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Cooperative Binding of ETS2 and NFAT Links Erk1/2 and Calcineurin Signaling in the Pathogenesis of Cardiac Hypertrophy

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BACKGROUND: Cardiac hypertrophy is an independent risk factor for heart failure, a leading cause of morbidity and mortality globally. The calcineurin/NFAT (nuclear factor of activated T cells) pathway and the MAPK (mitogen-activated protein kinase)/Erk (extracellular signal-regulated kinase) pathway contribute to the pathogenesis of cardiac hypertrophy as an interdependent network of signaling cascades. How these pathways interact remains unclear and few direct targets responsible for the prohypertrophic role of NFAT have been described.

METHODS: By engineering cardiomyocyte-specific ETS2 (a member of the E26 transformation-specific sequence [ETS] domain family) knockout mice, we investigated the role of ETS2 in cardiac hypertrophy. Primary cardiomyocytes were used to evaluate ETS2 function in cell growth.

RESULTS: ETS2 is phosphorylated and activated by Erk1/2 on hypertrophic stimulation in both mouse (n=3) and human heart samples (n=8 to 19). Conditional deletion of ETS2 in mouse cardiomyocytes protects against pressure overload–induced cardiac hypertrophy (n=6 to 11). Silencing of ETS2 in the hearts of calcineurin transgenic mice significantly attenuates hypertrophic growth and contractile dysfunction (n=8). As a transcription factor, ETS2 is capable of binding to the promoters of hypertrophic marker genes, such as *ANP*, *BNP*, and *Rcan1.4* (n=4). We report that ETS2 forms a complex with NFAT to stimulate transcriptional activity through increased NFAT binding to the promoters of at least 2 hypertrophy-stimulated genes: *Rcan1.4* and microRNA-223 (=n4 to 6). Suppression of microRNA-223 in cardiomyocytes inhibits calcineurin-mediated cardiac hypertrophy (n=6), revealing microRNA-223 as a novel prohypertrophic target of the calcineurin/NFAT and Erk1/2-ETS2 pathways.

CONCLUSIONS: Our findings point to a critical role for ETS2 in calcineurin/NFAT pathway-driven cardiac hypertrophy and unveil a previously unknown molecular connection between the Erk1/2 activation of ETS2 and expression of NFAT/ETS2 target genes.

Key words: calcineurin-NFAT pathway ■ cardiac hypertrophy ■ cardiomegaly ■ ETS2 ■ MAPK/Erk pathway ■ microRNA-223

Heart failure is a leading cause of morbidity and mortality around the world, one that is increasing in incidence and prevalence.[1] Whereas a number of efficacious therapies are available, including new ones emerging in recent years, the syndrome continues to have a major effect on individuals, society, and health care expenditures globally.

A major element of heart failure pathogenesis is hypertrophic growth of the myocardium. This growth response, left unchecked, culminates in heart failure.[2–4]

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Clinical Perspective

What Is New?

- ETS2 (a member of the E26 transformation-specific sequence [ETS] domain family) is activated by Erk1/2 (extracellular signal-regulated kinase) in both hypertrophied murine hearts and in human dilated cardiomyopathy.
- ETS2 is required for both pressure overload- and calcineurin-induced cardiac hypertrophy, responses involving signaling cascades distinct from, but interdependent with, Erk1/2 signaling.
- ETS2 synergizes with NFAT (nuclear factor of activated T cells) to transactivate Rcan1.4, an established downstream target of NFAT, and we identified microRNA-223 as a novel transcriptional target of NFAT/ETS2 in cardiomyocytes.
- In aggregate, these findings unveil a previously unrecognized molecular interaction between 2 canonical hypertrophic signaling pathways: MAPK (mitogen-activated protein kinase)–driven hypertrophy and calcineurin-driven hypertrophy.

What Are the Clinical Implications?

- Because pathologic cardiac hypertrophy is an established risk factor for heart failure development, our unveiling of novel signaling mechanisms is of potential clinical relevance.

Nonstandard Abbreviations and Acronyms

αMHC	α-myosin heavy chain
ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
βMHC	β-myosin heavy chain
ChIP	chromatin immunoprecipitation
CnA	Calcineurin A
ELF1	E74-like factor 1
Erk	extracellular signal-regulated kinase
ETS	E26 transformation-specific sequence
GATA4	GATA binding protein 4
GFP	green fluorescent protein
JNK	c-Jun N-terminal kinase
KO	knockout
MAPK	mitogen-activated protein kinase
MCIP	modulatory calcineurin-interacting protein
MEF2	myocyte enhancer factor-2
MEK	MAPK/Erk kinase
miRNA	microRNA
MKP3	MAPK phosphatase 3
NFAT	nuclear factor of activated T cells
NFATc1	nuclear factor of activated T cells, cytoplasmic 1
NRVM	neonatal rat ventricular myocyte
siRNA	small interfering RNA
sTAC	severe transverse aortic constriction

Suppressing cardiac hypertrophy in patients with heart failure enhances contractile function and improves clinical prognosis.[5] Our understanding of mechanisms governing disease-associated hypertrophic growth of the myocardium is incomplete, and elucidating them holds the prospect of identifying new molecular targets with potential relevance as innovative therapeutic strategies.

Two of the major cellular signaling pathways driving cardiac hypertrophy are the calcineurin/NFAT (nuclear factor of activated T cells) pathway and the MAPK (mitogen-activated protein kinase)/Erk (extracellular signal-regulated kinase) pathway.[6] Sustained activation of either pathway results in a hypertrophic response.[7] Sustained activation of calcineurin in the heart is sufficient to induce pathologic cardiac hypertrophy that progresses rapidly to dilated hypertrophy and heart failure.[7] In contrast, constitutive activation of Erk1/2 in the heart results in a milder hypertrophic response that does not appear to progress to failure.[8] Whereas it is clear that Erk1/2 can be prohypertrophic, it has been suggested that it may also drive protective mechanisms during hypertrophic stress.[9] Downstream targets of Erk1/2 that drive these events are unknown.

On activation by calcium, calcineurin binds to and dephosphorylates NFAT, resulting in the translocation of NFAT to the nucleus and activation of target genes.[6,10] Even though it is well-established that calcineurin/NFAT signaling is crucial for cardiac hypertrophy, few prohypertrophic target genes of NFAT have been identified. This may be attributable, at least in part, to NFAT's relatively weak DNA binding capability; often it cooperates with other transcription factors, such as MEF2 (myocyte enhancer factor-2) and GATA4 (GATA binding protein 4), to form heterodimers with stronger DNA binding affinity.[10,11]

In T cells, NFAT interacts with ETS1 and ELF1 (E74-like factor 1), 2 members of the E26 transformation-specific sequence (ETS) domain family of transcription factors.[12] In Th1 cells, ETS1 is required for the nuclear entry of NFAT and the recruitment of NFAT to the interleukin-2 promoter.[13] Like NFAT, ETS family members have been implicated in cardiac development,[14–16] and ETS2 binding sites are overrepresented in the promoters of genes upregulated in heart failure.[17] A role for ETS2 in hypertrophic remodeling of the heart has not been described.

METHODS

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing

these results or replicating these procedures by reasonable request directed to the corresponding author.

Mice

Mice were housed and fed under standard laboratory conditions with a 12 hours light/dark cycle from 6 AM to 6 PM. Floxed ETS2 mice were a gift from Dr Michael C. Ostrowski (Department of Biochemistry and Molecular Biology, Medical University of South Carolina).[18] To generate cardiomyocyte-specific ETS2 knockout (KO) mice, floxed mice were bred with α-myosin heavy chain (αMHC)–Cre transgenic mice. ETS2 KO mice were crossed with calcineurin/MCIP (modulatory calcineurin-interacting protein) double transgenic animals[19] to engineer ETS2 KO/calcineurin transgenic mice. All protocols were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center.

Pressure-Overload Hypertrophy Model and Echocardiography

Male wild-type, αMHC-Cre, floxed, and ETS2 KO mice (FVB/N mice; 8 to 10 weeks of age) were subjected to severe thoracic aortic constriction (sTAC) as described previously.[20,21] Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). The left chest was opened at the second intercostal space to identify the thoracic aorta. The thoracic aorta was ligated with a 28-gauge needle, and then the needle was removed, leaving a region of stenosis. Sham-operated mice underwent the same procedure without transverse aortic banding and served as controls.

Echocardiography was performed on conscious, gently restrained mice using a Vevo 2100 system (VisualSonics) with a MS400C scanhead as described previously.[22] M-mode recordings were obtained from a short axis view at the level of the papillary muscles. Left ventricular internal diameter (LVID) at both end-diastole (LVIDd) and end-systole (LVIDs) was measured at the time of the largest and smallest left ventricular areas, respectively. Fractional shortening was calculated as (LVIDd–LVIDs)/LVIDd ×100%. All parameters were measured at least 3 times; averages are presented.

Histologic Analysis

Mouse hearts were harvested and then fixed in 4% paraformaldehyde for 48 hours. Fixed hearts were embedded in paraffin and cut into 5-μm sections. Heart sections were stained with hematoxylin and eosin or wheat germ agglutinin to evaluate hypertrophic growth. Hematoxylin and eosin staining was performed using a standard method. For wheat germ agglutinin staining, deparaffinized and rehydrated heart sections were boiled in 10 mmol/L citrate buffer (pH 6.0) for 12 minutes, followed by blocking in 1% bovine serum albumin with 5% goat serum for 1 hour. Sections were then incubated with 10 μg/mL Alexa Fluor 594-conjugated wheat germ agglutinin (W11262; Thermo Fisher) for 1 hour at room temperature in the dark. After mounting with the ProLong gold antifade reagent (P36935; Thermo Fisher), sections were imaged with a fluorescence microscope (Leica). Circular to oval left ventricular myocytes were chosen from >5 mice. At least 10 fields per mouse were evaluated. Cell size was quantified using Image-Pro Plus software.

Plasmid and Adenovirus Constructs

The ETS2 plasmid, encoding the full-length human *ETS2* gene, was obtained from Addgene (28128). Truncated plasmids were constructed using Gibson assembly Master Mix (E2621; New England Biolabs). MicroRNA-223 (miR-223) reporter plasmid was obtained by subcloning the miR-223 promoter into pGL3-Basic vector. Luciferase assays were performed using the luciferase assay system (E1980; Promega). The Adeno-X Adenoviral System 3 kit (632267; Clontech) was used to generate an adenoviral vector expressing ETS2 (AdETS2) or mutated ETS2 (AdETS2 T72A and AdETS2 T72E).

