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Transcriptional Regulation During Development of the Ductus Arteriosus

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Abstract**—**The ductus arteriosus is a specialized blood vessel containing highly differentiated and contractile vascular smooth muscle, derived largely from neural crest cells, that is essential for fetal life but typically closes after birth. Impaired development of the ductus arteriosus or disruption of signaling pathways that initiate postnatal closure can result in persistent patency of the ductus arteriosus, the third most common congenital heart defect. We found that Tfap2β, a transcription factor associated with patent ductus arteriosus in humans, was uniquely expressed in mouse ductal smooth muscle. *Endothelin-1* and the hypoxia-induced transcription factor, *Hif2α* were also highly enriched in ductal smooth muscle at embryonic day 13.5 and were dependent on Tfap2β for their expression in this domain. Hif2α functioned as a negative regulator of Tfap2β-induced transcription by disrupting protein–DNA interactions, suggesting a negative feedback loop regulating Tfap2β activity. Our data indicate that Tfap2β, Et-1, and Hif2α act in a transcriptional network during ductal smooth muscle development and that disruption of this pathway may contribute to patent ductus arteriosus by affecting the development of smooth muscle within the ductus arteriosus. **(*Circ Res*. 2008;103:388-395.)**

Key Words: ductus arteriosus ■ transcriptional regulation ■ endothelin-1 ■ Tfap2β ■ hypoxia-inducible factor 1

Neural crest cells contribute to many embryonic structures and are among the first cells to differentiate into smooth muscle of the bilaterally symmetrical aortic arch arteries during cardiovascular development.[1] On extensive remodeling and contributions from progenitors of the second heart field,[2] the left sixth aortic arch artery contributes to a specialized vessel known as the ductus arteriosus (DA). The DA contains 1 of the most highly differentiated and contractile vascular smooth muscles.[3] Mammalian fetal circulation relies on this specialized vessel for blood to bypass the uninflated lung and enter the systemic circulation where oxygenation ultimately occurs in the placenta (Figure 1). At birth, closure of the DA separates the pulmonary and systemic circulations so that fully oxygenated blood is delivered to all of the organs after gas exchange in the lungs. Closure involves oxygen sensing by the specialized smooth muscle cells (SMCs) of the DA and a response to decreased circulating levels of the vasodilating hormone prostaglandin E_2. Failure of this process results in patent ductus arteriosus (PDA), the third most common form of congenital heart disease in humans.[4]

Although physiology of the DA has been well described, little is known about the transcriptional pathways that control development of its unique smooth muscle. In humans, PDA is associated with DNA-binding mutations in the gene encoding the neural crest-enriched transcription factor TFAP2β, however the mechanism for the ductal abnormality is unknown.[5,6] Although *TFAP2β* mutations are likely a rare cause of isolated PDAs, genetic causes of syndromic disease often provide unique insight into mechanisms of common disease. In mice, targeted deletion of *Tfap2β* causes apoptosis of renal epithelial cells and postnatal lethality, possibly attributable to polycystic kidney disease.[7] However, the effects on the DA have not been described.

Endothelin-1 (Et-1) may also play a role in the development of ductal smooth muscle (DSM). Et-1 is a 21 amino acid signaling peptide generally expressed in and secreted from vascular endothelial cells. Cleavage of the proform of Et-1 by the endothelin converting enzyme allows it to bind to its receptor, Et_A, expressed in adjacent vascular SMCs.[8] Et_A signaling is required for development of neural crest-derived structures, as targeted deletion of any of the components of the Et_A signaling pathway results in craniofacial, outflow tract, and aortic arch abnormalities.[9–11] Et_A signaling is important for differentiation of the neural crest-derived smooth muscle in the aortic arch and pharyngeal arches.[12,13]

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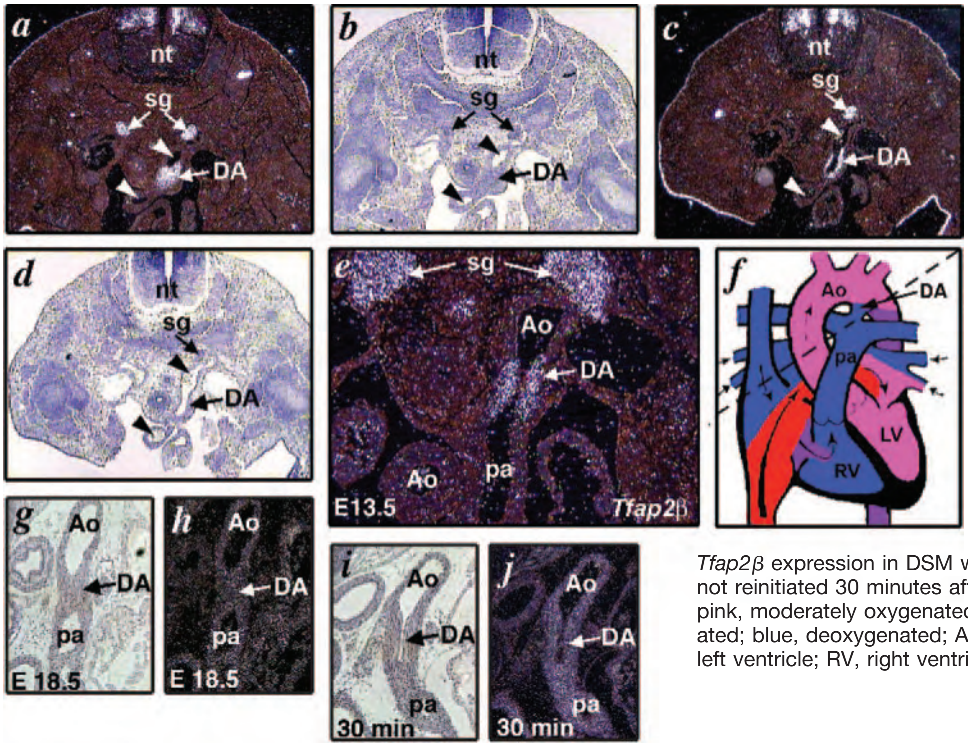

Figure 1. *Tfap2β* in DSM during development. Radioactive section in situ hybridization using a *Tfap2β* mRNA probe showed vascular expression specifically within the DA (arrows) at E13.5. a, c, and e, Multiple levels through the DA hybridized to a *Tfap2β* mRNA probe demonstrated that vascular expression of *Tfap2β* was restricted to the DA. Expression of *Tfap2β* was observed in both the sympathetic ganglia (sg) and portions of the neural tube (nt) as previously described. b and d, Corresponding light microcopy images. f, Schematic of fetal heart with dashed line indicating section through DA and arrows showing direction of blood flow. g through j, *Tfap2β* expression in DSM was diminished by E18.5 and was not reinitiated 30 minutes after birth. Red indicates oxygenated; pink, moderately oxygenated; purple, moderately deoxygenated; blue, deoxygenated; Ao, aorta; pa, pulmonary artery; LV, left ventricle; RV, right ventricle.

