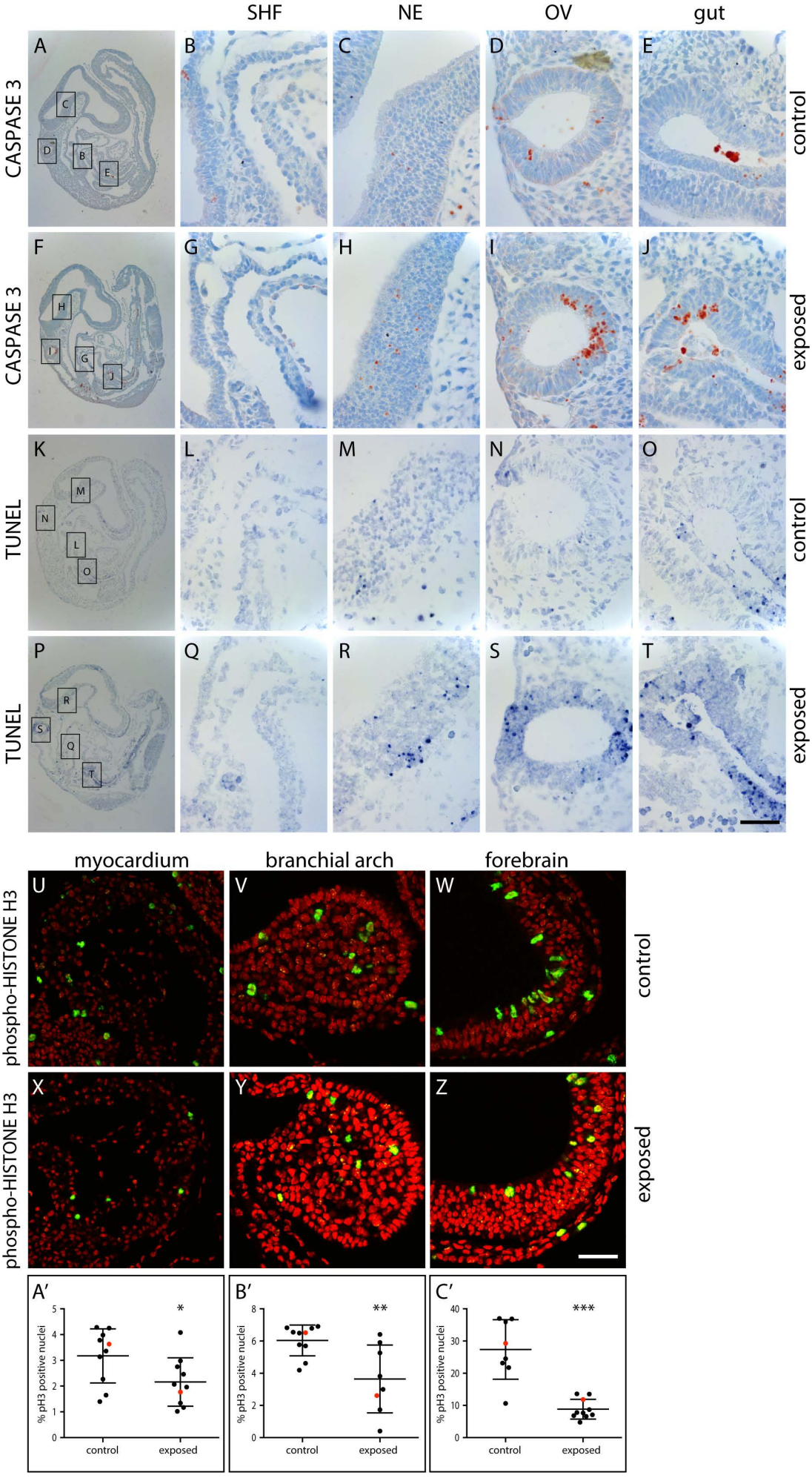

Figure S1. Effects of Low Oxygen Exposure of E9.5 Mouse Embryos on Apoptosis and Proliferation of Second Heart Field Cells. Related to Figure 1.
(A-J) Comparison of active/pro Caspase 3 protein levels (red) in control (A-E, n=10) and exposed (8 hours, F-J, n=10) E9.5 mouse embryos using immunohistochemistry on sagittal paraffin sections. (A and F) Low magnification images of representative sections. (B-E and G-J) High magnification images of the boxed regions of panels A and F showing the second heart field (SHF), neuroectoderm (NE), otic vesicle (OV) and gut. Sections were counterstained with haemotoxylin (light blue). (K-T) Comparison of TUNEL staining (dark blue) on adjacent sagittal paraffin sections of control (K-O, n=10) and exposed (P-T, n=10) E9.5 mouse embryos. (K and P) Low magnification images of representative sections. (L-O and Q-T) High magnification images of the boxed regions of