Supplemental Materials

Title: Epigenetic mechanisms underlying maternal diabetes-associated risk of congenital heart disease

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Supplemental Figure and Figure Legends:

Supplemental Figure 1: **Gene-environment interaction between MDM and Notch1.** (A-D) Representative images of wildtype (WT) and *Notch1*$^{+/-}$ E13.5 embryos from non-diabetic and diabetic mothers show no apparent growth retardation. (E, F) Three-dimensional reconstruction of E13.5 non-diabetic and diabetic WT heart (N=1 per group) using AMIRAv5.5 software showed the perimembranous VSD (*). AVC, Atrioventricular cushion (green); IVS, interventricular septum (red). (G) Breeding scheme showing non-diabetic *Notch1*$^{+/-}$ males crossed with diabetic (hyperglycemic, blood glucose >200mg/dl) WT females to test that resultant CHD phenotype is independent of maternal genetic background. (H-K) Representative histologic sections showing location of VSD (*) in E13.5 hearts (WT and *Notch1*$^{+/-}$) exposed to maternal diabetes as compared to non-diabetic controls. Scale bars: (A-D) 1 mm and (H-K) 200 µm. RA, right atrium, LA, left atrium; RV, right ventricle; LV, left ventricle.

Supplemental Figure 2: Maternal hyperglycemic stress results in spectrum of birth defects in developing embryos. (B-D) General appearance of E13.5 embryos exposed to increasing level of maternal glucose (N=3, range of maternal blood glucose (mg/dl) = 389-787 mg/dl at time of embryo collection) compared to non-diabetic wildtype (WT) control (A). Yellow arrowheads indicate craniofacial and neural tube defects (C, D). (E-L) Representative hematoxylin & eosin images of E13.5 heart sections demonstrate improper septation of outflow tract (I, K) compared to E and G, enlarged AV cushion (H, L, black arrowheads), septal defects (J, asterisk and black arrow) and myocardial wall thinning (H, J, L) compared to control embryos (E, F). Scale bars: (A-D) 1 mm and (E-L) 200 µm. RA, right atrium, LA, left atrium; RV, right ventricle; LV, left ventricle; Ao, Aorta; PT, Pulmonary trunk; CT, common trunk; AVC, Atrioventricular cushion; IVS, Intervetricular septum.

Supplemental Figure 3: Chromatin accessibility at *Notch1* and its downstream target loci. (A) Integrative Genome Viewer (IGV) tracks showing ATAC-seq signals for four open regions (R1-R4) at *Notch1* loci in NG (red) and HG (blue). (B-E) Chromatin accessibility at the proximal promoter regions of *Hey2*, *EfnB2*, *Nrg1* and *Jarid2* loci remain unaltered in HG compared in NG. Statistically significant changes in chromatin accessibility were found at the enhancer, downstream and intronic regions of *Hey2*, *Efnb2* and *Nrg1* respectively (peaks shown with asterisks).

Supplemental Figure 4: **Specificity of the probes and quantification of DAR-4M AM$^+$ and DHE$^+$ cells.** AVM cells treated with (A, D) DMSO, (B) 250µM DetaNONOate and (E) 300µM H_2O_2 served as negative and positive controls for DAR-4M AM and DHE staining, respectively, indicating the specificity of the dyes. (C, F) Quantification of mean fluorescence intensity of DAR4M$^+$ and DHE$^+$ cells in NG and HG ($N \geq 4$). Data presented as mean±SEM; *, p-value <0.05 by 2-tailed Student’s *t* test. Scale bars: 50µm (A, B) and 100µm (D, E). (G-I) and (J-L) Endothelial cell-specific NO production was measured using DAR4M AM (red) staining in E13.5 non-diabetic and diabetes exposed embryos (N=1 per group) co-stained with PECAM (green) and nuclei stained with DAPI (blue). Scale bars: 100µm (G, I, J, L) and 50µm (H, K).