Many studies also support a role for endothelin signaling in oxygen-induced constriction of the DA.[14–16] For example, in lambs, Et-1 release from ductal SMCs during the perinatal period is associated with constriction of the vessel at birth,[17] although its role in humans remains controversial.

Hypoxia-inducible factors (Hifs), which are basic helix–loop–helix (bHLH)/PAS domain-containing transcription factors, are stabilized during hypoxia and imported to the nucleus, where they become activated, heterodimerize with Arnt, another bHLH/PAS domain containing protein, and bind DNA to regulate the transcription of target genes.[18] In addition to regulating oxygen-sensitive cellular events, Hifs are required for closure of the ductus venosus, a hepatic fetal vessel, at birth.[19] However, the potential function of Hifs in the DA has not been explored.

We found that *Tfap2β* was enriched in DSM of the fetal mouse. *Hif2α* and *Et-1* were similarly enriched in embryonic DSM and relied on Tfap2β for transcriptional activation in this domain. Hif2α functioned in a negative feedback loop regulating Tfap2β activity consistent with the dose-sensitivity of TFAP2β in humans. The findings here suggest that a regulatory cascade involving Tfap2β, Hif2α, and Et-1 is involved in the specialized development of DSM cells and, consequently, regulation of fetal circulation in mammals.

Materials and Methods

Immunochemistry

Immunohistochemistry was performed following antigen retrieval in citrate solution (Biogenex) with Tfap2β antibody (Santa Cruz, 1:100 in PBS), biotinylated α-rabbit IgG (1:200 in PBS), streptavidin–HRP and diaminobenzidine chromagen (Vector Labs). Sections were counterstained with Mayer's hematoxylin.

Luciferase Reporter Assays

Luciferase assays were performed in human umbilical vein endothelial cells (HUVECs) or A10 cells (ATCC) transfected with indicated plasmids and *LacZ* expression vector using Fugene6 (Roche). The total amount of transfected DNA was held constant using empty vector. The reporter construct (kindly provided by N. Bishopric) consisted of a 669 bp *Et-1* promoter cloned upstream of luciferase. Assays were performed using the Luciferase Assay System (Promega). Results were normalized to β-gal activity detected with an *o*-nitrophenyl-galactopyranoside assay protocol.[20] Each transfection was performed a minimum of three times, and results are shown with standard deviations.

Embryo Harvesting and Histology

The care and use of animals were in accordance with institutional guidelines. *Tfap2β*$^{+/-}$ mice were generated by M. Moser, Max-Planck Institute of Biochemistry, Martinsreid, Germany. Mice of the appropriate genotype were intercrossed, and embryos or pups were collected at the indicated time and fixed in 4% paraformaldehyde overnight at 4°C. Genotype was determined by PCR.[7,10,21] Specimens were paraffin embedded and sectioned transversely. Surrounding landmarks were used to confirm comparable section angles. *Hif2α*$^{+/LacZ}$[21] embryos were harvested, fixed, and stained for β-gal activity.[22]

Radioactive In Situ Hybridization

^{35}S-Labeled antisense probes were synthesized from partial cDNAs of *Hif2α*, *Tfap2β*, *Et-1*, *Et*$_A$, or calponin. cDNAs were linearized and transcribed with the following restriction enzymes and RNA polymerases: *Hif2α*, *Bam*HI, SP6; *Tfap2β*, *Xba*I, T7; *Et-1*, *Eco*RV; SP6; *Et*$_A$, Pst1; SP6; calponin.[23] Radioactive section in situ hybridization was performed on paraffin mouse sections as described.[24]

Transfections, Chloramphenicol Acetyltransferase Assays, and Western Blot Analysis

Transfections and CAT assays were performed as described.[5] Each transfection was performed a minimum of three times, and results are shown with standard deviations. Tfap2β protein levels were measured by western analysis of cell lysates using a Tfap2β antibody, HRP-conjugated donkey anti-rabbit IgG, and Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

Electrophoretic Mobility-Shift Assays

Oligonucleotides were synthesized (Integrated DNA Technologies) as follows: control, 5′-GGGATCGAACTGACCGCCCGCGGC

CCGT-3′ and 5′-GGGACGGGCCGCGGGCGGTCAGTTCGATC-3′; mutant, 5′-GGGATTGTCAGACGTCTGTCGTCTGC-3′ and 5′-GGGCAGACGACAGACGTCTGACAAT-3′.

Oligonucleotides were annealed, radiolabeled with [α-^{32}P]dCTP using Klenow DNA polymerase, and purified on Sephadex G-25 spin columns (Roche). Proteins were produced using a TNT T7–coupled reticulocyte lysate system (Promega). DNA-binding assays were performed in gel shift binding buffer (Promega) in a total volume of 20 μL using the indicated volumes of each protein and 2 μL of labeled oligonucleotide at 50 000 cpm. The amount of reticulocyte lysate in each condition was constant. DNA–protein complexes were resolved on a 6% nondenaturing polyacrylamide gel, exposed to a Phosphor screen, and read in a PhosphorImager (Molecular Dynamics).

Hif2α Truncations

All truncations of Hif2α were generated by PCR and sequence-verified. Primer sequences are available on request.