panels K and P showing the second heart field (SHF), neuroectoderm (NE), otic vesicle (OV) and gut. (U-C') Comparison of phospho- histone H3 protein levels (green) in control (U-W, n=10) and exposed (X-Z, n=10) E9.5 mouse embryos using immunohistochemistry on sagittal paraffin sections. Nuclei were stained with TO- PRO-3 (red). Representative sections of myocardium (U,X), branchial arch (V,Y) and forebrain (W,Z) are shown. (A'-C') Quantification of percentage of phospho-histone H3 positive nuclei for each tissue. Red dots indicate the quantification of the images shown in panels (U-Z). Scale bars: 560 μm (A, F, K and P), 70 μm (B-E, G-J, L-O and Q-T), 110 μm (U,X) and 50 μm (V,W,Y,Z). Graph shows mean and standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

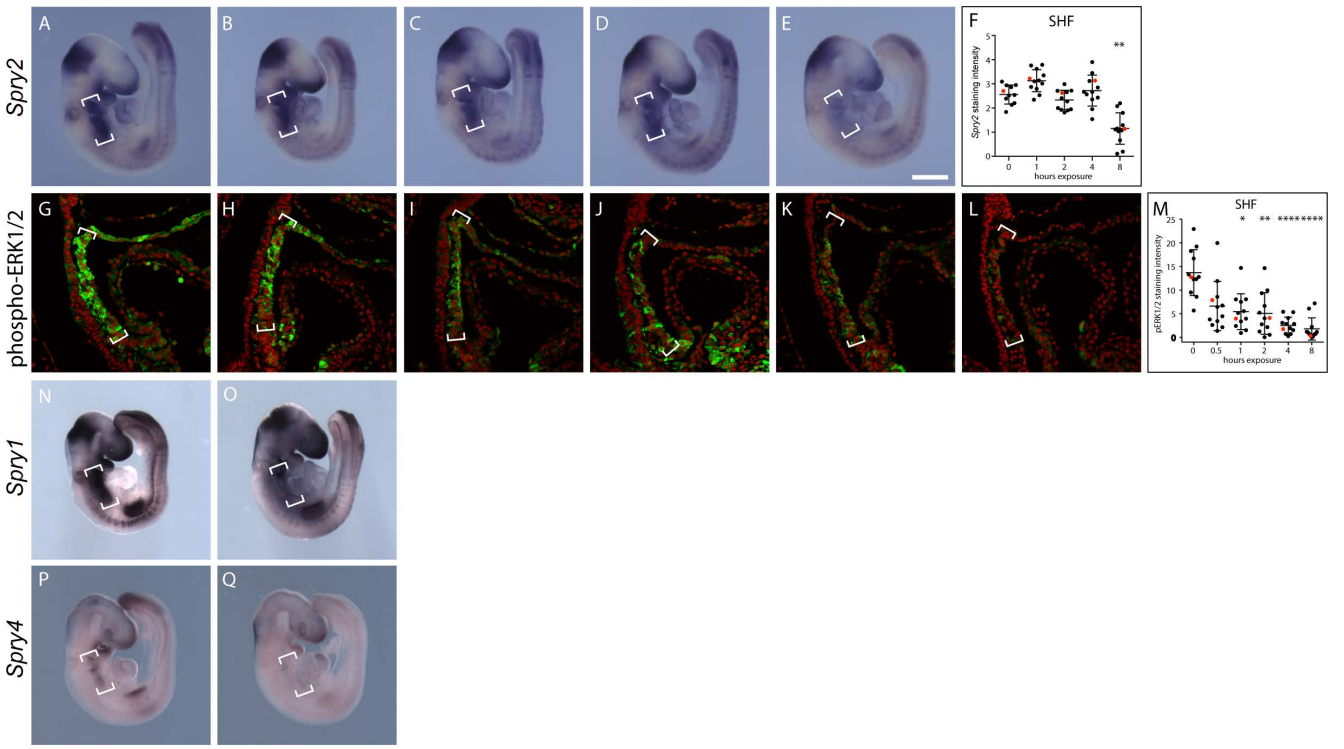

Figure S2. Effects of Low Oxygen Exposure of E9.5 Mouse Embryos on FGF Signaling Targets in the Second Heart Field. Related to Figure 2.