Supplemental Figure 5: Inability of N-acetyl cysteine mediated rescue of VSD phenotype in vivo: (A) Breeding scheme showing non-diabetic wildtype (WT) males crossed with non-diabetic (blood glucose = 165-189 mg/dl at time of embryo harvest) and diabetic (blood glucose=229-389 mg/dl at time of embryo harvest) WT females and subset of these females were treated with N-acetyl cysteine (NAC). (B) No significant rescue was observed in the incidence of VSD among diabetic groups with (31%) or without (37%) treatment of NAC. No VSD was observed in non-diabetic control groups (0%). (G-J) Histologic sections showing location of ventricular septal defects (VSD, *) in E13.5 hearts from two representative embryos exposed to maternal diabetes with or without NAC treatment. Non-diabetic wildtype (WT) control embryos are shown with normal ventricular septation. RA, right atrium, LA, left atrium; RV, right ventricle; LV, left ventricle. Scale bar: 200 μm.

Supplemental Figure 6: N-acetyl cysteine mediated rescue in vitro. AVM cells maintained in NG and HG were stained for (A-D) ROS generation using H_2DCFDA (green) and (E-H) NO detected using DAR4M AM (red) after treatment with 5mM NAC and compared to untreated controls. (I-L) and (M-P) Immunofluorescent staining demonstrate JARID2 (green) and active NOTCH1 (N1ICD, red) expression in presence and absence of NAC. Propidium iodide (red, A-D; I-L) and DAPI (blue, E-H; M-P) stain the nuclei. Scale bar: 50μm. (Q-T) Quantification of mean fluorescent intensity of DCFDA, DAR4M, JARID2 and N1ICD staining by ImageJ. N≥3, Data presented as mean±SEM; *, p-value <0.05 by 2-tailed Student's *t* test.

Supplemental Figure 7: RNAi transgene rescues shorter lifespan and cardiac phenotype in Drosophila maternal diabetic model. Survival curves for adult progenies those overexpressing heart specific *Jarid2*, *Numb*, *Notch* RNAi and *Jarid2* RNAi transgenes are shown. Color-coding identifies exposure to maternal diabetes and presence of transgenes.

Supplemental Figure 8: In vitro ATAC-seq highlights top GO enriched terms altered in hyperglycemic stress. (A) Gene ontology analysis performed on genes associated with peaks in "promoter" regions, with p-value <0.05 and >1.5 fold change, highlights the 25 most significant GO terms. Green: Cellular Architecture, Blue: Chromatin Regulation, Pink: Development, Grey: Other. (B) Circle plot generated to highlight the 12 GO terms that fall within Chromatin Regulation (Blue), Development (Pink), or Cellular Architecture (Green) classifications (C). Within the circle plot, the colored dots represent gene associated peaks that are more open in high glucose (red) or more open in normal glucose (blue) within each GO term. The size of the rectangle in the inner circle represents the p-value, with larger rectangles equating to lower p-values. The color of the rectangle represents the z-score, blue and red representing low and high z-scores respectively. The z-score used in this analysis refers to the following equation: z-score = (# of peaks up - # of peaks down)/ (sqrt of total count), allowing for an inference in the overall change of the biological function.

Supplemental Figure 9: Hyperglycemia reduces cardiomyocytes proliferation at E13.5 embryos. (A-H) Immunofluorescent staining using phospho-histone H3 (PHH3, Ser10, red) demonstrates decreased cardiomyocyte proliferation in the interventricular septum (IVS) of the diabetic E13.5 WT embryonic hearts compared to non-diabetic controls (N=3 each group). cTnT staining (green) is shown to label cardiomyocytes and corresponding nucleus stained with DAPI (blue). Scale bar (A-H): 20 µm.

(I) Quantitation of PHH3^{+} cardiomyocytes demonstrated downregulation of cardiomyocyte proliferation between two groups. N=5. Data presented as mean±SEM; * , p-value <0.05 by 2-tailed Student’s *t* test.

(J) Transcript expression of *CyclinD1* is reduced in diabetic WT E13.5 embryonic hearts with respect to non-diabetic controls (N=6 whole hearts pooled together/ group) as measured with qRT-PCR. Data shown as average± SD; *, p-value <0.05 by 2-tailed Student’s *t* test.