Cell Culture and Treatments

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day-old Sprague-Dawley rats using the Neonatal Cardiomyocyte Isolation kit (NC-6031; Cellutron). After 24 hours, medium was changed to 1% fetal bovine serum medium (Dulbecco Modified Eagle Medium/M199=3:1); the next day, it was changed to serum-free medium. NRVMs were then transfected with siRNAs (small interfering RNAs; Sigma) or infected with adenovirus. siRNA targeting ETS2 (SASI_Rn02_00225566 and SASI_Rn02_00225568), MKP3 (SASI_Rn01_00074707 and SASI_Rn01_00074714), and NFATc1 (number 1 sense siRNA sequence: 5′-AGACAGACAUCGGGAGAAA-3′; antisense siRNA sequence: 5′-UUUCUCCCGAUGUCUGUCU-3′; and number 2 sense siRNA sequence: 5′-CUACAUGGUUACUUGGAAA-3′; antisense siRNA sequence: 5′-UUUCCAAGUAACCAUGUAG-3′) were used. Mission siRNA universal negative control number 1 (SIC001; Sigma) was used as a negative control. Transfection was performed using siLentFect lipid reagent (1703361; Bio-Rad) in Opti-MEM medium (Invitrogen) for 6 hours. The medium was then changed to serum-free medium (Dulbecco Modified Eagle Medium/M199=3:1). After overnight serum starvation, cardiomyocytes were treated with 50 μmol/L phenylephrine to induce hypertrophy. For signaling inhibition experiments, 10 μmol/L U0126 (19-147; Sigma) and 1 μmol/L PD0325901 (13034; Cayman) were used to inhibit MEK (MAPK/Erk)–Erk1/2 signaling; 10 μmol/L SP600125 (S5567; Sigma) was used to inhibit JNK (c-Jun N-terminal kinase); 10 μmol/L SB203580 (S8307; Sigma) was used to inhibit p38; 1 μmol/L cyclosporin A (SML1018; Sigma) was used to inhibit calcineurin.

Adult cardiomyocytes were isolated from wild-type, αMHC-Cre, floxed, and ETS2 KO mouse hearts as described previously.[23] Briefly, hearts were perfused retrograde with Krebs-Ringer buffer (35 mmol/L NaCl, 4.75 mmol/L KCl, 1.19 mmol/L KH_2PO_4, 16 mmol/L Na_2HPO_4, 134 mmol/L sucrose, 25 mmol/L $NaCO_3$, 10 mmol/L glucose, and 10 mmol/L HEPES; pH 7.4) and then digested by collagenase II (0.8 mg/mL; Worthington). After removing atria and ventricles, cardiomyocytes were collected by centrifugation.

HEK-293A cells were maintained in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Transfections were performed using Fugene 6 (E2691; Promega) according to the manufacturer's instructions.

Immunofluorescence Analysis

NRVMs were rinsed twice with PBS and fixed with 4% paraformaldehyde for 15 minutes. Subsequently, cardiomyocytes

were permeabilizated with 0.1% Triton X-100 in PBS for 10 minutes and blocked with 3% bovine serum albumin for 1 hour. Then, NRVMs were incubated with anti-α-actinin (A7732; Sigma) antibody overnight at 4°C. After 3 washes, cells were incubated with secondary antibody for 1 hour and sealed with ProLong gold antifade mountant with DAPI (4′,6-diamidino-2-phenylindole; P36935; Thermo Fisher) on slides. Cell circularity index was calculated as $4\pi^{*}$area/perimeter.[2]

^{3}H-Leucine Incorporation

L-[3,4,5-^{3}H]-leucine (1 μmCi/mL; NET460A001MC; PerkinElmer) was added to the culture medium when NRVMs were treated with phenylephrine. After 48 hours, cardiomyocytes were rinsed twice with PBS and incubated with 10% trichloroacetic acid for 30 minutes at 4°C. After 2 washes with 95% ethanol, NRVMs were lysed with 0.5 N NaOH overnight at 37°C with shaking. Equal volume of 0.5 N HCl was then added, and the total content was mixed with scintillation solution (882475; EcoLite; MP Biomedicals) before radioactivity measurement.

Western Blotting

Proteins were prepared with radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.25% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (A32961; Thermo Fisher). Nuclear and cytoplasmic fractions were isolated using a kit according to the manufacturer's instructions (78835; Thermo Fisher). A total of 20 to 40 μg of proteins were separated using Criterion gels (Bio-Rad) and transferred to nitrocellulose membranes using the Trans-Blot Turbo Blotting System (Bio-Rad). The membranes were then blocked with 5% nonfat milk or bovine serum albumin, followed by incubation with primary antibodies overnight at 4°C. After 3 washes, the membranes were incubated with secondary antibody and scanned with an Odyssey CLx Imaging system (LI-COR Biosciences). The following antibodies were used: anti-ETS2 (sc-365666; Santa Cruz Biotechnology), anti-phospho-ETS2 (44-1105G; Thermo Fisher), anti-GAPDH (10R-G109a; Fitzgerald), anti-RCAN1.4 (D6694; Sigma), anti-MKP3 (sc-137246; Santa Cruz Biotechnology), anti-lamin A/C (2032; Cell Signaling), anti-NFATc1 (nuclear factor of activated T cells 1; MA3-024; Thermo Fisher), anti-GFP (green fluorescent protein; A-11120; Invitrogen), anti-FLAG (7425; Sigma), anti-Myc (2276; Cell Signaling), anti-Erk1/2 (9107; Cell Signaling), and anti-phospho-Erk1/2 (9101; Cell Signaling).

Quantitative Real-Time PCR

Total RNA from heart tissues was extracted using an Aurum Fatty and Fibrous Tissue Kit (732-6870; Bio-Rad) and RNA from NRVMs was isolated using a Quick-RNA MicroPrep Kit (R1051; Zymo Research). Reverse transcription was performed using iScript Reverse Transcription Supermix (Bio-Rad) according to the manufacturer's instructions. mRNA levels were determined by quantitative PCR using a LightCycler machine (Roche) and the iTaq Universal SYBR Green Supermix (Bio-Rad). All primer sequences are listed in Table I in the Data Supplement.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the EZ-ChIP kit (17-10086; Millipore). Briefly, cells were cross-linked with 1% formaldehyde for 10 minutes and harvested in SDS lysis buffer, followed by sonication to shear chromatin. Cross-linked protein/DNA was immunoprecipitated with anti-ETS2 (365666X; Santa Cruz Biotechnology), anti-GFP (A-11120; Invitrogen), or control immunoglobulin G. Promoter binding was determined by quantitative PCR.

Coimmunoprecipitation

Coimmunoprecipitation was performed as described previously.[24] Briefly, cells were lysed in immunoprecipitation buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 2 mmol/L EDTA, 1% NP40) supplemented with protease and phosphatase inhibitors (A32961; Thermo Fisher). Proteins were incubated with antibodies for 3 hours at 4°C. After that, 30 μL of preequilibrated Dynabeads protein G (10003D; Bio-Rad) was added and incubated overnight at 4°C. The beads were washed 5 times with immunoprecipitation buffer before eluting with 2× Laemmli loading buffer and analyzed by Western blotting.

miRNA Inhibitor Transfection and miRNA Isolation

We studied miR-223-5p, which, for sake of simplicity, we refer to as miR-223 throughout the article. A total of 50 nmol/L miR-223 inhibitor (MLTUD0340; Sigma) or the relevant control (NCSTUD002; Sigma) was transfected into NRVMs. Low-molecular-weight RNA from heart tissues and NRVMs was isolated using the mirVana miRNA Isolation Kit (AM1560; Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (4366596; Thermo Fisher). miR-223 levels were determined by quantitative PCR and normalized to U6.

Human Samples

Left ventricular tissues were harvested from patients with dilated cardiomyopathy. Control samples were obtained from healthy donors. Before cardiac tissue collection, informed consent was obtained from the prospective donors. Our study conformed to the principles outlined in the Declaration of Helsinki and was approved by the ethics review committee of Tongji Hospital. Details regarding the patients are provided in Table II in the Data Supplement.

Statistics

All results are presented as mean±SEM of at least 3 independent experiments. Normality of the distribution of data was assessed using the Shapiro-Wilk test, acknowledging the limitation that small sample sizes prevent us from making a reliable assessment of normality. Homogeneity of variance was assessed by the F test (for 2 groups) or Brown-Forsythe test (for ≥3 groups). Data with normal distribution and equal variances were analyzed by unpaired 2-tailed Student *t* test (for 2 groups) or 1-way ANOVA followed by Bonferroni post hoc test (for ≥3 groups). Otherwise, nonparametric statistical analyses were performed using the Mann-Whitney *U* test for 2 groups

or the Kruskal-Wallis test followed by the Dunn post hoc test for multiple comparisons (for ≥3 groups). All statistical analyses were performed by GraphPad Prism 8 (GraphPad Software, Inc).

RESULTS

ETS2 Is Activated in Hypertrophic Hearts

To determine whether ETS2 participates in hypertrophic growth of the heart, adult wild-type mice were subjected to severe transverse aortic constriction (sTAC), a surgical model of pressure overload–induced cardiac hypertrophy.[20] As expected, mice that underwent sTAC surgery displayed an initial hypertrophic response within 1 week that progressed to failure by 3 weeks. This response included increases in cardiac mass and cardiomyocyte size, hypertrophic marker gene expression, and progressive declines in contractile function as compared with age-matched, sham-operated animals (Figure IA–IE in the Data Supplement). In both early and late stages of the response, ETS2 was activated in ventricular tissue, as evidenced by an increase of ETS2 phosphorylation at threonine 72 (Figure 1A). There was no change in ETS2 protein or mRNA levels in whole tissue (Figure 1A and Figure IF in the Data Supplement). ETS2 phosphorylation was increased early in adult cardiomyocytes isolated from sTAC-subjected hearts, and high levels of p-ETS2 persisted in the heart until 3 weeks (Figure IG in the Data Supplement). This was associated with an increase in ETS2 protein levels, but not mRNA levels (Figure IG and IH in the Data Supplement), suggesting an increase in protein stability. Cellular fractionation revealed an increase in phosphorylated ETS2 in the nuclear fraction isolated from sTAC-treated hearts (Figure 1B and 1C). ETS2 steady-state protein levels were increased in both the cytoplasmic and nuclear fractions of adult cardiomyocytes isolated from sTAC-treated hearts, but phosphorylated ETS2 was increased only in the nucleus (Figure II in the Data Supplement).

Next, we tested whether these changes in ETS2 were mirrored in human heart failure. ETS2 abundance at both protein and mRNA levels was comparable in normal hearts and in hearts of patients with end-stage dilated cardiomyopathy (Figure 1D and Figure IJ and IK in the Data Supplement). However, ETS2 phosphorylation was increased 2-fold in dilated cardiomyopathy hearts (Figure 1D and Figure IJ in the Data Supplement). These results indicate that ETS2 is activated in the setting of hypertrophic growth of the myocardium and suggest that ETS2 may participate in its development.

ETS2 Deficiency Attenuates Cardiac Hypertrophy in Response to Pressure Overload

To test for a functional role of ETS2 in cardiac hypertrophy, we engineered cardiomyocyte-specific ETS2 KO mice by crossing ETS2-floxed (F/F) mice with αMHC-Cre mice. ETS2 transcript levels were decreased >70% in the hearts of KO mice compared with either Cre mice or F/F mice (Figure IIA in the Data Supplement). This decrease was specific to cardiomyocytes (Figure IIB and IIC in the Data Supplement). Eight-week-old ETS2 KO mice did not manifest cardiac morphologic or functional changes, suggesting that loss of ETS2 in these animals did not affect heart development (Figure 2A–2E).