(A-F) Comparison of gene expression levels of Spry2 in control (A, n=11) and exposed (B-E) E9.5 mouse embryos using whole-mount RNA in situ hybridization. Exposure times in hours were 1 (B, n=12), 2 (C, n=12), 4 (D, n=12) and 8 (E, n=12). (F) Quantification of levels of Spry2 transcript in the second heart field (SHF). Red dots indicate the values for the embryos depicted in panels A-E. (G-M) Comparison of phospho-ERK1/2 (green) protein levels in control (G, n=12) and low oxygen exposed (H-L) E9.5 mouse embryos using immunohistochemistry on sagittal paraffin sections. Exposure times in hours were 0.5 (H, n=12), 1 (I, n=12), 2 (J, n=12), 4 (K, n=12) and 8 (L, n=12). Nuclei were stained with TO-PRO-3 (red). (M) Quantification of levels of phospho-ERK1/2 in the SHF. Red dots indicate the values for the embryos depicted in panels G-L. (N-O) Comparison of gene expression levels of Spry1 in control (N, n=5) and 8 hour exposed (O, n=5) E9.5 mouse embryos using whole-mount RNA in situ hybridization. (P-Q) Comparison of gene expression levels of Spry4 in control (P, n=12) and 8 hour exposed (Q, n=14) E9.5 mouse embryos using whole-mount RNA in situ hybridization. In panels showing sections, rostral is to the top and caudal to the bottom. Location of the SHF is indicated by brackets. OFT = outflow tract, v = ventricle, a = atrium. Scale bars: 500 μm (A-E, N-Q) and 40 μm (G-L). Graph shows mean and standard deviation. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