Results

Tfap2β Is Expressed in DSM

We assayed *Tfap2β* mRNA expression in the embryonic mouse DA. At embryonic day (E)13.5, when remodeling of the outflow tract has occurred separating the pulmonary artery from the aorta, *Tfap2β* mRNA was strongly expressed in the DSM with sharp borders at the aortic and pulmonary artery ends (Figure 1). *Tfap2β* expression was extinguished in mouse DSM by E18.5 and was not reinitiated after birth (Figure 1g through 1j) but was maintained in other neural crest derivatives (data not shown). We also assayed Tfap2β protein levels in mouse DSM by immunohistochemistry and found that protein levels correlated well with mRNA levels (Figure I in the online data supplement, available at http://circres.ahajournals.org).

Tfap2β Affects DSM Development

Mice lacking Tfap2β die shortly after birth and have delayed closure of the DA (B. Gelb, unpublished observation). Based on the early and transient expression of *Tfap2β*, we hypothesized that loss of Tfap2β may result in a defect of embryonic DSM development. Histological analysis of hematoxylin/eosin-stained sections of wild-type and *Tfap2β*$^{-/-}$ embryos harvested at E13.5, E15.5, or E18.5 (Figure 2) revealed no difference in the morphology of DSM cells or in vascular wall thickness, nor did we observe differences in DA elastin deposition (supplemental Figure II). To distinguish developing DSM, we examined expression of calponin, a marker of highly differentiated, contractile SMCs (Figure 2). In both wild-type and *Tfap2β*$^{-/-}$ embryos from E13.5 through E15.5, radioactive in situ hybridization revealed higher levels of calponin mRNA expression in DSM than in the aorta or pulmonary artery (Figure 2g, 2h, 2k, and 2l). Preferential expression of calponin persisted at E18.5 in wild-type DSM (Figure 2o) but was lost in *Tfap2β*$^{-/-}$ embryos, in which the level of calponin expression was similar in DSM and adjacent smooth muscle (Figure 2p). These results indicate that, in the wild-type mouse, DSM likely matures earlier than aortic or pulmonary artery smooth muscle, consistent with previous reports on the developing human DA,[3] and suggest that *Tfap2β* may be necessary to maintain the highly differentiated state of DSM, although quantification of this difference is difficult.

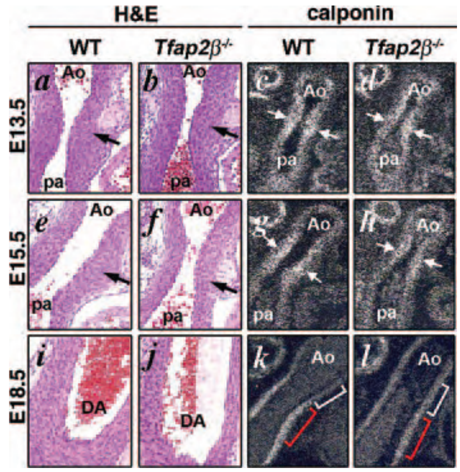

Figure 2. Histological analysis of wild-type and *Tfap2β*$^{-/-}$ DA. Comparison of hematoxylin/eosin-stained sections from E13.5 (top row), E15.5 (second row), or E18.5 (third row) wild-type (a, e, and i) or *Tfap2β*$^{-/-}$ (b, f, and j) embryos showed no difference in smooth muscle cell morphology or vessel wall thickness. Radioactive in situ hybridization using a calponin mRNA probe consistently revealed higher levels of calponin expression in the DSM compared to the aortic smooth muscle in both wild-type (c and g) and *Tfap2β*$^{-/-}$ (d and h) embryos harvested at E13.5 or E15.5. By E18.5, calponin expression in the *Tfap2β*$^{-/-}$ (l) DSM (red bracket) was equal to expression in the adjacent aorta (white bracket), whereas the wild type (k) continued to show higher calponin expression in the DSM compared to the aortic smooth muscle.

Et-1 and *Et*$_A$ Are Expressed in DSM

To understand the mechanism underlying the DA closure defect in *Tfap2β* mutants, we examined other genes involved in ductal development. Because endothelin signaling is important for development and closure of the DA, we examined mRNA expression of *Et*$_A$ and its major ligand, *Et-1*, in the aortic arch of E13.5 mouse embryos. *Et*$_A$ was expressed in SMCs throughout the great vessels but did not uniquely mark DSM cells (Figure 3a). *Et-1* was expressed in endothelial cells throughout the developing vasculature, as expected. Surprisingly, *Et-1* mRNA was also specifically expressed in DSM with distinct borders at the aortic and pulmonary artery junctions at E13.5 (Figure 3b).

To determine whether mouse DSM expresses *Et-1* perinatally, we examined *Et-1* expression in the DA of E18.5 mouse embryos and in mice harvested at 15 or 30 minutes after birth (Figure 3c through 3e). By E18.5, the DSM expression of *Et-1* was indistinguishable from background and was not reinitiated after birth, although endothelial expression remained. The DSM expression of *Et-1* during development likely results in especially high levels of endothelin signaling in the DA that may distinguish the DA from other vessels and may contribute to the unique differentiation of DSM and the oxygen-sensitivity of DSM later at the time of parturition.

Hif2α, a Potential Transcriptional Regulator of Et-1, Is Specifically Expressed in DSM During Development

Because *Et-1* expression is, in part, regulated by an upstream Hif response element,[25] we examined the expression of the genes encoding Hif1α, -2α, and -3α. At E13.5, *Hif1α* and