Supplemental Tables:

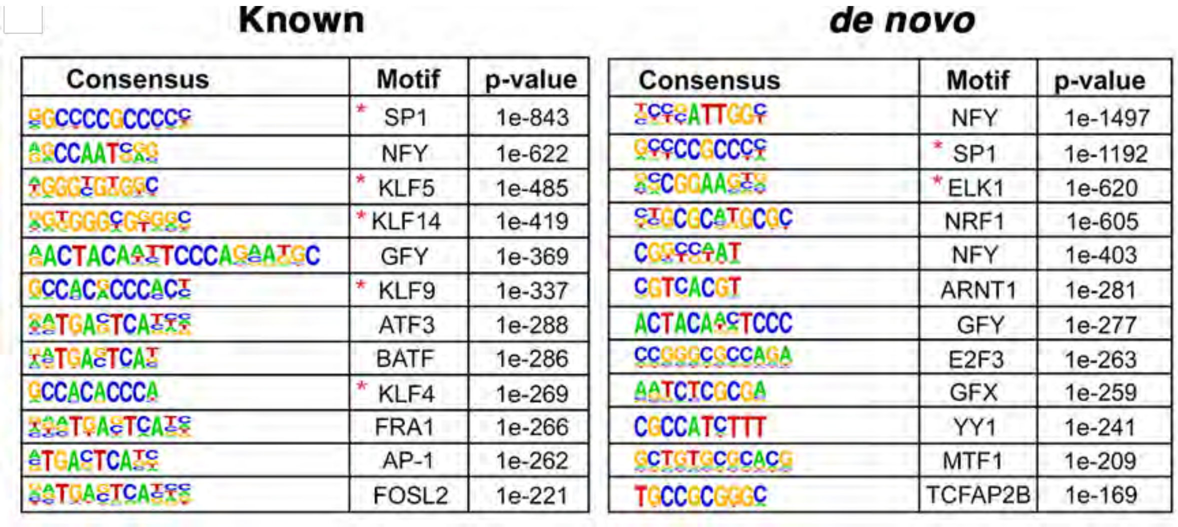
Supplemental Table 1: Incidence of VSD in E13.5 WT and *Notch1*$^{+/-}$ embryos derived from *Notch1*$^{+/-}$ male and WT diabetic female.

Parental Genotype	**Embryo Genotype**	**Embryos with VSD (%)**	**Fisher Exact p-value (non-diabetic vs. diabetic)**
Non-diabetic *Notch1*$^{+/-}$ (male)	*Notch1*$^{+/-}$	8/9 (88%)	0.035*
Diabetic WT (female)	WT	2/7 (28.7%)	

*** indicates statistically significant 2-tailed p value<0.05; WT= wildtype**

Supplemental Table 2: Full annotated ATAC-seq features in AVM cells treated with NG and HG

Supplemental Table 3: Motif enrichment analysis of NG ATAC-seq dataset with HG set as background identified overrepresented known and de novo motifs respectively.



Known

Consensus	Motif	p-value
GGCCCCGCCCCC	* SP1	1e-843
AGCCAATCGG	NFY	1e-622
AGGGTGTGGC	* KLF5	1e-485
GGTGGGCGGGGC	* KLF14	1e-419
AACTACAATTCCCAGAATGC	GFY	1e-369
GCCACACCCACT	* KLF9	1e-337
GATGACTCATCC	ATF3	1e-288
TATGACTCAT	BATF	1e-286
GCCACACCCA	* KLF4	1e-269
GGATGACTCATC	FRA1	1e-266
ATGACTCATC	AP-1	1e-262
GATGACTCATCC	FOSL2	1e-221

de novo

Consensus	Motif	p-value
TCCCATTGGC	NFY	1e-1497
GCCCCGCCCC	* SP1	1e-1192
GCCGGAAGTG	* ELK1	1e-620
CTGCGCATGCGC	NRF1	1e-605
CGGCCAAT	NFY	1e-403
CGTCACGT	ARNT1	1e-281
ACTACAACTCCC	GFY	1e-277
CCGGGCGCCAGA	E2F3	1e-263
AATCTCGCGA	GFX	1e-259
CGCCATCTTT	YY1	1e-241
GCTGTGCGCACG	MTF1	1e-209
TGCCGCGGGC	TCFAP2B	1e-169

Supplemental Table 4: Known and de novo transcription factor binding motifs enriched in NG compared to HG

Supplemental Table 5: TFBS profile for Nos3_R1

Supplemental Table 6: TFBS profile for Nos3_R2

Supplemental Table 7: TFBS profile for Nos3_R3

Supplemental Table 8: Incidence of VSD in E13.5 embryos with and without exposure to maternal diabetes and NAC treatment.