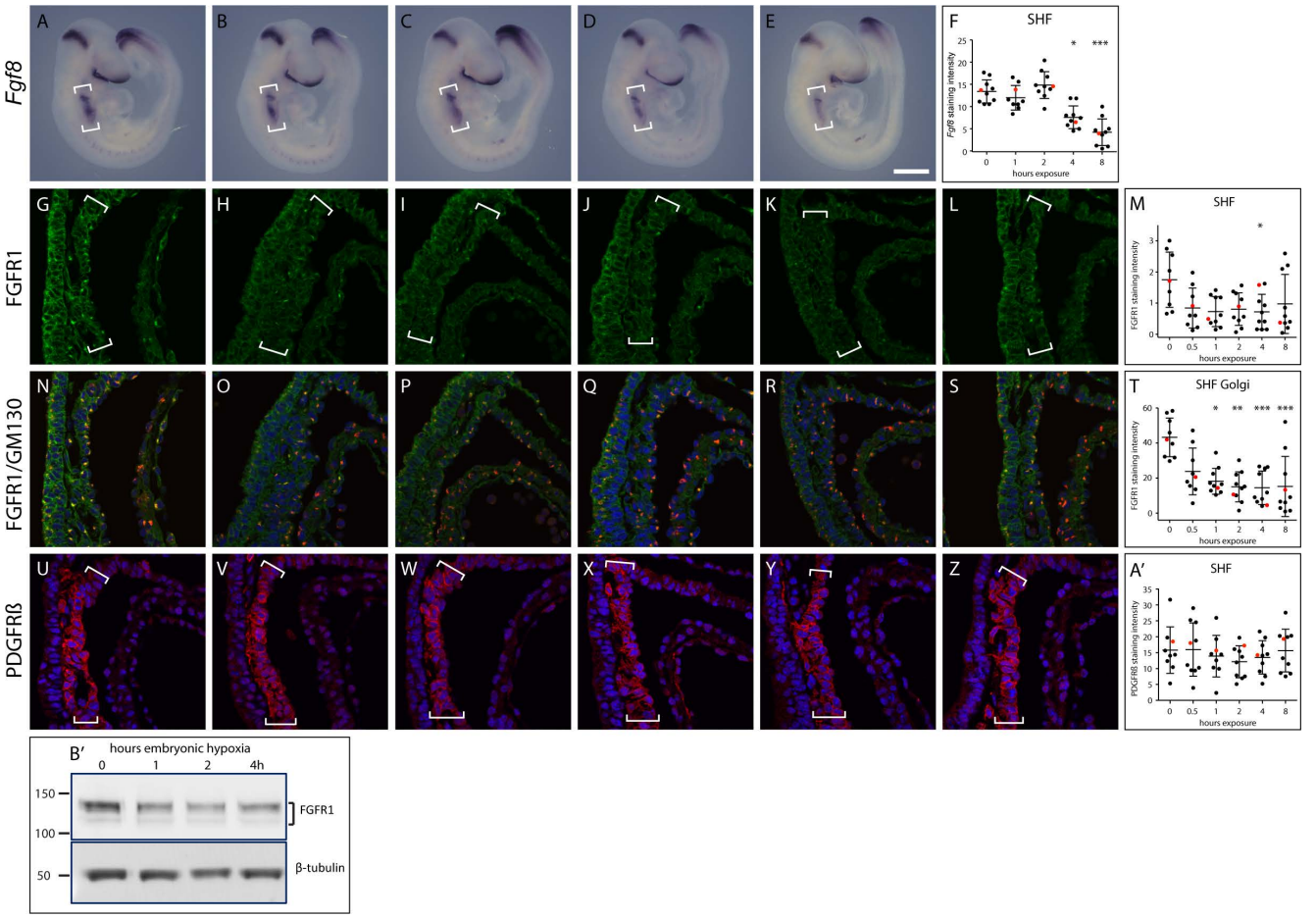

Figure S3. Effects of Low Oxygen Exposure of E9.5 Mouse Embryos on FGF Signaling Components in the Second Heart Field. Related to Figure 3.

(A-F) Comparison of gene expression levels of Fgf8 in control (A, n=10) and exposed (B-E) E9.5 mouse embryos using whole-mount RNA in situ hybridization. Exposure times in hours were 1 (B, n=9), 2 (C, n=10), 4 (D, n=10) and 8 (E, n=10). (F) Quantification of levels of Fgf8 transcript in the SHF. Red dots indicate the values for the embryos depicted in panels A-E. (G-M) Comparison of FGFR1 (green) protein levels in control (G, n=9) and low oxygen exposed (H-L) E9.5 mouse embryos using immunohistochemistry on sagittal paraffin sections. Exposure times in hours were 0.5 (H, n=9), 1 (I, n=10), 2 (J, n=10), 4 (K, n=10) and 8 (L, n=10). (M) Quantification of total levels of FGFR1 in the SHF. Red dots indicate the values for the embryos depicted in panels H-M. (N-S) Comparison of FGFR1 (green) protein levels in the Golgi of control (N, n=9) and low oxygen exposed (O- S) E9.5 mouse embryos using immunohistochemistry on sagittal paraffin sections. N-S are the same as G-L with the inclusion of staining for Golgi with GM130 (red) and nuclei with TO-PRO-3 (blue). (T) Quantification of levels of FGFR1 in SHF Golgi. Red dots indicate the values for the embryos depicted in panels N-S. (U-A') Comparison of PDGFRß (red) protein levels in control (U, n=9) and low oxygen exposed (W-Z) E9.5 mouse embryos using immunohistochemistry on sagittal paraffin sections. Exposure times in hours were 0.5 (V, n=10), 1 (W, n=9), 2 (X, n=10), 4 (Y, n=10) and 8 (Z, n=10). Nuclei were stained with TO-PRO-3 (blue). (A') Quantification of PDGFRß protein levels in the SHF. Red dots indicate the values for the embryos depicted in panels V-A'. (B') Example immunoblot showing total protein levels of FGFR1 in whole E9.5 embryos exposed to normoxia (0 hours), or 5.5% oxygen for 1-4 hours. Scale bars: 500 µm (A-E) and 40 µm (G-L, N-S and U-Z). In panels showing sections, rostral is to the top and caudal to the bottom. Location of the SHF is indicated by brackets. OFT = outflow tract, v = ventricle, a = atrium. Graph shows mean and standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