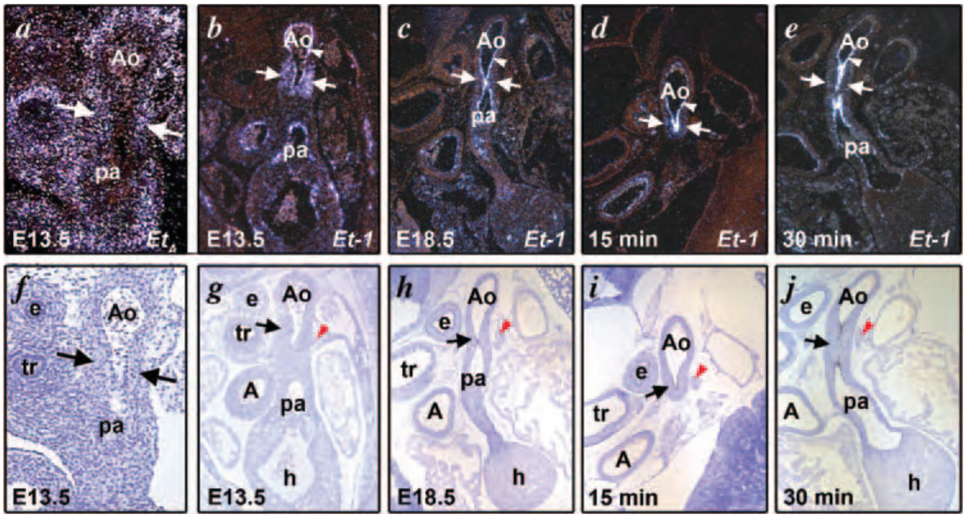

Figure 3. Et_A and *Et-1* expression in DSM during development. a, Radioactive section in situ hybridization using an Et_A mRNA probe showed uniform expression of Et_A in the smooth muscle throughout the aorta (Ao), pulmonary artery (pa), and DA (arrows). b, Radioactive section in situ using an *Et-1* mRNA probe revealed enriched DSM expression at E13.5. *Et-1* expression was absent in the DSM of animals harvested at E18.5 (c) and 15 (d) or 30 minutes (e) after birth, whereas endothelial expression (arrowheads) persisted. Corresponding bright field images (f through j) highlight anatomic landmarks used to locate the ductus arteriosus including the adjacent nerve bundle (red arrowhead). h indicates heart; A, ascending aorta; tr, trachea; e, esophagus.

Hif3α were ubiquitously expressed throughout the developing embryo (data not shown), whereas *Hif2α* was expressed primarily in vascular endothelial cells, as reported.[26] However, *Hif2α*, like *Et-1* and *Tfap2β*, was expressed specifically in the DSM (Figure 4a). Similar to *Et-1*, *Hif2α* expression declined in the DSM around birth but was maintained in the vascular endothelium (Figure 4b through 4d).

To visualize Hif2α expression at higher resolution, we obtained E13.5 $Hif2\alpha^{+/-}$ embryos whose targeted allele contains a *LacZ* cassette.[21] *LacZ* expression in the Hif2α domain, shown by β-galactosidase activity, was at highest levels in the DA (Figure 4e). Histological analysis of the outflow tract revealed that *LacZ* expression was restricted to vascular endothelial cells in most vessels but extended into the SMCs of the DA, consistent with the results of in situ hybridization for *Hif2α* mRNA (Figure 4f through 4h). *LacZ* expression was not observed in any other SMCs of the embryo at this stage.

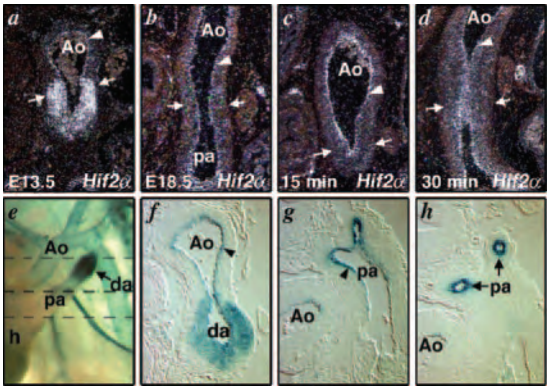

Figure 4. *Hif2α* expression in DSM during development. Radioactive section *in situ* using a *Hif2α* mRNA probe showed expression in DSM (arrows) and vascular endothelium (arrowheads) (*a-d*). LacZ-stained E14.5 $Hif2\alpha^{+/lacZ}$ embryo showed strong β-gal expression in the DA (e). Histological analysis of E14.5 LacZ-stained $Hif2\alpha^{+/lacZ}$ embryo along planes indicated by dashed lines confirmed strong DSM expression (f), whereas expression in the aorta (f), pulmonary trunk (g), and distal branches of the pulmonary artery (h) was confined to the vascular endothelium. Ao indicates aorta; pa, pulmonary artery; h, heart.

Tfap2β Is Required for DSM Expression of *Hif2α* and *Et-1*

Given the role of *Tfap2β* in DA development and coexpression with *Hif2α* and *Et-1*, we compared both *Hif2α* and *Et-1* expression in wild-type and $Tfap2\beta^{-/-}$ mouse embryos to determine whether lack of Tfap2β would affect their expression. At E13.5 in $Tfap2\beta^{-/-}$ mouse embryos, *Hif2α* and *Et-1* mRNA expression was lower in DSM of mutants compared to wild type (Figure 5). Maintenance of endothelial expression of both genes provided an internal control for signal intensity and surrounding landmarks demonstrate comparable histological levels and angles. Because calponin expression was maintained in the absence of Tfap2β, decreased *Hif2α* and *Et-1* expression was not attributable to a lack of DSM cells in the $Tfap2\beta^{-/-}$ embryos. This result demonstrated a dependence on *Tfap2β* specifically for DSM enhancement of *Hif2α* and *Et-1* expression.

Hif2α Regulates *Et-1* in Vascular SMCs

Because *Et-1* and *Hif2α* were specifically coexpressed in the DSM at E13.5, we investigated the possibility of an epistatic relationship between the 2 genes. An Hif response element (HRE) 118 bp upstream of the *Et-1* transcription start site is required for hypoxic induction of *Et-1* mRNA expression in cultured vascular endothelial cells.[27] Using this upstream region of the *Et-1* locus to drive expression of luciferase, we performed reporter assays to test whether Hif2α was able to activate transcription through this HRE in SMCs (Figure 6). All transfections were carried out with a mutant form of Hif2α that is not hydroxylated during normoxia, resulting in a stable form of the protein. Hif2α activated the reporter 6.5-fold in HUVECs, as expected. Hif2α also activated the reporter to a lesser extent in an A10 aortic SMC line and mutation of the HRE abolished this activation, indicating that Hif2α can act through the HRE to initiate transcription in cultured vascular SMCs (Figure 6). However, in vivo, we were able to detect some *Et-1* transcripts in DSM of *Hif2α* mutants before their death at E13.5, suggesting that other mechanisms may also regulate *Et-1* at this stage (data not shown).