Embryonic Genotype	**Maternal Environment**	**Embryos with VSD (%)**	**p-value (non-diabetic vs. diabetic)**	**p-value (diabetic vs. diabetic +NAC)**
WT	Non-diabetic	0/5 (0%)	NS	-
	Non-diabetic+ NAC	0/9 (0%)		
WT	Diabetic	3/8 (37%)	0.036*	NS
	Diabetic + NAC	5/16 (31%)	0.044*	

*** indicates statistically significant 2-tailed p value<0.05; WT= wildtype, NS=non-significant**

Supplemental Table 9: TFBS profile for Jarid2_R1

Supplemental Table 10: TFBS profile for Jarid2_R2

Supplemental Table 11: TFBS profile for Jarid2_R3

Supplemental Table 12: TFBS profile for Notch1_R1

Supplemental Table 13: TFBS profile for Notch1_R2

Supplemental Table 14: TFBS profile for Notch1_R3

Supplemental Table 15: TFBS profile for Notch1_R4

Supplemental Table 16: Differential peaks within 1.5kb upstream and downstream of TSS, P-value<0.05 and >1.5fold change between NG and HG

Supplemental Table 17: Gene Ontology enriched terms

Supplemental Table 18: ATAC-seq index primer sequences:

Index Primers	**Sequence 5’-3’**
Ad1_noMx	AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG