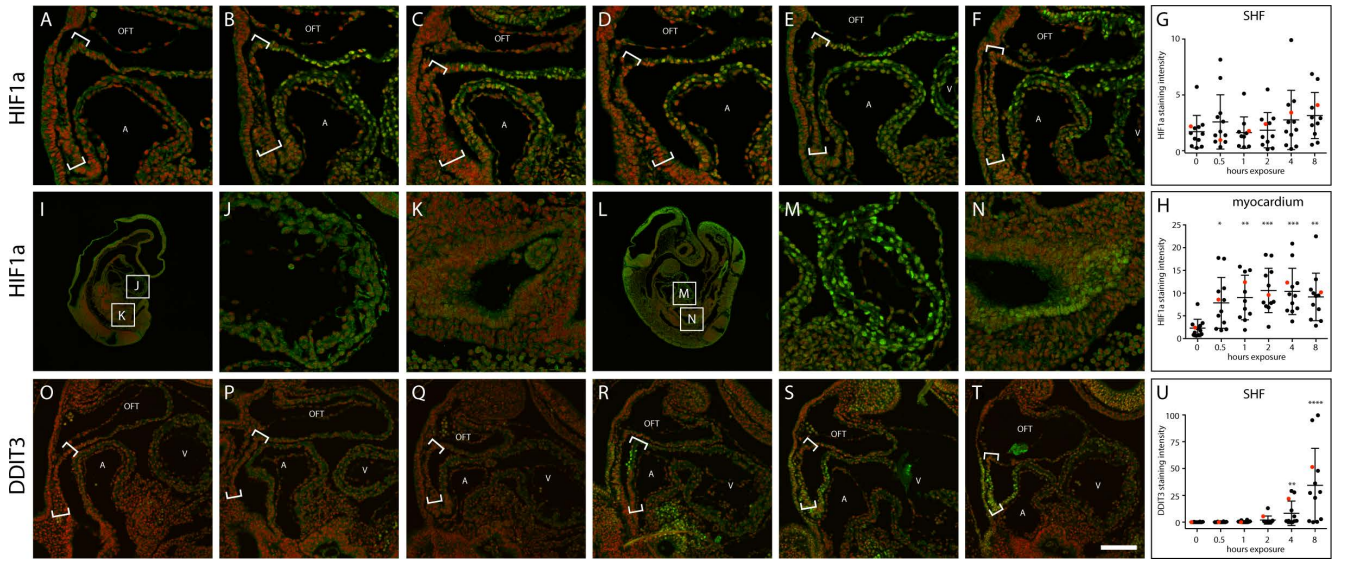

Figure S4. Cells in Embryos Exposed to Lowered Oxygen Levels in utero are Hypoxic and Induce the Unfolded Protein Response. Related to Figure 4.

(A-H) Comparison of HIF1α (green) protein levels in control (A, n=12) and exposed (B-F) E9.5 mouse embryos using immunohistochemistry on sagittal paraffin sections. Exposure times in hours were 0.5 (B, n=12), 1 (C, n=10), 2 (D, n=11), 4 (E, n=12) and 8 (F, n=11). Nuclei were stained with TO-PRO-3 (red). (G-H) Quantification of levels of HIF1α in the SHF (G) and myocardium (H). Red dots indicate the values for the embryos depicted in panels A-F. (I-N) Comparison of HIF1α (green) protein levels in control (I) and low oxygen exposed (L) E9.5 mouse embryos. Magnified views of the boxed areas in panels I and L showing HIF1α staining in myocardium (J and M) and gut (K and N). (O-U) Comparison of DDIT3 (green) protein levels in control (O, n=12) and low oxygen exposed (P-T) E9.5 mouse embryos using immunohistochemistry on sagittal paraffin sections. Exposure times in hours were 0.5 (P, n=12), 1 (Q, n=12), 2 (R, n=12), 4 (S, n=12) and 8 (T, n=12). Nuclei were stained with TO-PRO-3 (red). (U) Quantification of levels of DDIT3 in the SHF. Red dots indicate the values for the embryos depicted in panels O-T. In panels showing sections, rostral is to the top and caudal to the bottom. Scale bars: 80 μm (A-F), 570 μm (I and L), 70 μm (J-K, M- N) and 140 μm (O-T). Location of the SHF is indicated by brackets. OFT = outflow tract, v = ventricle, a = atrium. Graph shows mean and standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