To test for potential effect of ETS2 on development and progression of cardiac hypertrophy, we again turned

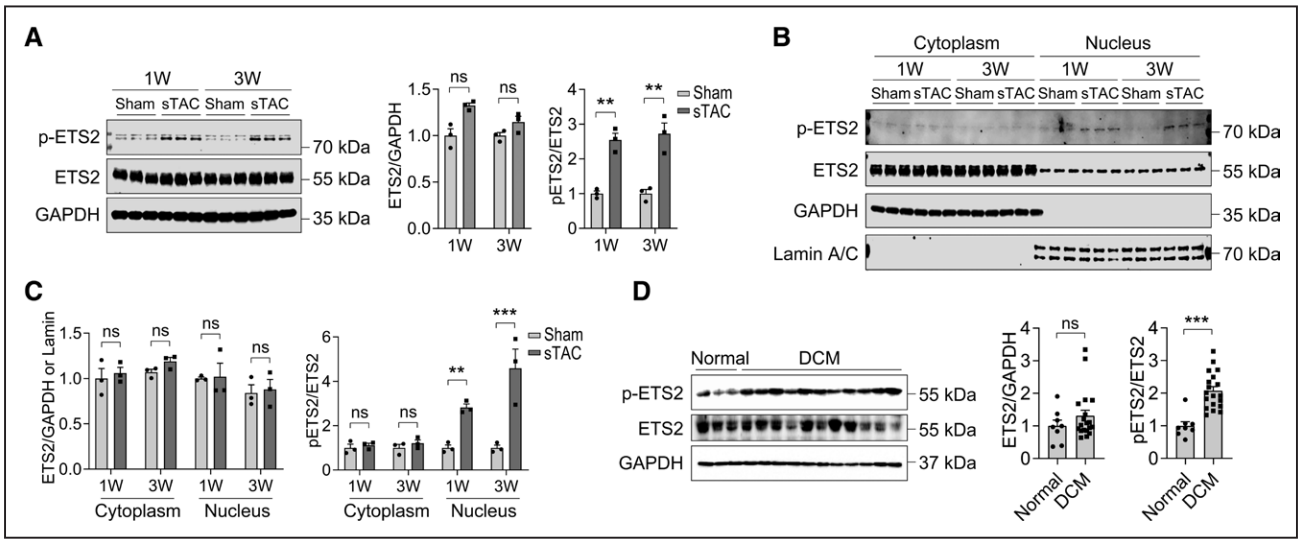

Figure 1. ETS2 is activated in hypertrophic hearts.
A, Western blot analyses and quantification showing ETS2 phosphorylation and protein levels in mouse hearts at 1 or 3 weeks after sham operation or severe thoracic aortic constriction (sTAC; n=3). **B** and **C**, Western blot analyses (**B**) and quantification (**C**) of ETS2 phosphorylation and protein levels in cytoplasmic and nuclear fractions extracted from sham-operated control hearts and sTAC-induced hypertrophied hearts (n=3). **D**, ETS2 phosphorylation is increased in hearts from patients with dilated cardiomyopathy (DCM; n=8 for normal controls, n=19 for DCM). $^{**}P<0.01$; $^{***}P<0.001$. ETS2 indicates a member of the E26 transformation-specific sequence (ETS) domain family; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ns, not significant; and sTAC, severe transverse aortic constriction.

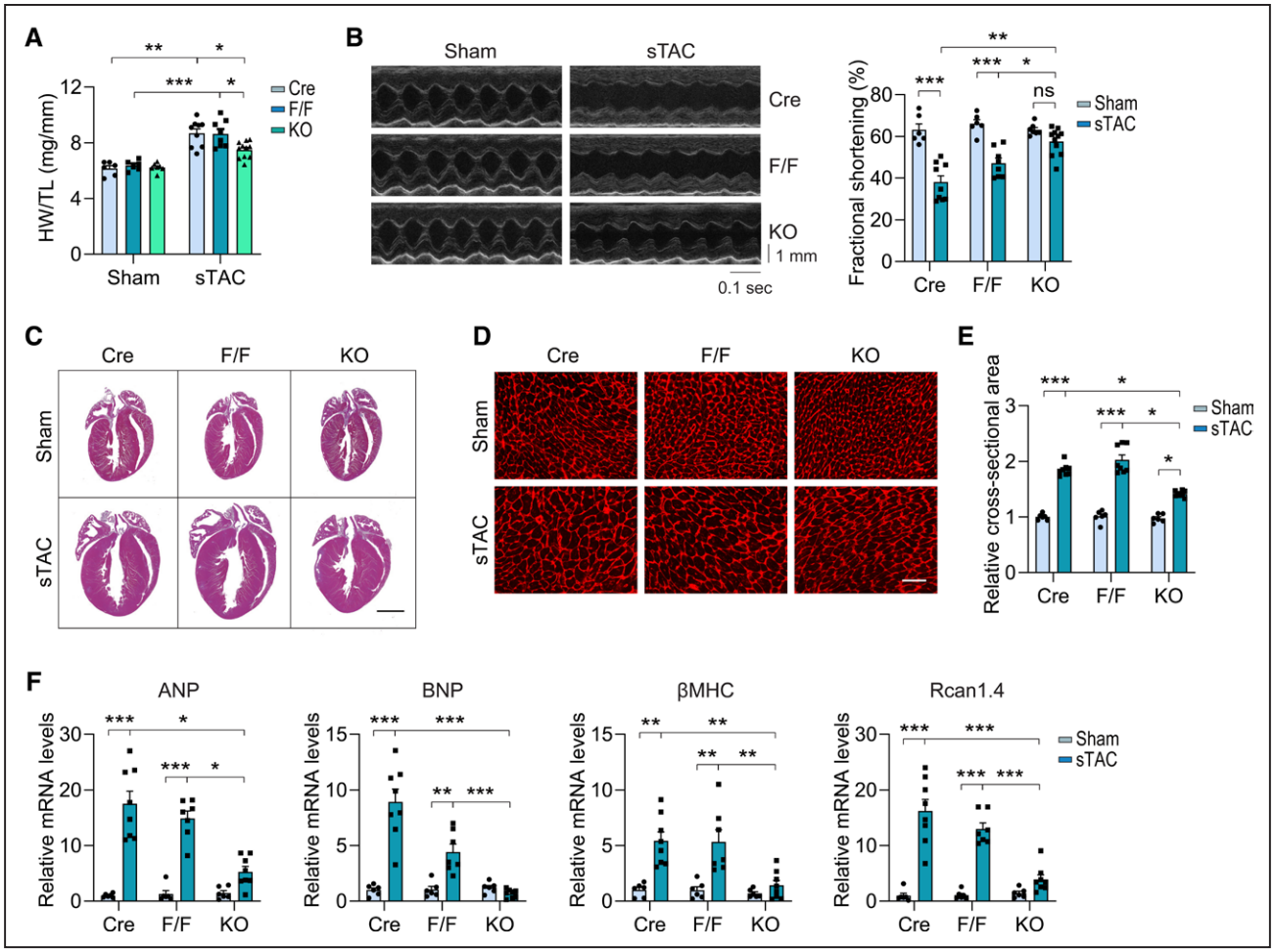

Figure 2. Cardiomyocyte-specific ETS2 (a member of the E26 transformation-specific sequence [ETS] domain family) knockout (KO) mice are protected from severe thoracic aortic constriction (sTAC)–induced cardiac hypertrophy.
A, The ratio of heart weight/tibia length (HW/TL) in α-myosin heavy chain–Cre (Cre), ETS2-floxed (F/F), and ETS2 knockout (KO) mice after 3 weeks of sham or sTAC (n=6 to 11). **B**, ETS2 KO mice showed enhanced contractile function after sTAC. Left, M-mode echocardiography images; right, fractional shortening (n=6 to 11). **C** and **D**, Histologic analyses of heart sections from Cre, F/F, and KO mice subjected to sham or sTAC for 3 weeks. Heart sections were stained with hematoxylin and eosin to analyze hypertrophic growth (**C**; scale bar, 2 mm) or stained with wheat germ agglutinin to demarcate cell boundaries (**D**; scale bar, 20 μm). **E**, Quantification of the relative cross-sectional area of the indicated groups (n=6 to 8). **F**, mRNA levels of hypertrophic marker genes (*ANP* [atrial natriuretic peptide], *BNP* [brain natriuretic peptide], *βMHC* [β-myosin heavy chain], and Rcan1.4) were decreased in ETS2 KO mice after sTAC (n=6 to 8). **P*<0.05; ***P*<0.01; ****P*<0.001. ns indicates not significant.

to the sTAC model. Animals underwent sham or sTAC surgery and cardiac phenotyping was carried out 3 weeks after surgery. As expected, control mice (F/F and Cre) developed significant increases in heart mass quantified as heart weight to body weight or heart weight to tibia length ratio as compared with sham controls (Figure 2A and Figure IID in the Data Supplement). However, this growth response was blunted in ETS2 KO hearts (Figure 2A and Figure IID in the Data Supplement). Functionally, sTAC elicited an increase in left ventricular diastolic and systolic dimensions in control mice, but resulted in no significant change in ETS2 KO mice (Figure IIE in the Data Supplement and Table III in the Data Supplement). Contractile function, quantified as % fractional shortening on echocardiography, was decreased in control animals after sTAC but remained preserved in ETS2 KO mice (Figure 2B and Table III in the Data Supplement).

Blunting of the hypertrophic response was also apparent at the cellular level, where silencing of ETS2 attenuated increases in cardiomyocyte cross-sectional area in response to sTAC (Figure 2C–2E). Furthermore, sTAC-triggered induction of hypertrophic marker gene expression, such as ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide), βMHC (β-myosin heavy chain), and Rcan1.4, was dramatically inhibited in the hearts of ETS2 KO mice (Figure 2F and Figure IIF in the Data Supplement). Taken together, these results indicate that ETS2 is required for the normal cardiac remodeling response to elevated afterload.

ETS2 Is Required for Calcineurin-Induced Cardiac Hypertrophy

Involvement of ETS2 in regulating Rcan1.4 expression (Figure 2F and Figure IIF in the Data Supplement), a

well-known target of calcineurin/NFAT signaling,[25] led us to consider whether ETS2 could play a role in calcineurin-induced cardiac hypertrophy. To this end, we bred ETS2 KO mice with α-MHC-calcineurin transgenic (CnA) mice.[7,19] These CnA transgenic mice harbor a constitutively active mutant of the calcineurin catalytic subunit expressed specifically in cardiomyocytes.[7] As expected, 4-week-old CnA transgenic mice manifested significant cardiac hypertrophy, with dramatically increased heart weight and severe declines in contractile performance (Figure 3A–3E and Table IV in the Data Supplement). In contrast, hearts of ETS2 KO/CnA transgenic mice were much smaller than those of CnA mice at the same age (Figure 3A and Figure IIG in the Data Supplement). Silencing of ETS2 also diminished calcineurin-induced increases in left ventricular internal dimensions with marked improvement in contractile function (Figure 3B and Figure IIH in the Data Supplement and Table IV in the Data Supplement). At the cellular level, calcineurin-driven increases in cardiomyocyte cross-sectional area and hypertrophic marker gene expression were blunted in ETS2 KO/CnA transgenic mice (Figure 3C–3F). Calcineurin-induced Rcan1.4 expression was repressed in the hearts of ETS2 KO/CnA transgenic mice (Figure 3F and 3G). Together, these results indicate that ETS2 is essential for calcineurin-dependent cardiac hypertrophy.