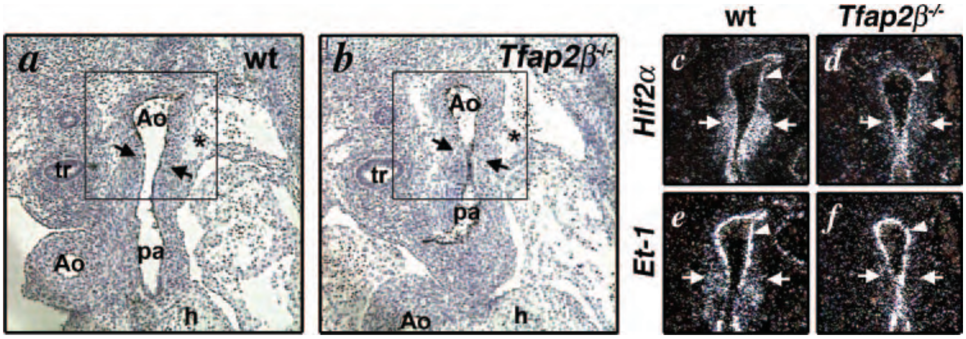

Figure 5. *Tfap2β* is required for DSM expression of *Hif2α* and *Et-1*. Bright field images of sections through the DA from E13.5 wild-type (a) and *Tfap2β*$^{-/-}$ (b) embryos. Note the comparable level and angle of section through the DA of the 2 embryos considering anatomic landmarks including the aorta (Ao), pulmonary artery (pa), trachea (tr), heart (h), and the nerve bundle adjacent to the DA (*). Radioactive in situ hybridization using *Hif2α* (c and d) or *Et-1* (e and f) mRNA probes on sections serial to those in a and b, focusing on the boxed regions to highlight the relevant anatomy. Expression of both genes was diminished in the DSM (arrows) of the *Tfap2β*$^{-/-}$ embryos but not in the endothelial cells (arrowheads).

Hif2α Blocks Transcriptional Activation by Tfap2β via the bHLH/PAS Domain

Because Hif2α and Tfap2β are both transcription factors enriched in DSM, we examined their potential synergistic or antagonistic interactions. Using a CAT reporter assay system, we found that Tfap2β could activate this reporter as described,[5] but cotransfection of Hif2α with Tfap2β blocked activation (Figure 7a).

Because Tfap2β and Hif2α both rely on the transcriptional coactivator p300, negative regulation of Tfap2β by Hif2α could be explained by Hif2α sequestering p300. To test this idea, excess p300 was cotransfected with Tfap2β, Hif2α, or both. Although overexpression of p300 was able to enhance the transactivation potential of Tfap2β, it could not rescue the negative regulation by Hif2α, indicating that sequestration of p300 was not the mechanism by which Hif2α negatively regulated Tfap2β activity. Because the decreased activity could also be caused by a decrease in Tfap2β expression, we assayed Tfap2β protein in the cell lysates used in the reporter assay. Tfap2β protein was actually increased on coexpression with Hif2α and p300, and yet, Tfap2β transcriptional activity declined (Figure 7a). Thus, Hif2α negatively regulated transactivation by Tfap2β independently of p300 sequestration or expression differences.

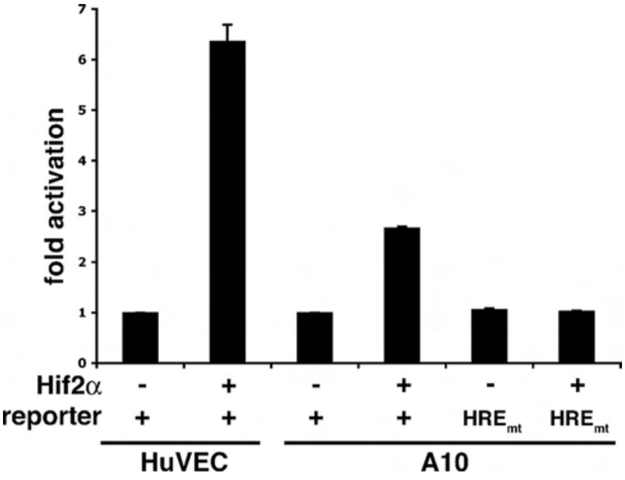

Figure 6. Hif2α activates an *Et-1* enhancer in cultured vascular smooth muscle cells. Luciferase assays using a luciferase reporter with an *Et-1* enhancer. Hif2α activated the reporter 6.5-fold in HUVECs and nearly 3-fold in A10 aortic smooth muscle cells. Hif2α did not activate the enhancer containing a mutated HRE (HRE$_{mt}$). Error bars indicate SD of results averaged from multiple experiments.

To determine which domains of Hif2α were responsible for its ability to block transcriptional activation by Tfap2β, we generated Hif2α truncations (Figure 7c) and tested them in the reporter assay described above (Figure 7b). The amino terminus of Hif2α contains a bHLH domain, important for DNA binding and dimerization, and 2 PAS domain repeats whose functions are unknown.[26] The carboxyl terminus contains transcriptional activation domains.[28] Tfap2β activity was blocked by 386 residues of the amino terminus containing the bHLH and PAS domains (ΔC386), but deletion of half of the second PAS domain (ΔC281) resulted in failure to block Tfap2β's activity suggesting that the PAS domains are required for this function. Deleting as few as 131 residues (ΔN131) from the amino terminus of Hif2α also resulted in the loss of its ability to block transcriptional activation by Tfap2β (Figure 7b and 7c). However, the PAS domains alone were not sufficient to elicit this effect, suggesting that the bHLH and PAS domains are required together. Western analysis of the cell lysates used to measure CAT protein levels revealed no significant difference in Tfap2β protein levels (data not shown), and immunocytochemical analysis showed that Hif2α mutants were appropriately localized to the nucleus (Figure 7d). Thus, changes in Tfap2β expression or Hif2α localization were not responsible for the observed differences. Although we cannot rule out problems with protein folding, these results indicated that the bHLH/PAS domains of Hif2α were both necessary and sufficient to disrupt Tfap2β-dependent reporter transactivation.