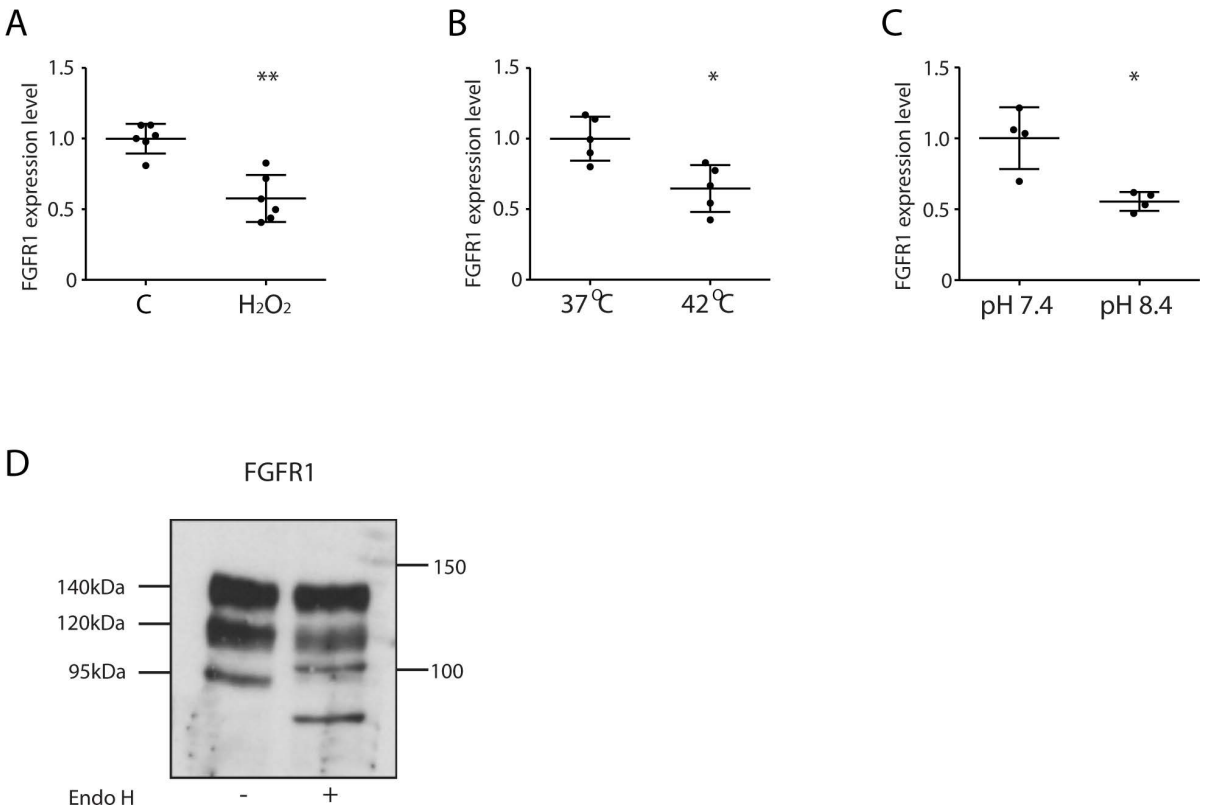

Figure S5. Quantification of FGFR1 Protein Levels in Embryos and Cells by Immunoblot. Related to Figure 5.

(A-C) Quantification graphs for FGFR1 in cells exposed for 2 hours to (A) 0.1 mM hydrogen peroxide, (B) heat shock at 420C, or (C) alkaline pH (culture under 0.1% CO2 atmosphere). (D) Immunoblot of endogenous FGFR1 in mouse muscle satellite C2C12 cells. Three bands are detected (140 kDa, 120 kDa and 95 kDa; left lane). Endoglycosidase H (Endo H; right lane) does not cleave the fully glycosylated 140 kDA polypeptide, but does cleave the partially glycosylated (120 kDa, 95 kDa) polypeptides. Molecular marker sizes are shown in kDa. Graph shows mean and standard deviation. * $p<0.05$, ** $p<0.01$.

Table S1. Short term Gestational Hypoxia Induces Heart Defects

				Type of heart defect observed								
Exposure stage	Embryos examined	Normal	Abnormal	VSD	muscular VSD	OA	DORV	TGA	ASD	PTA	SOTV	TVA
control	33	31	2	2	1	0	0	0	0	0	0	0
E7.5	27	27	0	0	0	0	0	0	0	0	0	0
E8.5	40	33	7	6	1	3	2	1	1	0	2	0
E9.5	48	27	21	18	1	7	10	3	3	1	1	1
E10.5	36	29	7	5	2	2	0	0	2	0	3	0

Note that a particular embryo may have more than one type of defect.

Pregnant mice were exposed to 5.5% oxygen for 8 hours on the indicated day of gestation. Mice were returned to normoxia, and embryos allowed to develop until E17.5, when heart morphology was analyzed. VSD: membranous ventricular septal defect; muscular VSD: muscular ventricular septal defect; OA: overriding aorta; DORV: double-outlet right ventricle; TGA: transposition of the great arteries; ASD: atrial septal defect; PTA: persistent truncus arteriosus; SOTV: straddling overriding tricuspid valve; TVA: tricuspid valve atresia