These data suggest that ETS2 participates in both afterload-driven hypertrophic growth of the heart and that driven by calcineurin. To elucidate the role of ETS2 in hypertrophic remodeling of the heart, we turned to NRVMs in culture. Similar to our observations with hypertrophic stress in vivo, phenylephrine increased cardiomyocyte ETS2 phosphorylation (Figure 4A). ETS2 steady-state protein levels, but not mRNA levels, were increased in NRVMs exposed to phenylephrine for 6 hours or 24 hours (Figure 4A and Figure IIIA in the Data Supplement). Phenylephrine-induced increases in ETS2 protein abundance were detected in both the cytoplasm and nucleus, but increased ETS2 phosphorylation was detected only in the nucleus (Figure 4B).

ETS2 Is Activated by Erk1/2 in Response to Phenylephrine

It has been reported that ETS2 is phosphorylated in macrophages by MAPKs Erk1/2 and JNK.[26–28] To test their involvement in cardiomyocytes, we exposed NRVMs to phenylephrine, an established hypertrophic growth cue acting via MAPKs,[29–31] and used U0126, SP600125, and SB203580, specific inhibitors of MEK,[30] JNK,[32] and p38,[33] respectively. Whereas JNK and p38 inhibition had no significant effect, inhibition of MEK-Erk1/2 signaling resulted in a dramatic decrease in ETS2 phosphorylation (Figure 4C and Figure IIIB in the Data Supplement). ETS2 protein levels were also decreased by Erk1/2 inhibition, without change in ETS2 mRNA levels (Figure 4C and Figure IIIC in the Data Supplement). This result was corroborated independently with another MEK inhibitor,[34] PD0325901 (Figure IIID and IIIE in the Data Supplement). These findings suggest that ETS2 is a target of Erk1/2 in cardiomyocytes, and ETS2 phosphorylation may be important for its protein stability.

Given our observation that ETS2 participates in calcineurin-induced cardiac hypertrophy, we examined ETS2 phosphorylation levels in NRVMs exposed to adenovirus expressing CnA. We observed an increase in ETS2 phosphorylation in AdCnA-infected myocytes, but not in AdLacZ control myocytes (Figure IIIF and IIIH in the Data Supplement). Thus, ETS2 is phosphorylated (activated) in the settings of calcineurin-driven and Erk1/2-driven hypertrophic remodeling, lending additional credence to the notion of crosstalk between these pathways.

To test whether ETS2 is required for phenylephrine- or CnA-induced cardiomyocyte hypertrophy, or both, we silenced ETS2 in NRVMs by siRNA transfection using 2 sequence-independent RNA interference constructs (Figure 4D) and exposed the cells to phenylephrine or infected them with CnA adenovirus. As expected, both phenylephrine-treated and AdCnA-infected NRVMs displayed a robust hypertrophic response, as indicated by increased cardiomyocyte size and protein synthesis (Figure 4E–4G and Figure IIII–IIIK in the Data Supplement). These increases, triggered by either growth cue, were significantly suppressed by ETS2 knockdown (Figure 4E–4G and Figure IIII–IIIK in the Data Supplement). ETS2 silencing also inhibited expression of the hypertrophic marker genes *ANP* and *BNP*, although no change in βMHC expression was observed (Figure 4H and Figure IIIL in the Data Supplement). Expression of Rcan1.4 was significantly decreased after ETS2 knockdown, confirming our in vivo observations (Figure 4I and Figure IIIL–IIIN in the Data Supplement).

To explore the role of Erk1/2 phosphorylation in this process, we used U0126, a specific inhibitor of MEK-Erk1/2 signaling.[30] As reported previously, inhibition of Erk1/2 decreased the phenylephrine-induced hypertrophic response in NRVMs (Figure IVA–IVC in the Data Supplement).[30] Erk1/2 inhibition also reduced expression of the hypertrophic marker genes *ANP*, *BNP*, and *Rcan1.4* to an extent similar to that occurring with *ETS2* gene silencing (Figure IVD–IVF in the Data Supplement). Unlike ETS2 inhibition, βMHC levels were also reduced with Erk1/2 inhibition, suggesting a broader role for Erk1/2 signaling under these conditions (Figure IVD in the Data Supplement).

ETS2 Activation by Erk1/2 Establishes an Inhibitory Feedback Loop Through MKP3

Findings reported thus far reveal that ETS2 is required for disease-related hypertrophic remodeling of the myo-

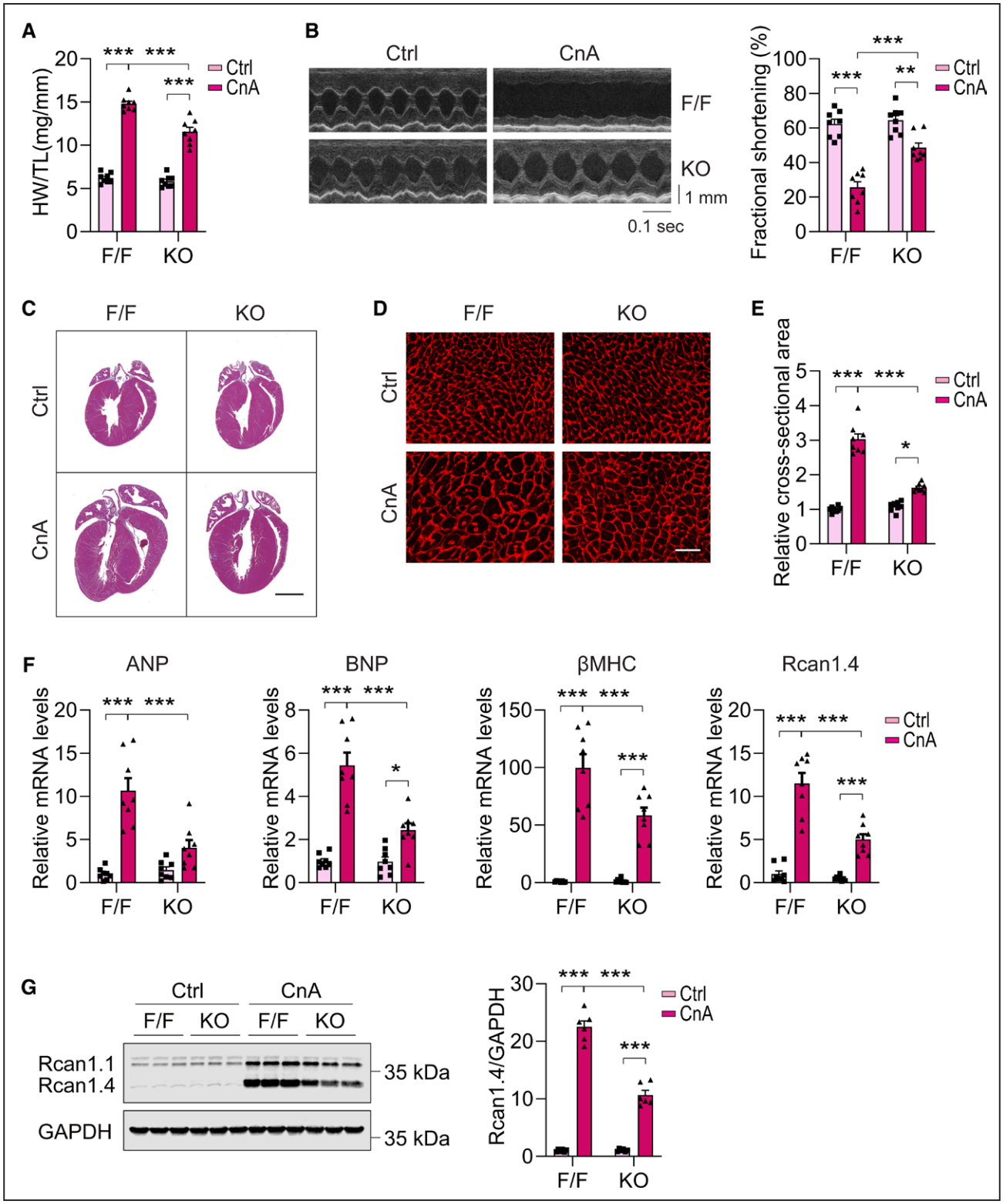

Figure 3. ETS2 (a member of the E26 transformation-specific sequence [ETS] domain family) knockout (KO) suppresses calcineurin-induced cardiac hypertrophy.

A, Heart weight/tibia length (HW/TL) ratio of ETS2$^{F/F}$ and KO mice crossed with calcineurin transgenic mice (CnA; n=8). **B**, Echocardiographic analyses of cardiac function of ETS2$^{F/F}$ and KO mice crossed with calcineurin transgenic mice. Left, M-mode echocardiographic images; right, fractional shortening (n=8). **C** and **D**, Histologic analyses of heart sections from ETS2$^{F/F}$ and KO mice crossed with calcineurin transgenic mice. Heart sections were stained with hematoxylin and eosin (**C**; scale bar, 2 mm) or wheat germ agglutinin (**D**; scale bar, 20 μm). **E**, Quantification of the relative cross-sectional area of the indicated groups (n=8). **F**, mRNA levels of hypertrophic marker genes (n=8). **G**, Western blot analyses and quantification of Rcan1.4 protein levels in indicated groups (n=6). *P<0.05; **P<0.01; ***P<0.001. ANP indicates atrial natriuretic peptide; βMHC, β-myosin heavy chain; BNP, brain natriuretic peptide; and Ctrl, control.

