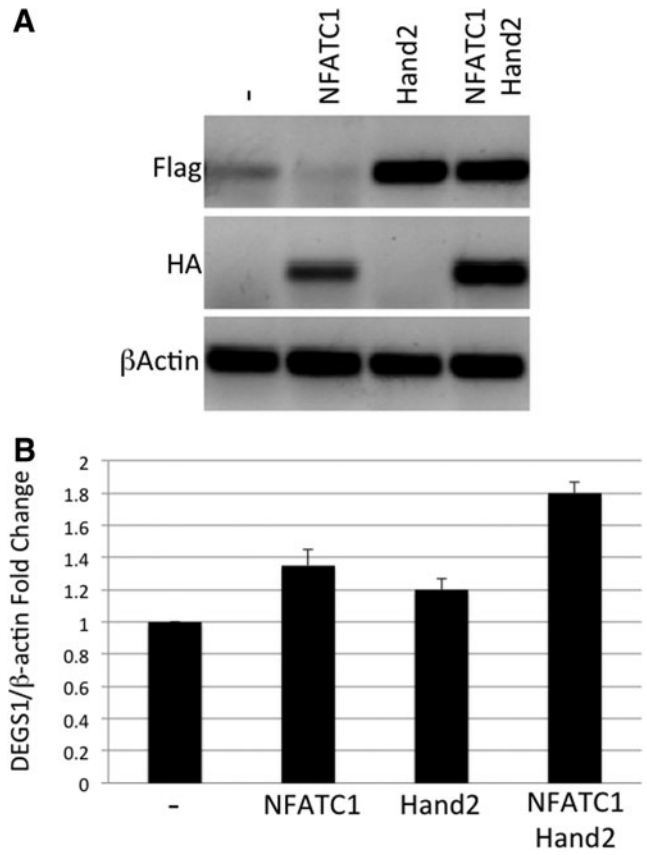

FIG. 5. *DEGS1* upregulation by the coexpression of NFATC1 and Hand2. AD293 cells were transiently transfected with, mock empty vector, Flag-NFATC1, and HA-Hand2. Whole cell extracts were prepared 48 h post-transfection and were validated to express tagged NFATC1 and Hand2 proteins by Western blots **(A)**. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to evaluate the expression of *DEGS1* in the transfected cells using ACTB as a reference housekeeping gene **(B)**.

proteasome-dependent protein turnover. Diverse stresses, including chemotherapeutic drugs, UV light, and DTT can induce this turnover (Sridevi *et al.,* 2009). This fact could definitely contribute to the turnover of DHC-DS and would require further study.

Combinatorial interaction between NFATC and Hand2 *regulates the expression of* DEGS1

The involvement of *LASS* genes in early response to acute and chronic hypoxia cannot, however, be extended to the late stages of adaptation of the right ventricles, since ceramide species are all downregulated and the only measurable event was the accumulation of DHC-16-Cer at week 8. This led us to propose a possible regulatory step in the *de novo* synthesis of ceramide independent of the action of the Lass genes. We measured the expression levels of the enzyme DHC-DS (DEGS1), which is responsible for converting DHC-16-Cer to the Cer-16 species (Schulze *et al.,* 2000). Whereas the level of *DEGS1* expression significantly increased in the hypoxic left ventricle as compared to control at week 8, *DEGS1* expression significantly decreased by nearly 50% in the hypoxic right ventricle as compared to control at week 8. Given that DHC-16-Cer has been demonstrated to have no effect on cell survival (Geley *et al.,* 1997), we believe that this enzyme forms a regulatory step in the ceramide synthesis pathway. By altering the expression of this enzyme, the cell can control the production of ceramide, which in turn can control resistance to cell death as was shown in *Caenorhabditis elegans* (Menuz *et al.,* 2009). It was thus important to link the decreased expression of *DEGS1* to upstream regulators involved in the right ventricle function. The decreased calcineurin expression prevents the translocation of NFATC1 from the cytoplasm to the nucleus. We therefore searched for potential NFATC-binding sites on the *DEGS1* murine promoter. We found a consensus NFATC-binding site next to an E-box and showed that NFATC1 and Hand2 can form a stable ternary complex. Nuclear extracts from the right ventricles at week 8 did show a decrease in the expression of the different complexes formed with the probe harboring both binding sites (data not shown). The interaction, which was, however, simulated *in silico*, was proven to be strong enough to be detected by Gel Shift assays. The biological effects of this described interaction were reflected in the induction of *DEGS1* gene expression as observed by luciferase and Q-PCR experiments. It is noteworthy that examples of such interactions are very rare and suggest, as in the case of Nkx2-5/Tbx5, a common pathway in gene regulation conserved during evolution in physiological and pathological conditions (Bruneau *et al.,* 2001).

Previous results strongly suggested that NFATC3 plays a role in the chronic hypoxia-induced vascular changes that underlie pulmonary hypertension (de Frutos *et al.,* 2007, 2009). Our results showed that a myocardium's acute and chronic response to hypoxia in terms of changes in the NFATC-DNA-binding protein is the opposite to what is seen in other models of cell injury. This points out to a novel mechanism by which, the decrease in NFATC can negatively regulate the expression of genes necessary for cell survival. Targeted deletion of NFATC3 or NFATC4 in the ventricles would thus be of great value to understand the role of NFAT proteins in chronic and acute hypoxia.

As for *HAND2*, the observed downregulation at all ages and especially at week 8 in the right ventricle is also seen in rodent hypertrophy and human cardiomyopathy as a possible leeway to reinitiate the fetal gene program and activate the adaptive physiological changes that allow the heart to compensate by hypertrophy (Thattaliyath *et al.,* 2002). However, this is the first report demonstrating a physical and functional interaction between NFATC and bHLH proteins in the regulation of gene expression. Identifying target genes for both proteins at different stages of cardiac development and/or pathological conditions will help understand the mechanisms underlying such interactions.

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