SUPPLEMENTAL MATERIALS AND METHODS

Optical projection tomography

For analysis of E10.5 OFTs, embryo torsos immunostained with MF 20 were dehydrated in methanol and cleared for at least 24 hours in 1:2 benzoic acid/benzyl benzoate (BABB). These were superglued directly to the mounting post of the OPT scanner. For whole E17.5 hearts, hearts were dissected from Bouin's fixed embryos stored in 70% ethanol, rehydrated to water, then dehydrated in methanol and cleared in BABB for at least 24 hours. Single hearts were placed in a cut-off and sealed glass Pasteur pipette, and attached to the mounting post. OPT scanning was performed using a custom microscope controlled by purpose-built software OPTimum (James Springfield, Institute for Molecular Biosciences, University of Queensland, Australia). 800-1,200 images in the FITC channel were collected per sample. These were reconstructed using nRecon (Bruker microCT, Belgium). Data were rendered in Amira 5.5.0 (FEI Visualization Services Group, USA). E17.5 heart morphology was analyzed directly in Amira. To quantitate OFT dimensions, reconstructions of OFTs were first rotated to the same orientation using Amira. 2D views were then exported in PNG format, and the OFT angle and length measured using ImageJ 1.49 (NIH, USA).

Immunohistochemistry

For immunohistochemistry, embryos were harvested at E9.5, fixed overnight in 4% paraformaldehyde at 4^{O}C, paraffin embedded and sectioned in the sagittal plane. To minimize inter-slide staining variation, tissue arrays were made by putting single sections from 12-20 different embryos on a single slide, and slides were processed using a Shandon Sequenza® Immunostaining Center (Thermo Scientific). Antigen retrieval for all antibodies except active/pro Caspase 3 was done using TE buffer (Moreau et al., 2014). For active/pro Caspase 3, slides were boiled in 10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0 for 25 minutes. Slides were mounted in Mowiol® with DABCO (Allan, 1999). Images were captured on either a LSM 7 Duo or a LSM 710 upright confocal microscope (Carl Zeiss).uFor detecting FGFR1 or HIF1α in paraffin sections, biotinylated donkey anti-rabbit secondary and streptavidin Cy3 tertiary reagents were used. For detecting active/pro Caspase 3 and Hypoxyprobe™ in paraffin sections, ImmPRESS™ secondary reagent and 3-amino-9-ethylcarbazole (AEC for Caspase 3; Sigma Aldrich) or 3, 3'-diaminobenzidine (DAB for Hypoxyprobe™; Sigma Aldrich) was used.

Table S2. Antibodies used for immunodetection

Target	Name	Catalog number	Species and type	Supplier	Dilution for IHC	Dilution for IB
Primary antibodies						
active/pro Caspase 3		ab13847	rabbit polyclonal	Abcam	1:200	na
Myosin II heavy chain	MF 20		mouse monoclonal	Developmental Studies Hybridoma Bank	1:100	na
phospho-histone H3	(Ser 10)-R	sc-8656	rabbit polyclonal	Santa Cruz Biotechnology	1:200	na
phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	D13.14.4E	4370	rabbit monoclonal	Cell Signaling Technology	1:200	1:1,000
p44/42 MAPK (Erk1/2)		9102	rabbit polyclonal	Cell Signaling Technology	na	1:1,000
FGFR1	D8E4	9740	rabbit monoclonal	Cell Signaling Technology	1:400	1:1,500
GM130		610822	mouse monoclonal	BD Transduction Laboratories	1:400	na
PDGFRß	958	sc-432	rabbit polyclonal	Santa Cruz Biotechnology	1:50	na
Hypoxyprobe ™ FITC	4.3.11.3		Mouse monoclonal	Hypoxyprobe, Inc.	1:400	na
HIF1α		NB100-449	rabbit polyclonal	Novus Biologicals	1:400	na
DDIT3	F-168	sc-575	rabbit polyclonal	Santa Cruz Biotechnology	1:50	1:500
phospho eIF2α (Ser51)	D9G8	3398	rabbit monoclonal	Cell Signaling Technology	na	1:1,000
ß-tubulin	E7		mouse monoclonal	Developmental Studies Hybridoma Bank	na	1:500
FLAG®	M2	F1804	mouse monoclonal	Sigma-Aldrich	na	1:1,000
Secondary Antibodies						
Donkey anti-		711-165-		Jackson	1:500	na

rabbit Cy™3		152		ImmunoResearch		
Donkey anti-goat AlexaFluor® 488		A-11055		Life Technologies	1:500	na
Donkey anti-mouse AlexaFluor ® 488		715-545-151		Jackson ImmunoResearch	1:500	na
Goat anti-mouse IRDye® 800CW		926-32210		LI-COR	na	1:10,000
Goat anti-rabbit AlexaFluor® 680		A-21109		Life Technologies	na	1:5,000
Donkey anti-rabbit biotinylated		711-065-152		Jackson ImmunoResearch	1:500	na
ImmPRESS™ (Peroxidase) Polymer anti-mouse Ig Reagent		MP-7402		Vector Laboratories	1:1	na
ImmPRESS™ (Peroxidase) Polymer anti-rabbit Ig Reagent		MP-7401		Vector Laboratories	1:1	na
Tertiary reagents						
Steptavidin Cy™3		GTX85902		GeneTex	1:1,000	na
Nuclear stain						
TO-PRO®-3 Iodide		T3605		Life Technologies	1:10,000	na