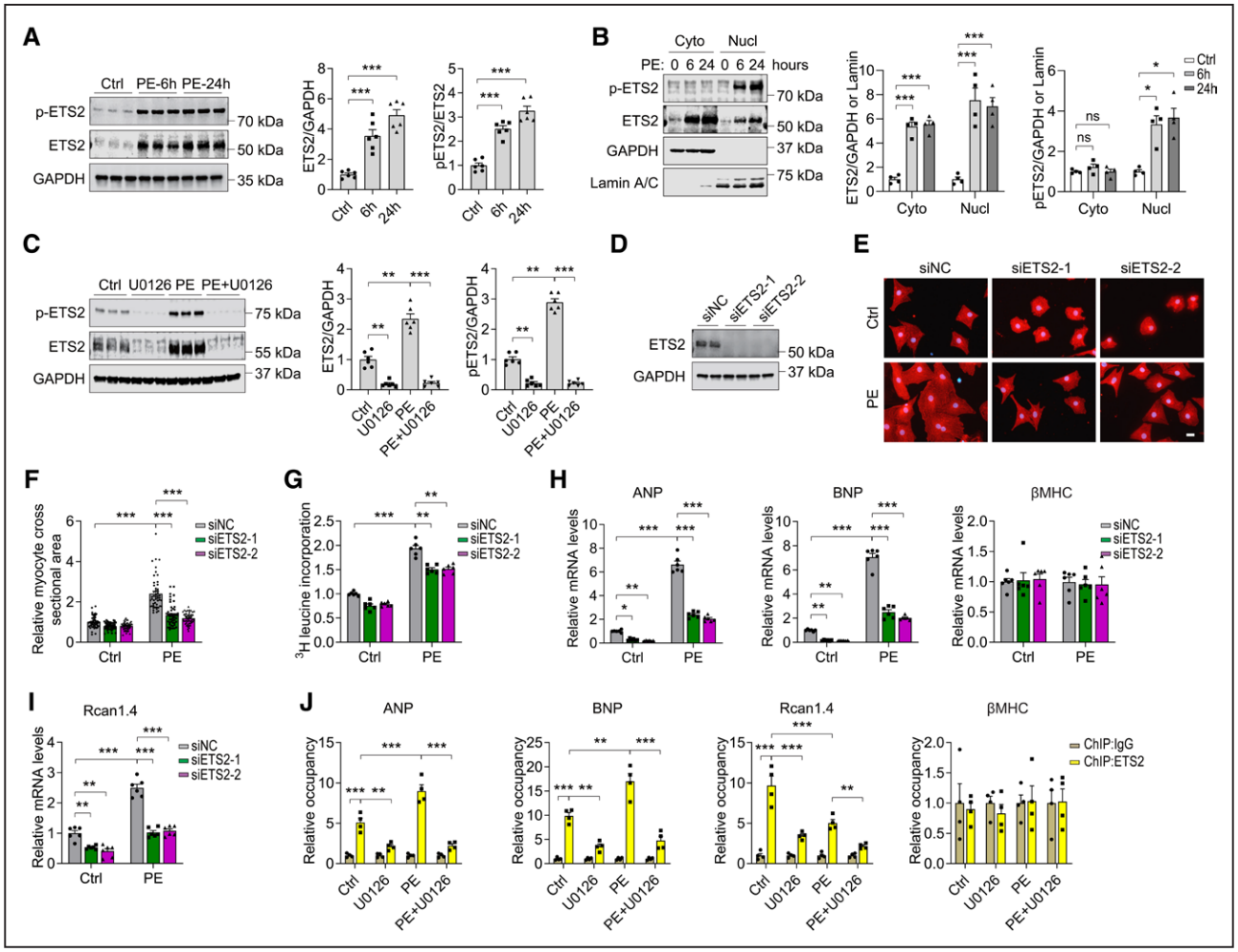

Figure 4. ETS2 (a member of the E26 transformation-specific sequence [ETS] domain family) knockdown attenuates phenylephrine-induced hypertrophy in vitro.

A, Representative Western blots and quantification showing ETS2 phosphorylation and protein levels in isolated neonatal rat ventricular myocytes (NRVMs) treated with 50 μmol/L phenylephrine (PE) for 6 or 24 hours (n=6). **B**, Western blot analyses and quantification of ETS2 phosphorylation and expression in cytoplasmic and nuclear fractions extracted from PE-treated NRVMs (n=4). **C**, ETS2 phosphorylation and protein levels in NRVMs treated with PE or MEK inhibitor U0126, or both, for 24 hours (n=6). **D**, ETS2 was silenced in NRVMs by small interfering RNA (siRNA) transfection. **E**, Representative immunofluorescence images of α-actinin staining in NRVMs transfected with siRNA control (siNC) or siRNA targeting ETS2 (siETS2) and then treated with PE for 48 hours. Scale bar, 20 μm. **F**, Quantification of relative cardiomyocyte surface area (n=50). **G**, ETS2 knockdown inhibits PE-induced protein synthesis in NRVMs (n=6). **H**, mRNA levels of ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide), and βMHC (β-myosin heavy chain) in NRVMs transfected with siNC or siETS2 and then treated with PE for 24 hours (n=6). **I**, Rcan1.4 mRNA levels in NRVMs treated as in **H** (n=6). **J**, Chromatin immunoprecipitation (ChIP) analysis to detect ETS2 binding to the promoters of the indicated genes. ChIP was performed with an ETS2-specific antibody or immunoglobulin G control antibody in control NRVMs or NRVMs treated with PE or U0126, or both, for 6 hours. The occupancy of ETS2 at promoters is shown relative to background signals with immunoglobulin G control antibody (n=4). **P*<0.05; ***P*<0.01; ****P*<0.001. βMHC indicates β-myosin heavy chain; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; Ctrl, control; Cyto, cytoplasm; ETS2, a member of the E26 transformation-specific sequence (ETS) domain family; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ns, not significant; and nucl, nucleus.

cardium culminating from 2 distinct signaling pathways. Next, we set out to determine whether ETS2 is sufficient to induce cardiac hypertrophy. To address this, we engineered an adenovirus expressing ETS2 (Figure VA in the Data Supplement) and infected NRVMs. In control NRVMs, overexpressed ETS2 was located in the cytoplasm, but phenylephrine treatment resulted in its translocation to the nucleus (Figure VB in the Data Supplement). ETS2 overexpression alone had no effect on cell size, protein synthesis, or hypertrophic marker gene expression (Figure VC–VH in the Data Supplement). Because phosphorylation of threonine 72 is crucial for ETS2 activity[26,35] and is increased by hypertrophic stimuli (Figure 1A and Figure 4A), we engineered a phosphomimetic mutant by changing tyrosine 72 to a glutamine (T72E; Figure VA in the Data Supplement). The phosphomimetic was localized to the cytoplasm and did not induce hypertrophy or affect hypertrophic gene expression at baseline (Figure VB–VH in the Data Supplement).

We next evaluated whether ETS2 overexpression alters the hypertrophic response triggered by growth cues. We expressed in NRVMs wild-type, the T27E phos-

phomimetic, or the T27A phospho-null mutant ETS2 and induced hypertrophy with phenylephrine. At baseline, none of the constructs had an effect on hypertrophy (Figure VC–VE in the Data Supplement). On growth induction with phenylephrine, the T27A phospho-null mutant manifested a hypertrophic response similar to the GFP control (Figure VC–VE in the Data Supplement). Cells expressing either the wild-type or T27E construct did not manifest an exacerbated response, but rather showed an unexpected reduction of the hypertrophic response (Figure VC–VE in the Data Supplement). Even though phenylephrine-induced increases in ANP and BNP mRNA levels were amplified by overexpression of wild-type ETS2 or the T72E constitutively active mutant, cardiomyocyte size, protein synthesis, and Rcan1.4 expression were decreased under these conditions (Figure VC–VH in the Data Supplement). In aggregate, these results suggest that ETS2 overexpression inhibits hypertrophic growth in NRVMs in a manner that requires T27 phosphorylation.

To pursue this surprising result, we tested whether overexpression of ETS2 might be altering Erk1/2 signaling through a negative feedback loop. Consistent with this, we found that both wild-type ETS2 and the T27E mutant, but not the T27A mutant, repressed Erk1/2 phosphorylation in response to phenylephrine (Figure VIA in the Data Supplement).

In both 3T3 fibroblasts and cancer cells, ETS2 has been reported to bind to the promoter of MKP3 (MAPK phosphatase 3), an established mediator of Erk1/2 dephosphorylation.[36–38] We observed an increase in MKP3 expression in NRVMs treated with phenylephrine (Figure VIB and VIC in the Data Supplement). ETS2 was required for this, as ETS2 knockdown diminished MKP3 expression both at baseline and on phenylephrine treatment (Figure VID and VIE in the Data Supplement). Assessment of promoter binding by ChIP revealed that ETS2 occupies the MKP3 promoter in NRVMs (Figure VIF in the Data Supplement).

To test whether MKP3 has a role in ETS2-mediated Erk1/2 inhibition in cardiomyocytes, we first examined MKP3 expression in ETS2-overexpressing NRVMs. We found that whereas MKP3 message levels increase ≈50% on exposure to phenylephrine, overexpression of wild-type ETS2 and the T27E mutant, but not T27A ETS2, further increased that response 2- and 3-fold, respectively, over untreated cells (Figure VIB and VIC in the Data Supplement). We next silenced MKP3 in ETS2-overexpressing NRVMs (Figure VIG in the Data Supplement) to determine whether ETS2 overexpression inhibits Erk1/2 via MKP3. We found that MKP3 knockdown blocked the inhibitory effect of ETS2 on Erk1/2 signaling (Figure VIH in the Data Supplement). ETS2 overexpression promoted phenylephrine-induced hypertrophy in MKP3-silenced NRVMs (Figure VII–VIK in the Data Supplement). Together, these results indicate that whereas activation of ETS2 by Erk1/2 can drive cardiomyocyte hypertrophy, ETS2 also increases MKP3 expression to inhibit Erk1/2 in a negative feedback loop.

Recognizing that ETS2 is a transcription factor, we next examined whether ETS2 binds to the promoter of hypertrophy-related genes in cardiomyocytes. ChIP analyses revealed that ETS2 binds to the promoters of ANP, BNP, and Rcan1.4, but not βMHC (Figure 4J). Phenylephrine increased the ability of the ETS2 antibody to immunoprecipitate the promoters of ANP and BNP, whereas pulldown of the Rcan1.4 promoter was decreased in phenylephrine-treated NRVMs (Figure 4J). Erk1/2 inhibition led to a significant decrease in ETS2 binding to all promoters, highlighting the importance of Erk1/2 phosphorylation of ETS2 in this pathway (Figure 4J).

ETS2 Synergizes With NFAT to Transactivate Rcan1.4

The observation that ETS2 binds to the Rcan1.4 promoter led us to investigate the relationship between ETS2 and NFAT transcriptional activity more directly. We took advantage of a plasmid harboring the Rcan1.4 promoter linked to a luciferase coding region and performed reporter assays in HEK293 cells by adding plasmids expressing exogenous ETS2 and NFATc1. Transfection of ETS2 on its own increased the transcriptional activity of the Rcan1.4 promoter ≈5-fold (Figure 5A), whereas an ETS2 mutant lacking the DNA binding domain had no effect on Rcan1.4 promoter activity (Figure 5A). NFATc1 alone transactivated the Rcan1.4 promoter ≈15-fold, whereas coexpression of ETS2 and NFATc1 resulted in a ≈30-fold increase in Rcan1.4 luciferase activity (Figure 5A). This synergy in activating the Rcan1.4 promoter was dependent on the DNA binding domain of ETS2 (Figure 5A). We then tested another NFAT target gene, miR-23a. In contrast to the Rcan1.4 promoter, ETS2 had no effect on expression driven by the miR-23a promoter, and whereas NFATc1 activated this promoter, ETS2 did not alter this expression level (Figure VIIA in the Data Supplement). These data point to the existence of a specific subclass of NFAT/ETS2 target genes.