Ad2.1_TAAGGCGA	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT
Ad2.2_CGTACTAG	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
Ad2.3_AGGCAGAA	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
Ad2.4_TCCTGAGC	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
Ad2.5_GGACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGT
Ad2.6_TAGGCATG	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT

Supplemental Table 19: Oligonucleotide sequences for gene expression

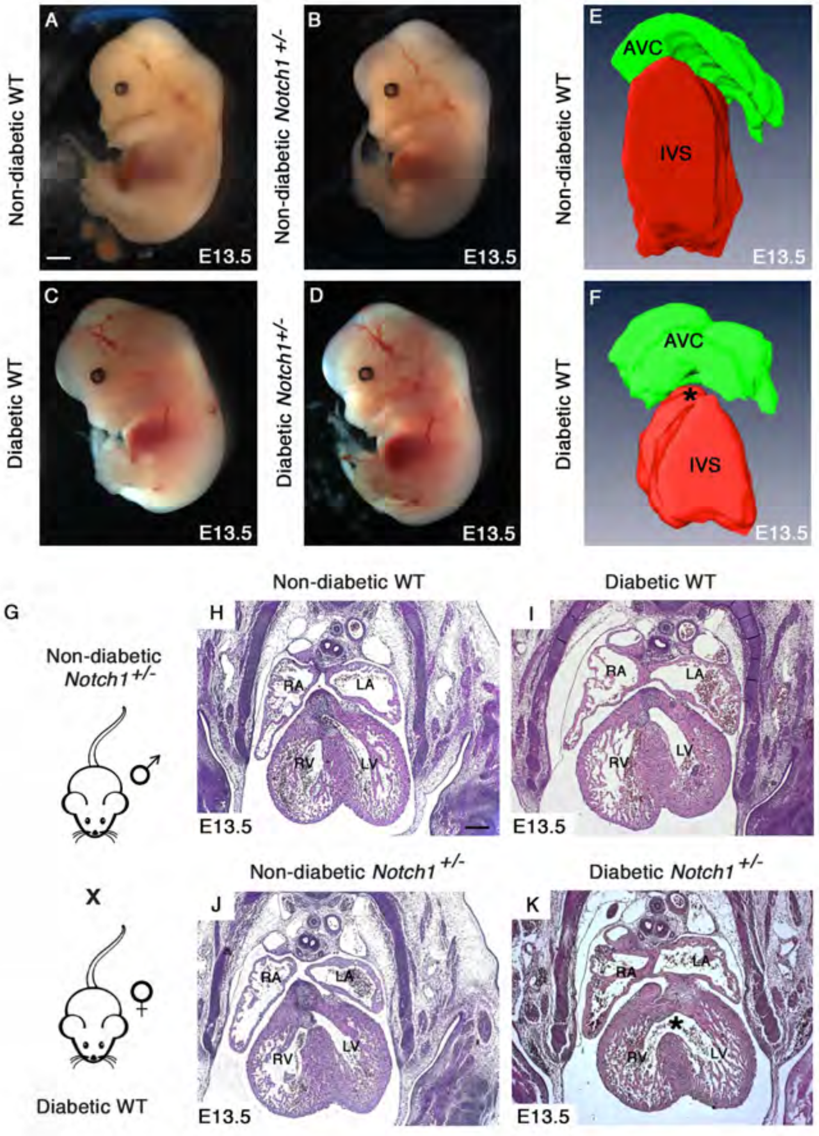
Gene name	Primer Name	Sequence 5’-3’
18S	mus_18S F.P	ACGACCCATTCGAACGTCTGC
	mus_18S R.P	GGACTCATTCCAATTACAGGG
Gapdh	mus_Gapdh F.P	GAAGGGCTCATGACCACAGT
	mus_Gapdh R.P	GATGCAGGGATGATGTTCTGG
Notch1	mus_Notch1 F.P	CCAGGAAAGAGGGCATCAGA
	mus_Notch1 R.P	ACACTTCCAGCGTCTTTGGG
Hey1	mus_Hey1 F.P	CCAGACTACAGCTCCTCAGATA
	mus_Hey1 R.P	CGCCGAACTCAAGTTTCCATT
Hey2	mus_Hey2 F.P	CCTGGTCTCTCATCTCAGCA
	mus_Hey2 R.P	GGCCAGAGAGGAAGTCATTG
Nrg1	mus_Nrg1 F.P	AAATCGCCCCCTTCGGAAAT
	mus_Nrg1 R.P	GTCACAAGAAGCAGAGGCCT
EfnB2	mus_EfnB2 F.P	CATCACTTTGGTGGTGCTGC
	mus_EfnB2 R.P	CCATTGTTGTTGCCACCTCG
Bmp10	mus_Bmp10 F.P	TGACCCTTTGCTGGTTGTGT