RNA *in situ* hybridization probes

cDNA probes used were as follows: *Spry1* (Addgene plasmid # 22091, Minowada et al., 1999)), *Spry2* (Addgene plasmid # 22097, Minowada et al., 1999), *Spry4* (Addgene plasmid # 22093, (Minowada et al., 1999), and *Fgf8* (Mahmood et al., 1995).

Cell culture and immunoblots

Mouse muscle satellite C2C12 cells (ATCC) were cultured in IMDM medium with glutamine (Life Technologies) and 10% fetal bovine serum (Life Technologies) on 40 mm Steriplan®

glass petri dishes (DURAN) in 5% CO_2. C2C12 cells were verified as free from mycoplasma contamination using the MycoAlert Detection Kit (Lonza). Hypoxic exposure of cells was done using a H35 Hypoxystation (Don Whitley Scientific). Culture medium was equilibrated in 0.1% O_2 overnight prior to use. Culture dishes were transferred to the workstation, rinsed once with hypoxia-equilibrated medium, and incubated in fresh hypoxia-equilibrated medium for the indicated times. For the study of UPR inhibitors, cells were incubated for 2 hours in the normoxic or hypoxia-equilibrated medium containing 20 μM IRE1 inhibitor 4μ8c (Sigma Aldrich) or 1 μM PERK inhibitor GSK2606414 (Selleck Chemicals) or equivalent volume of DMSO as a control. Whole cell protein extracts and immunoblots were performed by standard methods, with detection using either HRP-labeled secondary antibodies and SuperSignal West Pico chemiluminescent substrate (Thermo Fisher) or fluorescently-labeled secondary antibodies and the Li-Cor Odyssey Infrared Imaging System.

Quantification methods

Staining intensity of immunohistochemistry on paraffin sections was quantified using ImageJ 1.49 (NIH, USA). Briefly, LSM files generated by confocal microscopy were imported into ImageJ. The region of interest (ROI) was manually defined, and the image was threshholded to remove background. Identical threshholding values were used for all images of each experiment. The signal above threshold was quantified, and normalized to ROI area. For quantitation of FGFR1 proteins levels in the Golgi, stacks of confocal LSM images from sections co-stained with FGFR1 and GM130 were imported into ImageJ. The ROI was manually defined in the GM130 channel, and all signal outside this area was removed. The GM130 channel was threshholded to remove background, using identical values for all images, and the intensity of GM130 staining quantified. The area of GM130 staining in the ROI was used to make a selection mask, which was then applied to the FGFR1 channel. The FGFR1 channel was threshholded to remove background using identical values for all images, and the intensity of FGFR1 staining in the GM130-expresing domain of the ROI was quantified. For proliferation measurement, the percentage of phospho-histone H3 positive cells in each embryo was calculated as the ratio of combined total number of phospho-histone H3 positive cells against the combined total number To-Pro3+ nuclei from three independent sections from the same embryo. Staining intensity of RNA wholemount *in situ* hybridization images was also quantified using ImageJ. Stained embryos were photographed using a M125 dissection microscope with DFC450 camera (Leica microsystems GmbH, Germany), using

identical lighting and camera settings for all embryos stained for the same transcript. TIFF files were imported into ImageJ and changed to 8-bit grayscale. The ROI was manually defined, threshholded to remove background with identical settings for all images, and the integrated density in the ROI measured. Immunoblots analyzed by chemiluminescence were digitized using a FLA-5100 scanner (Fujifilm, Japan), and converted to TIFF format using ImageJ. Immunoblots analyzed by Odyssey Imager (LI-COR) were scanned at high resolution and saved as TIFF files. In both cases, bands were quantitated using Gelanalyzer (gelanalyzer.com).

Analysis of embryonic apoptosis

Two adjacent sagittal sections of each embryo (n=10 control and n=10 exposed) were stained for active/pro Caspase 3 and the other for TUNEL. These were taken from roughly the same sagittal plane from every embryo to ensure a similar area was surveyed across all embryos. One view field per section was counted for each of the SHF, OFT, ventricular myocardium, posterior part of the neural tube, otic vesicle and gut.

SUPPLEMENTARY REFERENCES

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