Promoter binding is an important regulatory mechanism governing transcriptional activity. Therefore, ChIP was used to determine whether ETS2 influences the binding of NFATc1 to the Rcan1.4 promoter. Plasmid encoding full-length NFATc1 fused to GFP was transfected into HEK293 cells and a GFP antibody was used for subsequent ChIP assay. As expected, both ETS2 and NFATc1 bound to the Rcan1.4 promoter (Figure 5B). Although NFATc1 did not affect ETS2 binding to the Rcan1.4 promoter, ETS2 increased the binding of NFATc1 to the Rcan1.4 promoter ≈4-fold (Figure 5B).

Synergy between ETS2 and NFATc1 led us to consider whether there is an interaction between these 2 molecules. Immunoprecipitation of a flag-tagged ETS2 revealed significant pulldown of the coexpressed

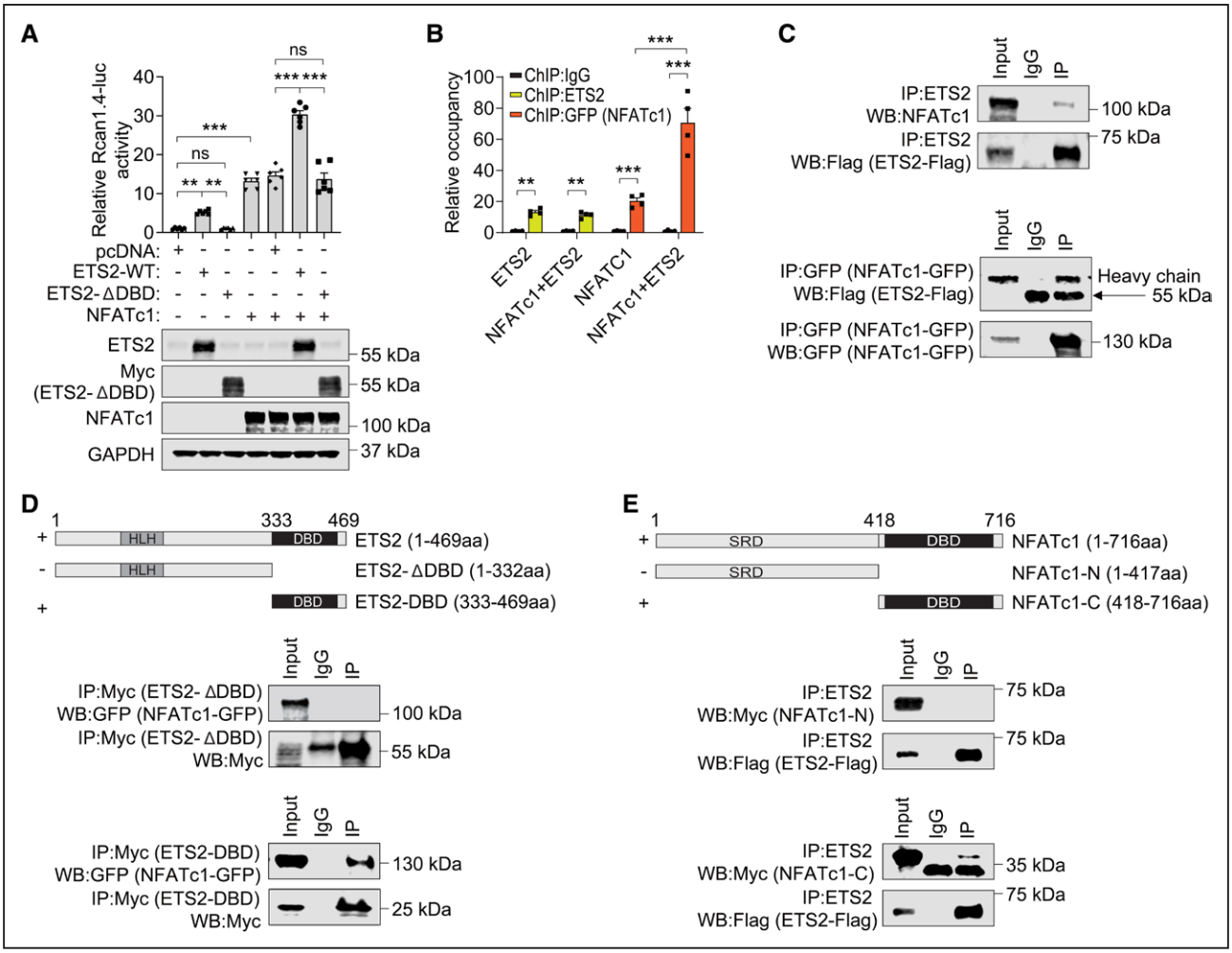

Figure 5. ETS2 (a member of the E26 transformation-specific sequence [ETS] domain family) cooperates with NFAT (nuclear factor of activated T cells) to regulate Rcan1.4 transcription.

A, Luciferase activity in HEK-293 cells transfected with an Rcan1.4 luciferase reporter plasmid, along with expression plasmids of ETS2, ETS2 lacking DNA binding domain (ETS-ΔDBD), and NFATc1 (n=6). **B**, Chromatin immunoprecipitation (ChIP) analysis using an ETS2-specific antibody or a GFP antibody to detect ETS2 or NFATc1 binding to the Rcan1.4 promoter. HEK-293 cells were transfected with an ETS2 expression plasmid, a plasmid encoding full-length NFATc1 fused to green fluorescent protein (NFATc1-GFP), and an Rcan1.4 luciferase reporter plasmid. A total of 48 hours after transfection, cells were collected for ChIP analysis (n=4). **C**, ETS2 interacts with NFATc1. HEK-293 cells were transfected with expression plasmids of NFATc1-GFP and flag-tagged ETS2, then an ETS2-specific antibody and a GFP antibody were used for immunoprecipitation (IP) analysis, respectively. **D**, NFATc1 binds to ETS2 DBD. HEK-293 cells were transfected with expression plasmids of NFATc1-GFP and truncated ETS2, which has a myc tag. A total of 48 hours after transfection, cells were collected for IP analysis. **E**, ETS2 binds to NFATc1 DNA binding domain. HEK-293 cells were transfected with expression plasmids of flag-tagged ETS2 and truncated NFATc1, which has a myc tag, and then an IP assay was performed. $^{**}P<0.01$; $^{***}P<0.001$. HLH indicates helix–loop–helix domain; IgG, immunoglobulin G; ns, not significant; SRD, serine-rich domain; and WB, Western blot.

NFATc1 protein. Likewise, immunoprecipitation of the GFP-tagged NFATc1 resulted in pulldown of ETS2 protein (Figure 5C). To map the domain required for the interaction between ETS2 and NFATc1, truncated ETS2 or NFATc1 was expressed in HEK293 cells. We found that ETS2 and NFATc1 bound to each other's DNA binding domain (Figure 5D and 5E). Immunoprecipitation of endogenous ETS2 in NRVMs triggered significant pulldown of NFATc1, and phenylephrine treatment resulted in greater pulldown of NFATc1 protein (Figure VIIB in the Data Supplement). Furthermore, the interaction between ETS2 and NFATc1 was detected exclusively in the nucleus in phenylephrine-treated NRVMs (Figure VIIC in the Data Supplement). These results indicate that ETS2 physically and functionally interacts with NFATc1 to transactivate Rcan1.4 gene expression, suggesting cooperation between Erk1/2 and calcineurin in downstream targeting of a subset of hypertrophic genes.

MiR-223 Is a Downstream Target of ETS2 and NFAT in Cardiomyocytes

ETS proteins recognize sequences with a GGAA/T core motif, which is similar to NFAT binding sites

(GGAAA).[39,40] Thus, we postulated that ETS2 and NFAT may share additional targets that are relevant to their roles in cardiomyocyte hypertrophy. To test this, we queried the Gene Transcription Regulation Database (http://gtrd20-06.biouml.org/) to identify genes harboring ETS2 or NFATc1 binding sites. Our search revealed 4124 genes harboring ETS2 binding sites and 15 124 genes harboring NFATc1 binding sites (Figure VIID and Spreadsheet I in the Data Supplement). Approximately 90% (3710) of the genes in the ETS2 group were also found in the NFATc1 group, including Rcan1 (Spreadsheet I in the Data Supplement). This high degree of intersection suggests ETS2 and NFATc1 share a large number of downstream target genes. To test this, we queried https://ccg.epfl.ch/ssa/findm.php to find target genes that harbor ETS2 and NFATc1 binding motifs in close proximity. The entire sequence range was set to −500 to approximately +100, and 759 genes were found, including Rcan1 (Spreadsheet II in the Data Supplement).

Because miR-223 is a downstream target of ETS2 in myeloid cells,[41] and miR-223 is able to induce cardiac hypertrophy and heart failure,[42] we tested whether ETS2 regulates miR-223 expression in the heart. miR-223 levels were significantly decreased in ETS2 KO mice on hypertrophic stimulation (Figure 6A and 6B). Consistent with this, knockdown of ETS2 in NRVMs also downregulated miRNA-223 expression after phenylephrine treatment (Figure 6C), as did inhibition of Erk1/2 (Figure VIIE in the Data Supplement). ChIP assays revealed that ETS2 binds to the miR-223 promoter in cardiomyocytes and phenylephrine treatment increased ETS2 binding (Figure 6D). Silencing NFATc1 also dramatically decreased miR-223 levels in NRVMs (Figure 6E).

To further tease apart the relationship among ETS2, NFAT, and miR-223, luciferase reporter assays were performed. We constructed a reporter vector harboring the full-length miR-223 promoter and found that ETS2 induced a small (≈2-fold) increase in reporter activity, whereas NFATc1 induced activity ≈10-fold over baseline (Figure 6F). The combination of ETS2 and NFATc1 resulted in >30-fold increase in miR-223 promoter activity (Figure 6F), a response that was abolished by deletion of the ETS2 DNA binding domain (Figure 6F).

To corroborate putative synergy between ETS2 and NFATc1, we performed an additional luciferase reporter assay using a vector harboring ≈700 bp of the miR-223 promoter, thus removing the ETS2 binding site. ETS2 had no effect on the activity of this reporter, whereas NFATc1 activated it (Figure VIIF in the Data Supplement). ETS2 increased NFATc1-mediated induction of miR-223 reporter activity (Figure VIIF in the Data Supplement), a response that was abolished by deletion of the ETS2 DNA binding domain, which is also the binding site of NFATc1 (Figure VIIF in the Data Supplement). ChIP assays revealed that both ETS2 and NFATc1 bind to the miR-223 promoter (Figure 6G). Much like our findings with the Rcan1.4 promoter, NFATc1 did not influence ETS2 binding to the miR-223 promoter, but ETS2 dramatically increased NFATc1 binding to the miR-223 promoter (Figure 6G). These results suggest ETS2 synergizes with NFAT to regulate expression of miR-223.