	mus_Bmp10 R.P	ATCGGGCCCACTGAAGAAAG
Jarid2	mus_Jarid2 F.P	CTGGCCTTCACTCTTCTGCA
	mus_Jarid2 R.P	GCAGATCTGGCACCTCCTTT
Cyclin D1	mus_CyclinD1 F.P	GCCGAGAAGTTGTGCATCTA
	mus_Cyclin D1 R.P	GTTCACCAGAAGCAGTTCCA
Nos3	mus_Nos3 F.P	ATTGGCATGAGGGACCTGTG
	mus_Nos3 R.P	GGTGTCCAGATCCATGCACA

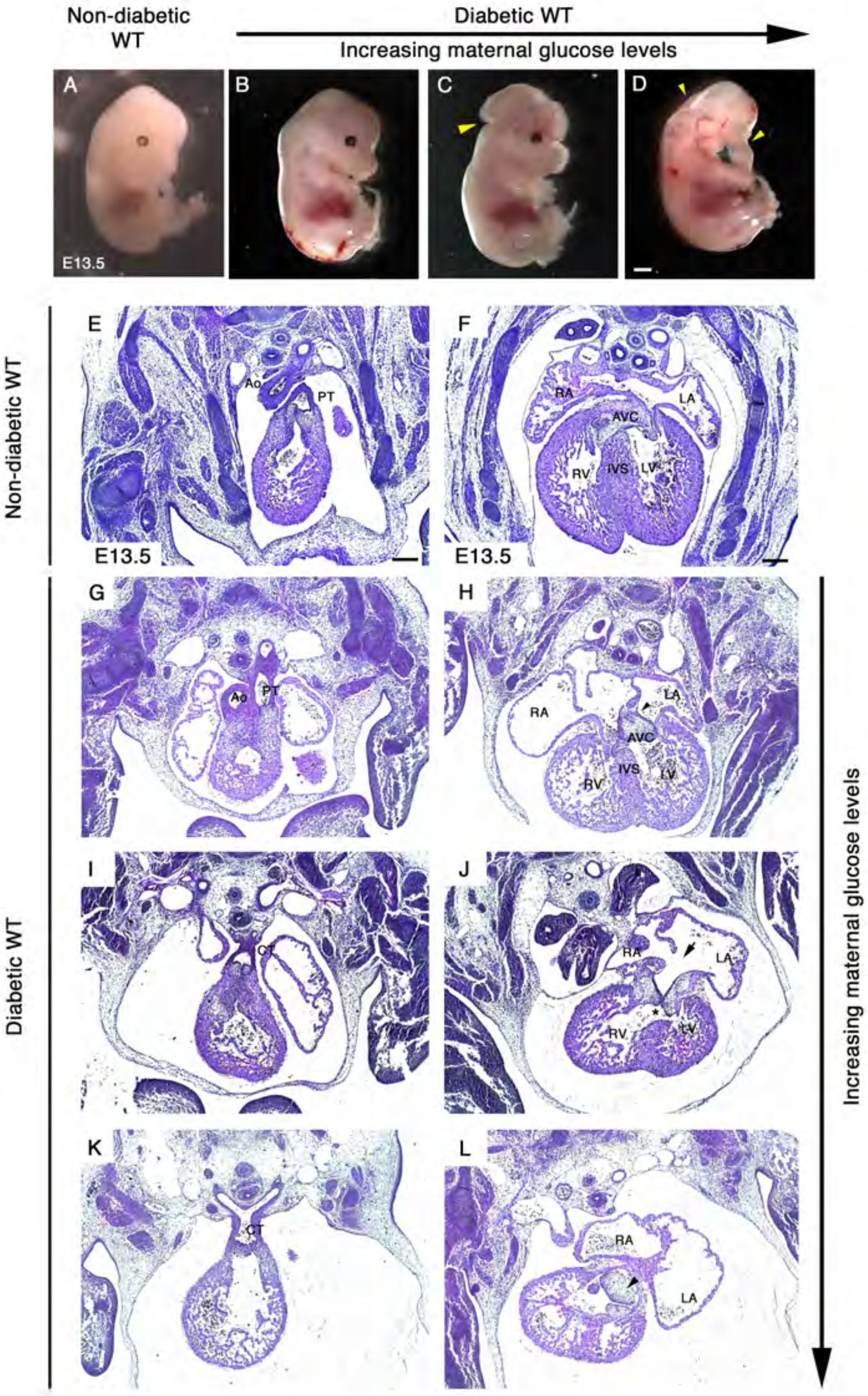
Supplemental Table 20: Oligonucleotide sequences for ChIP qPCR

Gene name	**Primer Name**	**Sequence 5'-3'**
Nos3	Nos3_R1F.P	GAAACTCTGCCTCTGTCCGA
	Nos3_R1R.P	TCACAGTGTCCAGAAGAGGG
	Nos3_R2F.P	TGAGTCACTTCAGAAGGCCA
	Nos3_R2R.P	CTAGCTGCCGTTGTGATTCC
	Nos3_R3F.P	GAGTATGAGAGAGGCCTCCA
	Nos3_R3R.P	GTGGAAGGGCTCTAGTGTGT
Notch1	Notch1ChIP_+1150 F.P	ACTGCCTGCCCTTTCCTTAA