Hif2α Disrupts Tfap2β-DNA Interaction

The negative regulation of Tfap2β by Hif2α could occur through 1 of at least 2 mechanisms: Hif2α could form a complex on DNA with Tfap2β, thereby prohibiting transactivation by Tfap2β; alternatively, Hif2α could disrupt DNA binding by Tfap2β altogether, thus preventing transactivation. To distinguish between these 2 possibilities, we performed electrophoretic mobility-shift assays with Tfap2β or Hif2α protein and an oligonucleotide containing a Tfap2 consensus binding site. Tfap2β specifically retarded oligonu-

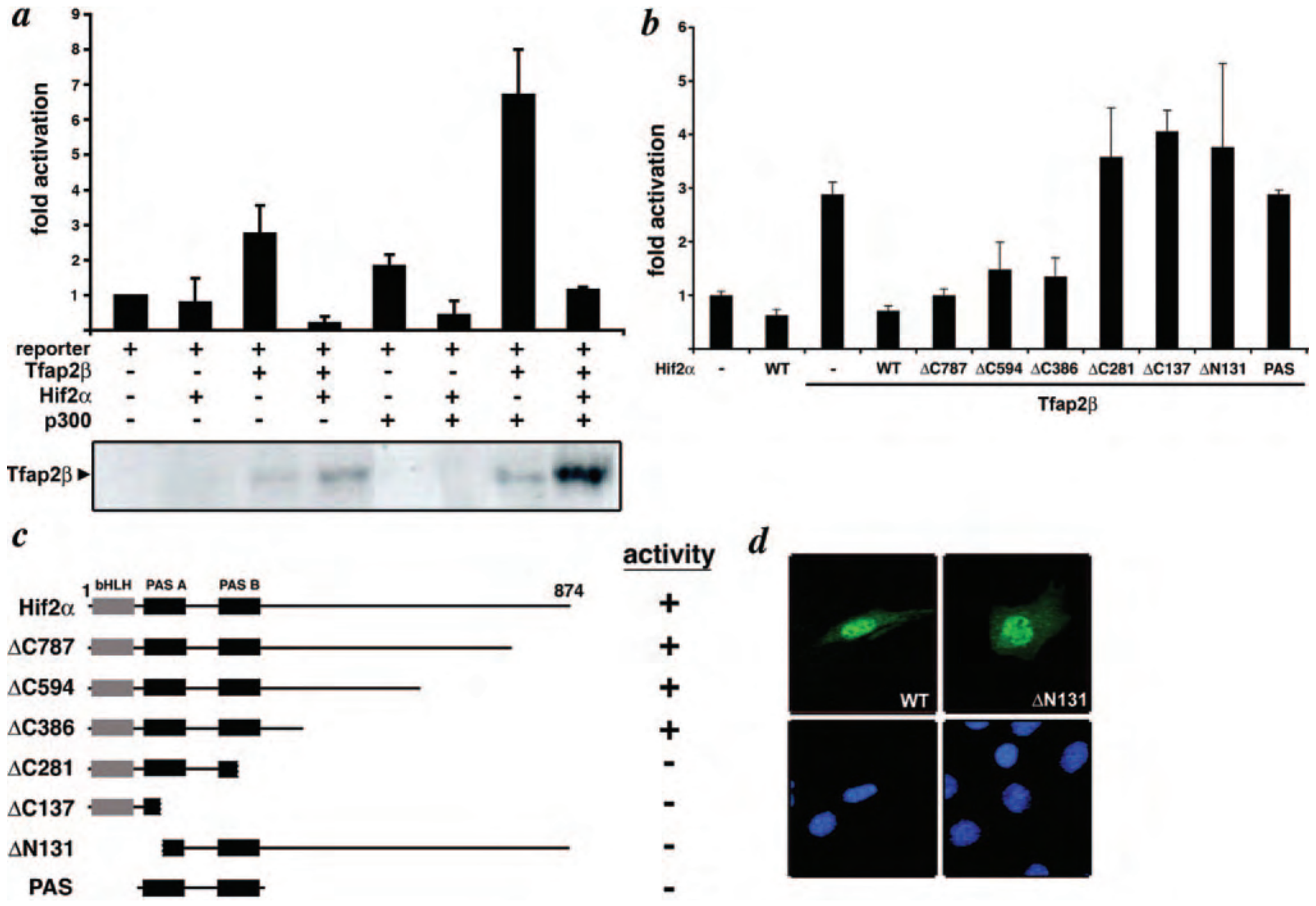

Figure 7. Hif2α negatively regulates Tfap2β via the bHLH/PAS domain. a and b, ELISAs measuring CAT levels in cell lysates. NIH-3T3 cells were transfected with a CAT reporter containing 3 copies of a Tfap2 binding element. Error bars indicate SD of results averaged from several experiments. a, Tfap2β, Hif2α, or p300 were cotransfected as indicated. Tfap2β activated the reporter and Hif2α decreased this effect. Cotransfection of p300 did not rescue the decrease. Western blot analysis of Tfap2β in each sample is shown. b, Tfap2β, Hif2α, or truncations of Hif2α were cotransfected as indicated. c, Schematic showing truncations of Hif2α used in reporter assays and summarized activation data. d, Immunocytochemistry detecting overexpressed wild-type Hif2α or mutant (represented by ΔN131) in NIH-3T3 cells, demonstrating proper localization to the nucleus (green). Corresponding DAPI staining (blue).

cleotide migration, but addition of Hif2α caused a dose-dependent decrease in the amount of DNA bound to Tfap2β (Figure 8). We did not observe a supershift of the Tfap2β-DNA complex arguing against a Tfap2β-Hif2α-DNA complex, repressing Tfap2β. Thus, negative regulation of Tfap2β by Hif2α was likely attributable to a decrease of site-specific DNA binding by Tfap2β in the presence of Hif2α. Because Tfap2β is required for DSM expression of Hif2α, the negative feedback regulation of Tfap2β by Hif2α may allow finer control of Tfap2β-dependent gene expression during development.

Discussion

This study shows that *Tfap2β*, *Hif2α*, and *Et-1* are coexpressed in mouse DSM and that Tfap2β is necessary for proper DSM expression of both *Hif2α* and *Et-1*. Although DSM expression of *Tfap2β* occurs well before birth, we show that it is essential for the gene regulation associated with smooth muscle differentiation, suggesting a role for *Tfap2β* in development or maturation of DSM. Also, we found that Hif2α positively regulated *Et-1* in cultured vascular SMCs but negatively regulated Tfap2β activity through inhibition of sequence-specific DNA binding by Tfap2β. These data suggest a negative feedback loop through which Hif2α may titrate expression of Tfap2β target genes during DSM development.