To further corroborate miR-223 as a downstream target of the calcineurin/NFAT/ETS2 pathway, we examined the effect of calcineurin on miR-223 levels. We found that calcineurin induced miR-223 expression in NRVMs, whereas cyclosporin A, an inhibitor of calcineurin,[7] blocked the induction of miR-223 by calcineurin, as did inhibition of Erk1/2 (Figure 6H). Calcineurin significantly increased the luciferase activity driven by the miR-223 promoter (Figure VIIG in the Data Supplement).

We next tested whether miR-223 is required for calcineurin-induced hypertrophy. We found that an miR-223 antagomir attenuated calcineurin-mediated hypertrophy as measured by decreases in cell cross-sectional area, protein synthesis, and cell circularity index (Figure 6I and 6J and Figure VIIH–VIIK in the Data Supplement). Together, these results unveil miR-223 as a downstream target of both the Erk1/2 and calcineurin/NFAT pathways, with the 2 downstream effectors, ETS2 and NFAT, functioning in synergy to transactivate miR-223 to promote hypertrophy.

DISCUSSION

We set out to elucidate the role of ETS2 in hypertrophic remodeling of the heart. We report that ETS2 is activated by Erk1/2 in both hypertrophied murine hearts and in human dilated cardiomyopathy. ETS2 is required for both pressure overload- and calcineurin-induced cardiac hypertrophy, responses involving signaling cascades distinct from, but interdependent with, Erk1/2 signaling. We discovered that ETS2 synergizes with NFAT to transactivate Rcan1.4, an established downstream target of NFAT. We identified miR-223, an miRNA that has been suggested previously to induce cardiac hypertrophy, as a novel transcriptional target of NFAT/ETS2 (Figure 7). In aggregate, these findings unveil a previously unrecognized molecular interaction between 2 canonical hypertrophic signaling pathways: MAPK-driven hypertrophy and calcineurin-driven hypertrophy.

ETS2

Several transcription factors, including GATA4, MEF2, NKX2.5, and NFAT, play critical roles in hypertrophic remodeling of the myocardium.[43] Responding to upstream signals, they regulate hypertrophic gene expression and induce cardiomyocyte growth. We identify the transcription factor ETS2 as a previously unrecognized target of Erk1/2 in the setting of hypertrophic stress. On activation by Erk1/2, ETS2 binds to the promoters of

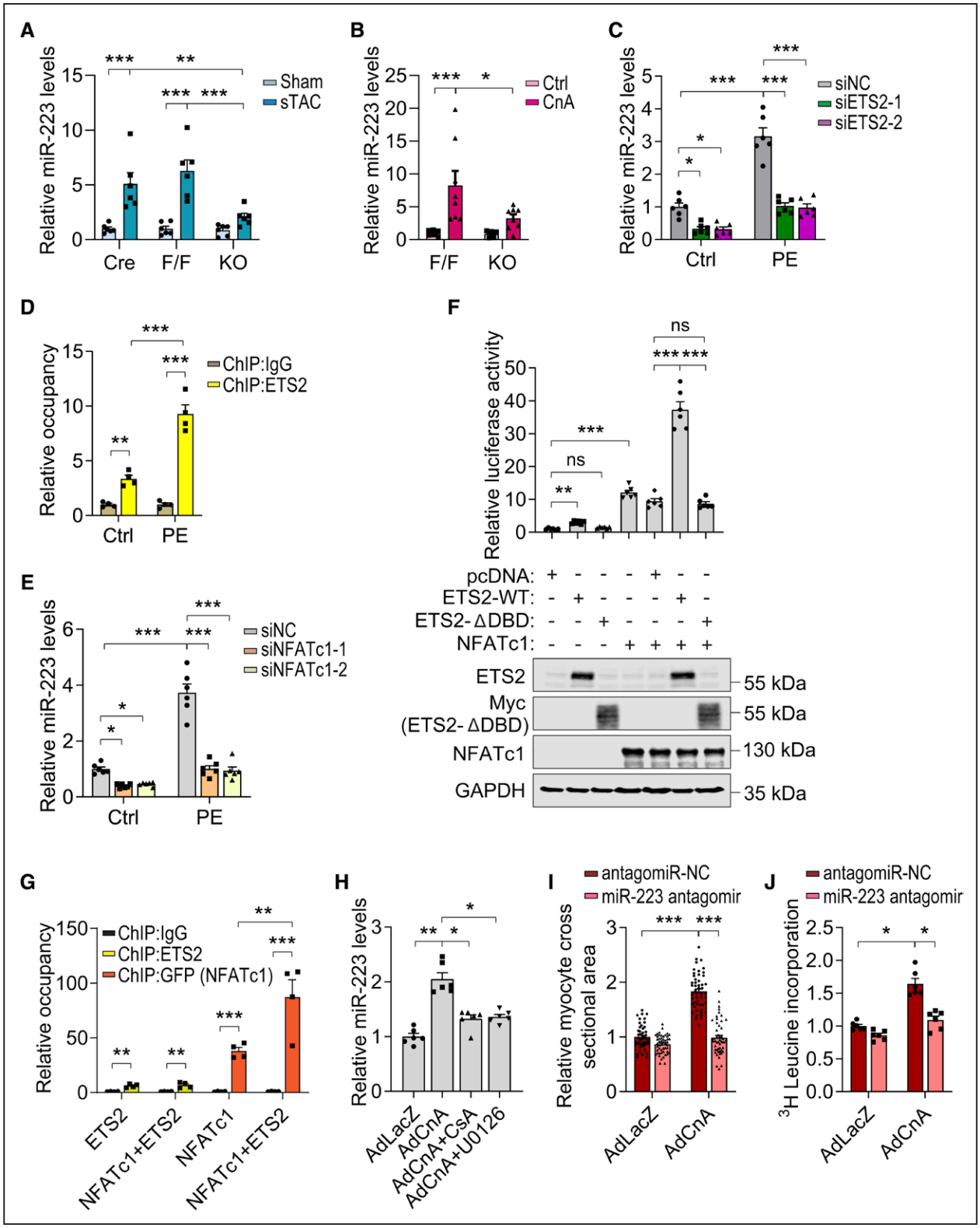

Figure 6. MicroRNA (MiR)–223 is required for calcineurin-NFAT (nuclear factor of activated T cells)/ETS2 (a member of the E26 transformation-specific sequence [ETS] domain family) pathway.

A, MiR-223 levels in the hearts of Cre, F/F, and knockout (KO) mice subjected to sham or severe transverse aortic constriction (sTAC) for 3 weeks (n=6). **B**, MiR-223 levels in the hearts of ETS2$^{F/F}$ and KO mice crossed with calcineurin transgenic mice (n=8). **C**, MiR-223 levels in neonatal rat ventricular myocytes (NRVMs) transfected with small interfering RNA (siRNA) control (siNC) or siRNA targeting ETS2 (siETS2) and then treated with phenylephrine (PE) for 24 hours (n=6). **D**, Chromatin immunoprecipitation (ChIP) analysis using an ETS2-specific antibody to detect ETS2 binding to miR-223 promoter in control NRVMs or NRVMs treated with PE for 6 hours (n=4). (*Continued*)

Figure 6 Continued. **E**, MiR-223 levels in NRVMs transfected with siNC or siRNA targeting NFATc1 (siNFATc1; n=6). **F**, Luciferase activity in HEK-293 cells transfected with a luciferase reporter plasmid harboring a full-length miR-223 promoter, along with expression plasmids of ETS2, ETS2 lacking DNA binding domain (ETS-ΔDBD), and NFATc1 (n=6). **G**, ChIP analysis using an ETS2-specific antibody or a GFP (green fluorescent protein) antibody to detect ETS2 or NFATc1 binding to the miR-223 promoter. HEK-293 cells were transfected with a miR-223 luciferase reporter plasmid, along with expression plasmids of ETS2 and NFATc1-GFP. A total of 48 hours after transfection, cells were collected for ChIP analysis (n=4). **H**, MiR-223 levels in indicated NRVMs. NRVMs were infected with an adenovirus expressing constitutively activated calcineurin (AdCnA) or a control adenovirus (AdLacZ). Before harvest, calcineurin-overexpressing NRVMs were treated with cyclosporin A (CsA) or U0126 for 6 hours (n=6). **I**, Quantification of the relative cardiomyocyte cross-sectional area. NRVMs were transfected with antagomir control (antagomir-NC) or miR-223 antagomir and then infected with AdCnA for 48 hours (n=50). **J**, Protein synthesis in NRVMs treated as in (**I**; n=6). **P*<0.05; ***P*<0.01; ****P*<0.001. Ctrl indicates control; IgG, immunoglobulin G; and ns, not significant.

hypertrophy-related genes, activating their transcription. Silencing of ETS2 in the heart attenuates pressure overload–induced hypertrophic growth and significantly improves cardiac function.

ETS2, a member of the ETS-domain family, is expressed in many tissues, including the heart, throughout embryonic and neonatal development, extending into adulthood.[44] ETS2 plays an important role in regulating cell growth, differentiation, cell death, and tissue development.[44] Similar to some other cardiac transcription factors, ETS2 is essential for proper myocardial development in *Ciona intestinalis* and chick embryos.[15,16] By contrast, ETS2 KO mice develop and grow normally with no detectable abnormalities in cardiac function and morphology under physiologic conditions, suggesting that ETS2 is not required for normal mouse postnatal cardiac development.

To test for a role of ETS2 in disease-related stress, we used a model of severe pressure overload (sTAC[20]). We chose this strategy, as opposed to standard transverse aortic constriction, because FVB mice tend to be less susceptible to afterload stress as compared with C57BL/6 mice. In these experiments, control mice developed cardiac hypertrophy by 3 weeks but not contractile dysfunction. ETS2 deficiency, however, significantly attenuated sTAC-induced cardiac remodeling; ETS2 KO mice manifested smaller hearts and cardiomyocytes, enhanced contractile function, and decreased expression of hypertrophic marker genes compared with control mice exposed to sTAC.

Erk1/2 in Cardiac Hypertrophy

The role of Erk1/2 in the development of cardiac hypertrophy is controversial. Whereas in vitro NRVM models point to a role for Erk1/2,[45] work in animals has yielded conflicting results.[46] Cardiomyocyte-restricted transgenic expression of MKP3 decreases Erk1/2 autophosphorylation with no observable effect on heart mass or cardiomyocyte cross-sectional areas after transverse aortic constriction, but decreased contractile function, suggesting a protective role for Erk1/2.[46] Subsequent work, however, suggested that a different autophosphorylation site on Erk1/2 contributed to hypertrophic signaling.[47] Whereas both studies examined gross changes in hypertrophy after transverse aortic constriction, neither examined the hypertrophic genes described here.