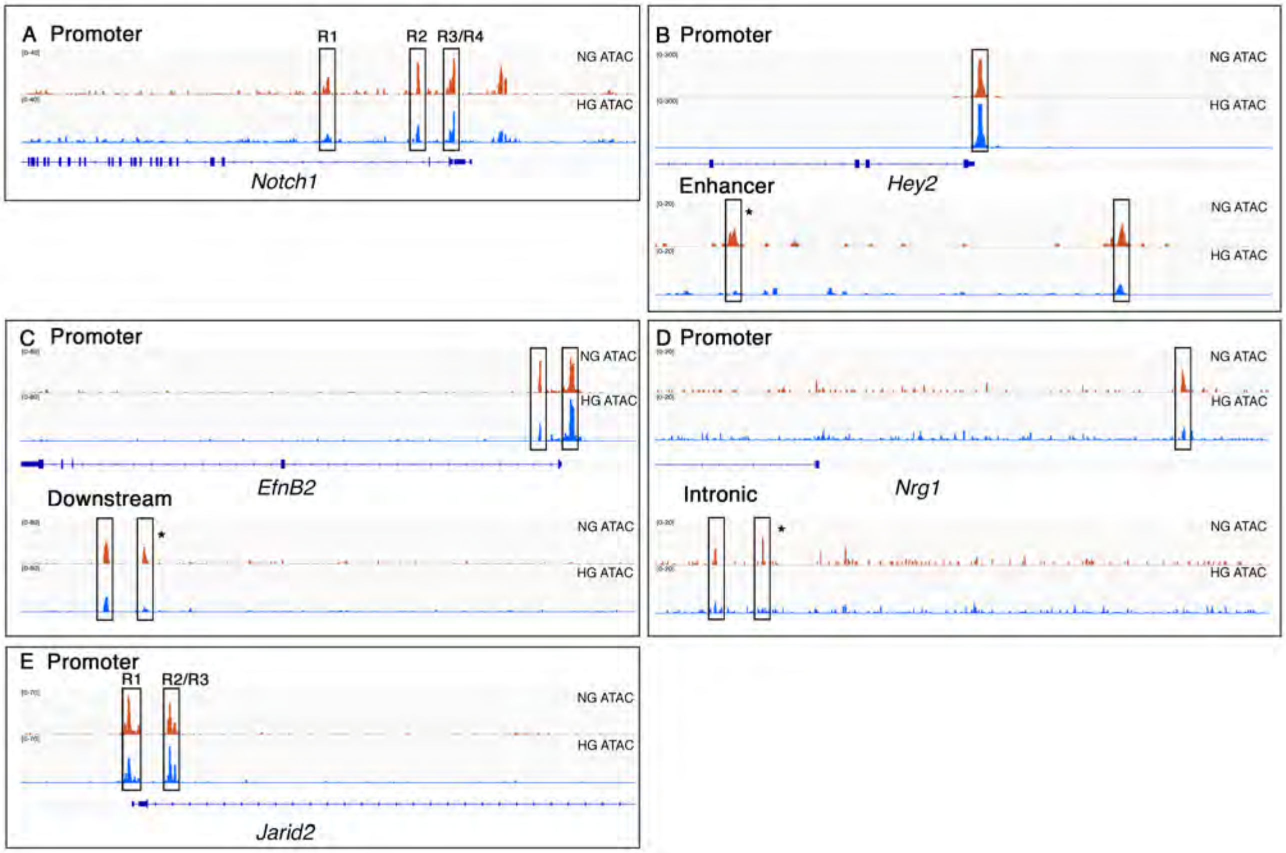
Notch1ChIP_+1150 R.P	CCCACGCCATCTTAAAGAGC