Tfap2β and PDA

In humans, heterozygous mutations resulting in single amino acid substitutions within the DNA-binding or transactivation domains of TFAP2β are associated with Char syndrome and result in facial dysmorphism, PDA, and hand anomalies.[5,6] These mutant forms of TFAP2β dimerize normally with other Tfap2 proteins causing dominant-negative effects. In contrast, targeted deletion of *Tfap2β* in mice results in a null mutation. Although the expression of other Tfap2 proteins in DSM and other tissues of *Tfap2β*$^{-/-}$ embryos has not been examined, compensation by the remaining Tfap2 family members may account for the reduced severity of Char syndrome characteristics in *Tfap2β*$^{-/-}$ pups, such as facial dysmorphism, which has not been noted, and hand anomalies, which occur with incomplete penetrance.[29]

Interestingly, targeted deletion of smooth muscle myosin in mice, which prevents general smooth muscle contraction, results in postponement but not failure of DA closure,[30] whereas targeted deletion of several genes encoding elements

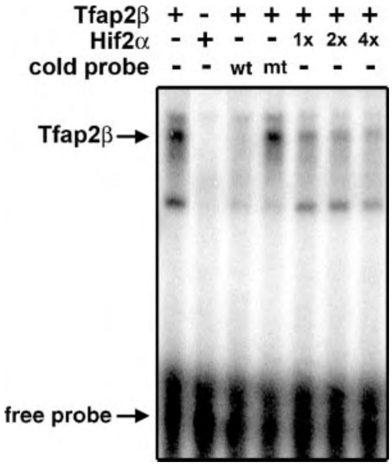

Figure 8. Hif2α blocks DNA binding by Tfap2β. Electrophoretic mobility-shift assays were performed using in vitro transcribed and translated Tfap2β or Hif2α protein and a ^{32}P-labeled oligonucleotide containing a Tfap2-binding site; 100× cold wild-type oligo (wt) competed for Tfap2β binding, whereas 100× cold mutant oligo (mt) failed to compete, demonstrating sequence-specific binding to the probe by Tfap2β. Hif2α failed to shift the probe but reduced the amount of oligo shifted in the presence of Tfap2β in a dose-dependent manner.

of the prostaglandin pathway, including the receptor, EP_4, and the cyclooxygenases, Cox-1 and Cox-2, results in PDA.[31–34] The relatively early expression of Tfap2β in DSM led us to explore its role in DSM development, leaving the relationship of the Tfap2β/Hif2α/Et-1 developmental axis to genes encoding proteins important for oxygen sensing or DA constriction unknown. However, our data support the idea that earlier transcriptional events governed by Tfap2β activity may be important to prepare the DSM to respond to peripartum signals. For example, thickening of the subendothelial layer of the DA occurs during late gestation and may be a prostaglandin-dependent event stimulating migration of DSM cells, which is required for timely closure of the DA at birth.[35–37]

Role of *Et-1* and *Hif2α* in DSM Differentiation

Classical endothelin signaling occurs in a paracrine fashion: endothelial cells release endothelin ligands and bind their cognate receptors on the surface of neighboring SMCs, exerting a variety of effects, including proliferation. However, autocrine endothelin signaling can be stimulated in vitro and is uniquely associated with differentiation of vascular SMCs, rather than proliferation.[38] We have shown that DSM activates a unique transcriptional program under the influence of Tfap2β, resulting in *Et-1* expression. Given the role of Et-1 in smooth muscle differentiation, and its importance for delineation of neural crest-derived structures in general, it is possible that the DA defect in $Tfap2\beta^{-/-}$ mice is attributable, in part, to the downregulation of *Et-1* in the DSM of these animals. Consistent with this, we have recently identified a heterozygous nonsense mutation in the gene encoding the endothelin receptor A in a patient with PDA (V. Garg and D. Srivastava, unpublished observation, 2003). This mutation introduced a premature stop codon (S382X), not found in 300 control chromosomes, truncating the receptor and eliminating its cytoplasmic tail. A similar truncation renders the receptor nonfunctional in in vitro studies,[39] but conclusive evidence of a role for Et-1 signaling in human PDA will require further genetic analyses.

Et-1 is expressed in the DSM of near-term fetal lambs, and its release depends on oxygen availability.[17] In mice, we found that *Et-1* is not expressed perinatally within DSM, although it was highly expressed in endothelial cells throughout the great vessels. This may indicate a species-specific difference in the dependence on endothelin signaling during DA closure. However, both species may require Et-1 expression in DSM during development, not to induce constriction, but to pattern the neural crest-derived DSM in preparation for its physiological changes at birth.

Although a relationship between Hif2α and Et-1 is well established, our data suggest that the role of Hif2α in the DA includes refining the transactivation potential of Tfap2β by negatively regulating Tfap2β-DNA binding. To date, neither Hif2α nor Tfap2β are known to directly control transcription of genes governing smooth muscle differentiation and determining the in vivo role of Hif2α in the DA beyond midgestation will await the development of the tissue-specific deletion of *Hif2α* in DSM.

Clinical Implications

Closure of the DA is vital for healthy extrauterine life. However, in the case of particular congenital heart defects that obstruct or disrupt blood flow to the lungs or body, DA patency is essential for systemic and pulmonary blood flow. Understanding the transcriptional regulation of normal DA development, maturation, and closure by factors such as Tfap2β, Et-1, and Hif2α, may provide additional targets for rational drug design to either close or open the DA, particularly in premature infants.

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Disclosures

None.