ETS2 is a known downstream target of Erk1/2.[26] Our data demonstrate that ETS2 is phosphorylated in the setting of hypertrophic stress and that this phosphorylation requires Erk1/2 activity. In vitro, we demonstrate a requirement of ETS2 phosphorylation by Erk1/2 for development of hypertrophy and activation of ETS2 downstream gene targets. In vivo, we show that ETS2 plays an important role in stimulating cardiac hypertrophy and in the downstream activation of these hypertrophic response genes. Given that Erk1/2 may be targeting many factors, some pro- and some antihypertrophic, conflicting findings reported regarding Erk1/2-induced hypertrophy may not be surprising. ETS2 targets ANP, BNP, and Rcan1.4, all known to inhibit the hypertrophic response, while activating miR-233, which induces the hypertrophic response.[48,49] In the case of ETS2, the overall effect is prohypertrophic. Clearly the Erk1/2 pathway and ETS2 seem to play especially prominent roles in phenylephrine-induced hypertrophy, but given the multiple stimuli activated in the heart exposed to pressure-overload stress, it is not surprising that the protection observed in ETS2 KO mice is less robust. Because ETS2 often works in concert with other transcription factors, it will be interesting to test whether specific interactions influence the spectrum of prohypertrophic versus protective downstream targets.

Crosstalk Between MAPK-Driven and Calcineurin-Driven Cardiac Hypertrophy

We show that ETS2 is also required for calcineurin-mediated cardiac hypertrophy. It is widely recognized that calcineurin/NFAT coupling contributes to pathologic cardiac hypertrophy and progression to heart failure.[7] Inhibition of the calcineurin/NFAT pathway has been shown to be an effective strategy for preventing hypertrophic growth.[49–52] The ETS family of transcription factors share an evolutionarily conserved ETS domain that binds to purine-rich DNA sequences with a central GGAA/T core consensus, which is similar to NFAT binding sites (GGAAA).[39,40] Two ETS family members, ETS1 and ELF1, exhibit binding sites adjacent to, or overlapping with, NFAT in several inducible lymphoid genes, such as interleukin-2, interleukin-3, and granulocyte-macrophage colony-stimulating factor.[53–57] Like NFAT, ETS2 transactivates Rcan1.4, and overlapping binding sites of ETS2 and

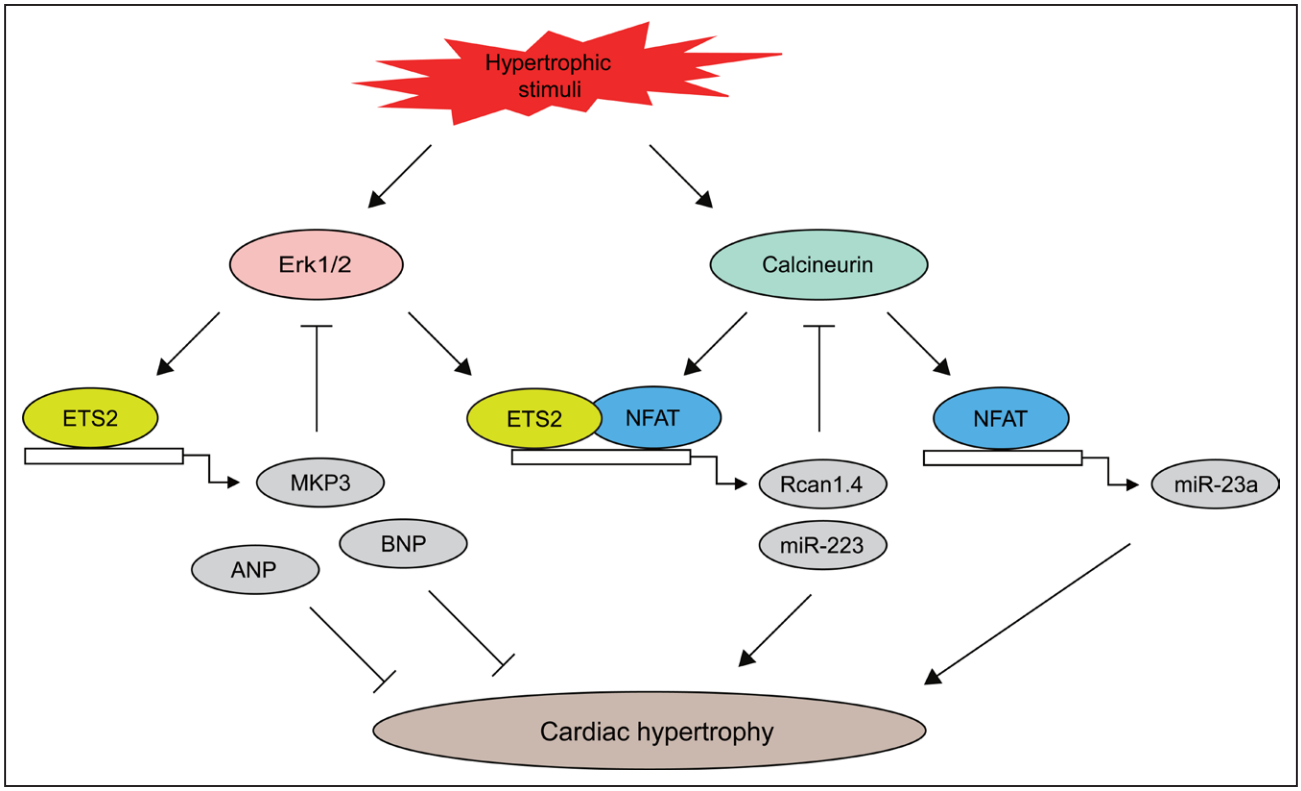

Figure 7. Schematic illustrating signaling pathways regulated by ETS2 (a member of the E26 transformation-specific sequence [ETS] domain family) in cardiac hypertrophy.

Hypertrophic stress activates calcineurin and Erk1/2 Erk (extracellular signal-regulated kinase), which, in turn, activate nuclear factor of activated T cells (NFAT) and ETS2, respectively. ETS2 not only binds to the promoters of Rcan1.4 and microRNA (MiR)–223 in cardiomyocytes, it also synergizes with NFAT to transactivate Rcan1.4 and miR-223, and thus promotes cardiac hypertrophy. On the other hand, ETS2 binds to the MKP3 promoter to upregulate MKP3 expression in response to hypertrophic stress, thereby inhibiting Erk1/2 signaling. ANP indicates atrial natriuretic peptide; BNP, brain natriuretic peptide; Erk, extracellular signal-regulated kinase; ETS2, a member of the E26 transformation-specific sequence (ETS) domain family; MKP3, MAPK phosphatase 3; and NFAT, nuclear factor of activated T cells.

NFAT are found within the Rcan1.4 promoter. Moreover, ETS2 is also required for calcineurin-induced cardiac hypertrophy. We report that deletion of ETS2 in the heart of calcineurin transgenic mice dramatically attenuated cardiac dysfunction and hypertrophic growth. Our findings further demonstrate that ETS proteins share targets with NFAT and in some cases they work cooperatively.

Because of weak DNA binding capability, NFAT often forms heterodimers with other transcription factors to direct gene transcription. We show that ETS2 binds directly to the Rcan1.4 promoter and cooperates with NFAT to transactivate Rcan1.4 expression. This effect is specific for only some NFAT target genes, as ETS2 had no effect on the induction of transcription by NFAT on miR-23a. Likewise, NFAT has been suggested to bind to GATA4 or AP-1 (activator protein-1) to activate the βMHC promoter[58] and ETS2 does not appear to bind to or activate the βMHC promoter. These data point to a subset of targets specific for ETS2/NFAT signaling.

ETS2 has been reported to bind to the miR-223 promoter to regulate its expression in myeloid cells.[41] In cardiomyocytes, miR-223 has been shown to be a positive regulator of cardiac hypertrophy.[42] miR-223 transgenic mice develop cardiac hypertrophy and heart failure spontaneously, whereas cardiomyocyte silencing of miR-223 protects against pathologic hypertrophic growth.[42] Our report shows that miR-223 is a downstream target of ETS2/NFAT in cardiomyocytes. Knockdown of NFAT in NRVMs significantly decreased miR-223 levels. There are several NFAT binding sites within the miR-223 promoter, and NFAT binds to the miR-223 promoter. We show that ETS2 synergizes with NFAT to transactivate miR-223 expression. We further show that calcineurin induces the transcriptional activation of miR-223, and miR-223 is essential for calcineurin-induced cardiac hypertrophy. In aggregate, these findings point to miR-223 as a prohypertrophic target of the Erk1/2-ETS2/calcineurin/NFAT pathway.

Negative Feedback Signaling

Our data also uncovered a negative feedback loop involving ETS2. ETS2 overexpression prevented phenylephrine-induced hypertrophic growth in NRVMs, an event stemming from ETS2-dependent inhibition of Erk1/2 signaling, which is critical for cell growth. We go on to demonstrate that ETS2 upregulates MKP3 to inhibit Erk1/2 phosphorylation in NRVMs. MKP3 is

a MAPK phosphatase, a molecule that functions as a negative regulator of MAPK signaling. MKP3 specifically binds to Erk1/2 to inhibit Erk1/2 phosphorylation.[59] Meanwhile, Erk1/2 directly phosphorylates ETS2 to promote ETS2 binding to the MKP3 promoter in 3T3 fibroblasts and cancer cells.[37,38,60] Our study reveals that this feedback loop involving Erk1/2, ETS2, and MKP3 also exists in cardiomyocytes and helps to explain discrepancies previously reported in the literature. ETS2 promotes phenylephrine-induced cardiac hypertrophy in MKP3-silenced NRVMs. Thus, ETS2 has dual roles in cardiac hypertrophy. On one hand, ETS2 synergizes with NFAT to activate miR-223 to induce hypertrophy. On the other hand, it inhibits Erk1/2 signaling to suppress hypertrophic remodeling. Therefore, our data suggest that under physiologic conditions ETS2 is pro-hypertrophic, but when overexpressed it can tip the balance to suppress hypertrophy.

Functional crosstalk between calcineurin/NFAT and MEK-Erk1/2 signaling is widely recognized. Calcineurin is reported to activate Erk1/2 in the heart.[61] Earlier work suggested that MEK-Erk1/2 signaling is essential for calcineurin/NFAT-induced hypertrophy.[62] Inhibiting Erk1/2 by overexpressing MKP3 led to a significant decrease in NFAT DNA binding activity.[62] Our data revealing that ETS2 stimulates NFAT binding to the Rcan1.4 promoter provides a molecular explanation for how Erk1/2 modulates calcineurin/NFAT activity.

Summary and Perspective

Our findings demonstrate that ETS2 is required for hypertrophic remodeling of the myocardium triggered by pressure overload, Erk1/2, and calcineurin. Our data support a model in which ETS2 is activated by Erk1/2 and interacts with and recruits NFAT to specific gene promoters, including those of Rcan1.4 and miR-223. Our findings uncover miR-223 as a novel target of the calcineurin/NFAT/ETS2 pathway and provide new evidence for crosstalk between calcineurin/NFAT and Erk1/2 signaling. Together, these findings reveal a previously unrecognized molecular interaction between 2 canonical hypertrophic signaling pathways: MAPK-driven hypertrophy and calcineurin-driven hypertrophy.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Materials

Data Supplement Figures I–VII
Data Supplement Tables I–IV
Data Supplement Spreadsheets I and II

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