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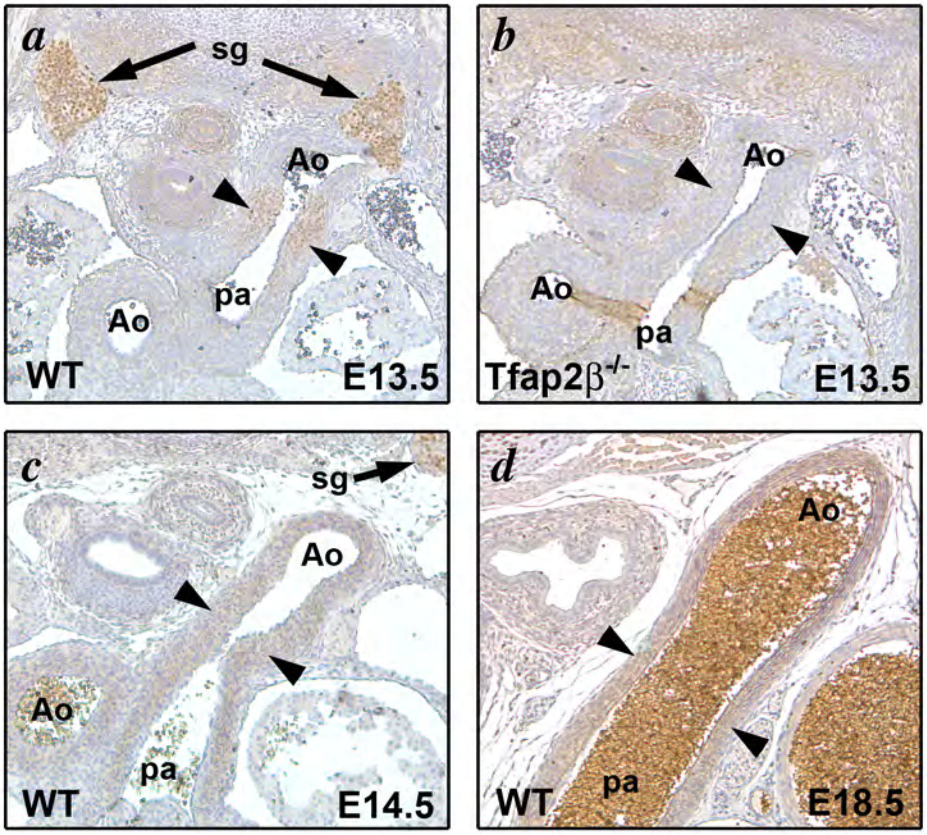

Supplemental Figure 1
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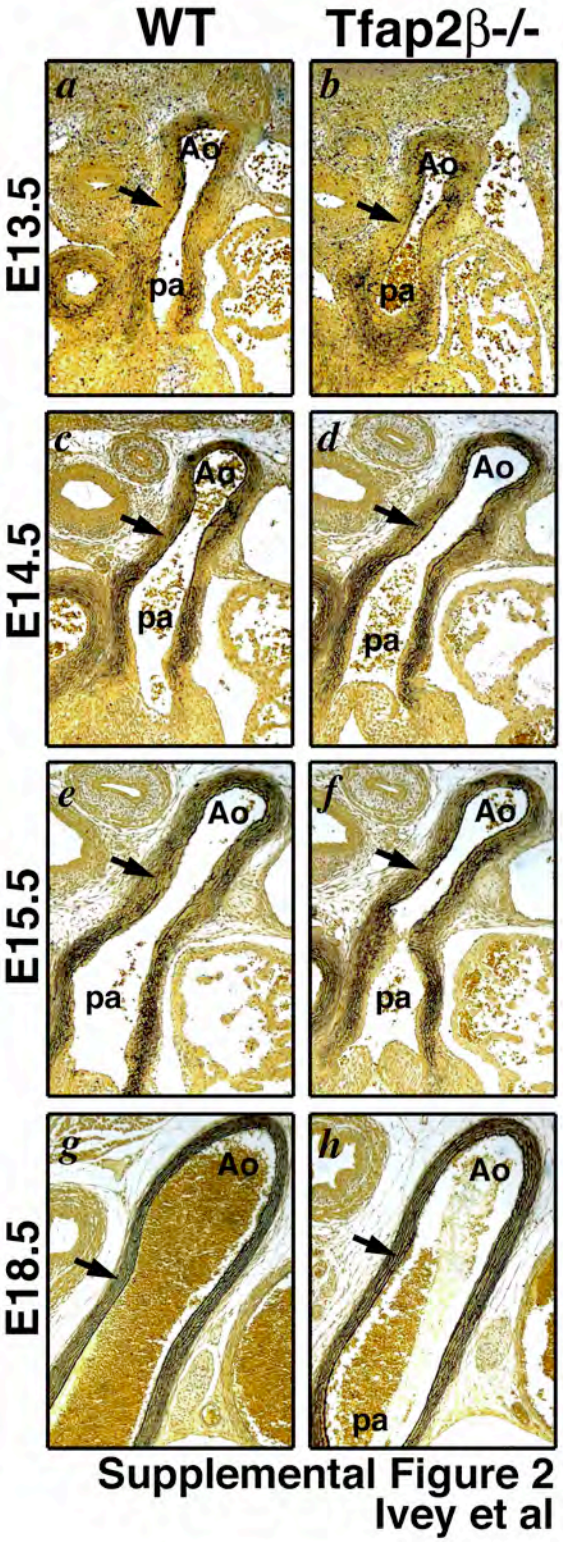
Supplemental Figure 1. Tfap2β protein is expressed in DSM during mid-gestation. Immunohistochemistry using a Tfap2β antibody was performed on sections through the DA of (*a*) E13.5 wild-type (WT), (*b*) E13.5 Tfap2β$^{-/-}$,(*c*) E14.5 wild-type, or (*d*) E18.5 wild-type mouse embryos. Positive immunostaining was detected specifically in the DA (arrowheads) and sympathetic ganglia (sg) of the wild-type at E13.5 (*a*). DA expression of Tfap2β protein was diminished at E14.5 (*c*) and E18.5 (*d*), although expression in other structures, such as the sympathetic ganglia was still maintained. Ao, aorta; pa, pulmonary artery.



Supplemental Figure 2. Elastin deposition is comparable in the DA of wild-type and Tfap2$\beta^{-/-}$ embryos. Elastin staining on sections through the ductus arteriosus of wild-type (*a,c,e,g*) or Tfap2$\beta^{-/-}$ (*b,d,f,h*) mouse embryos at various stages of embryonic development. At each stage, elastin deposition in the ductus arteriosus (arrows) is similar in the wild-type and knockout embryos. Ao, aorta; pa, pulmonary artery.