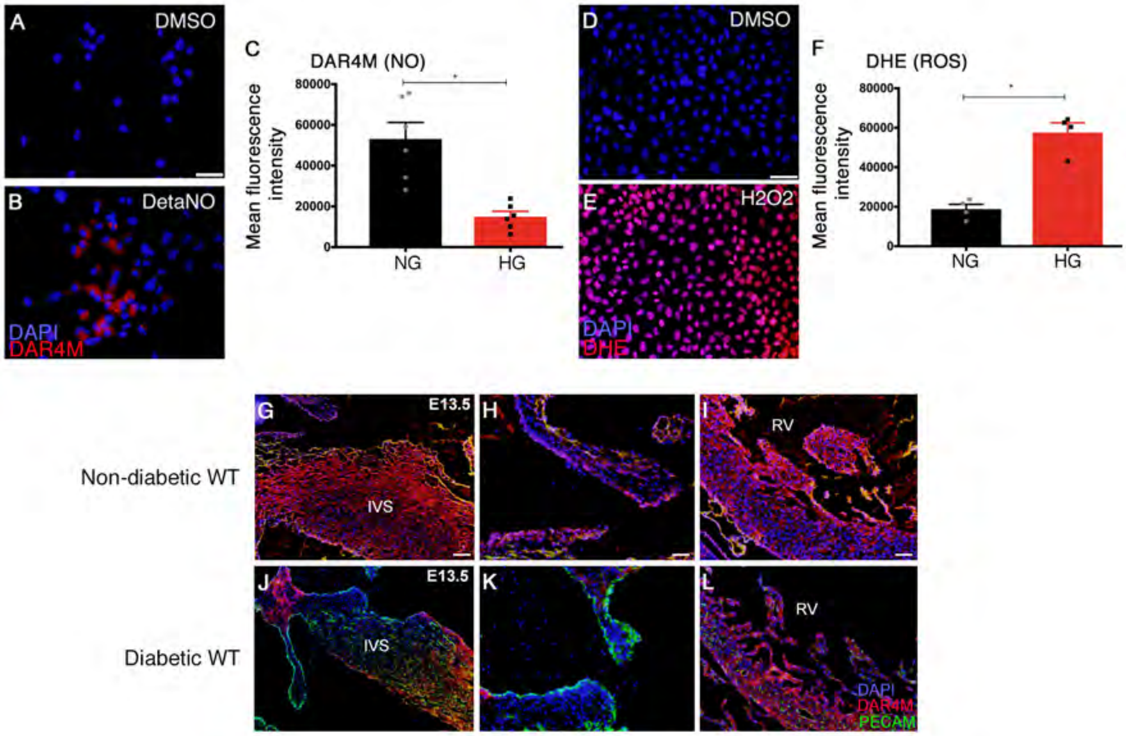
Supplemental Figure 1



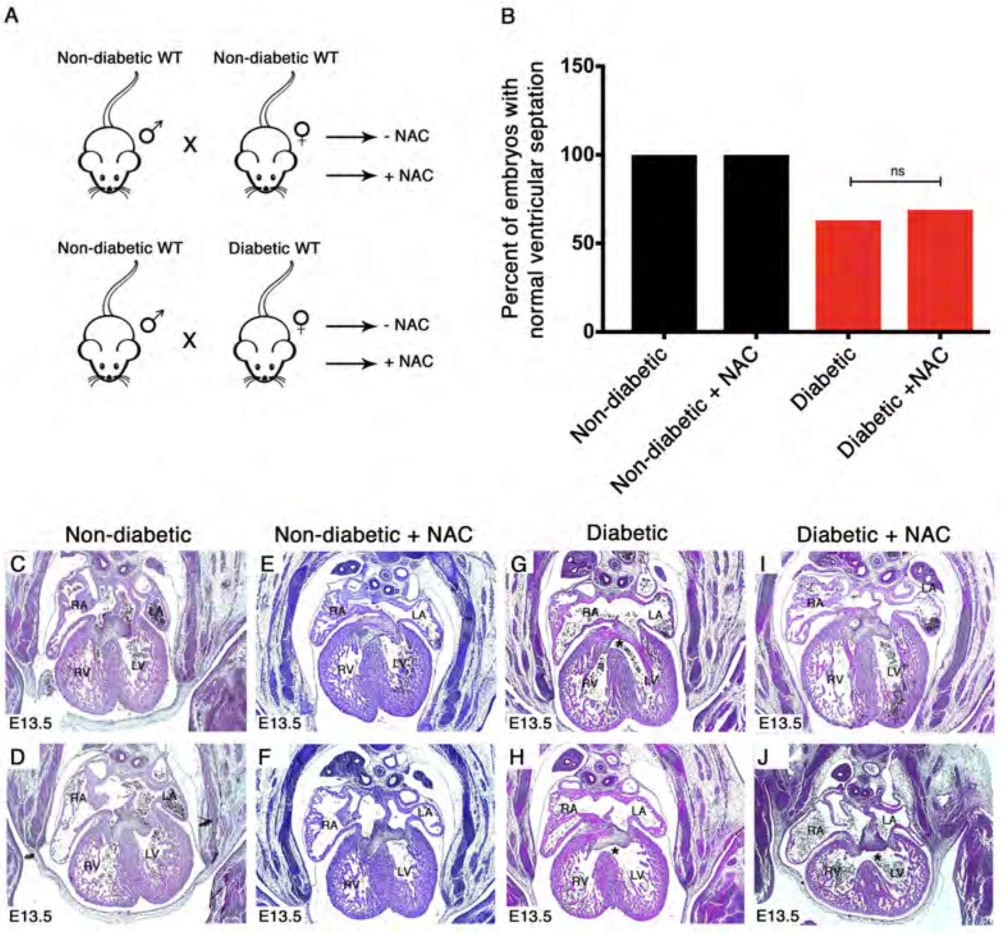
Supplemental Figure 2



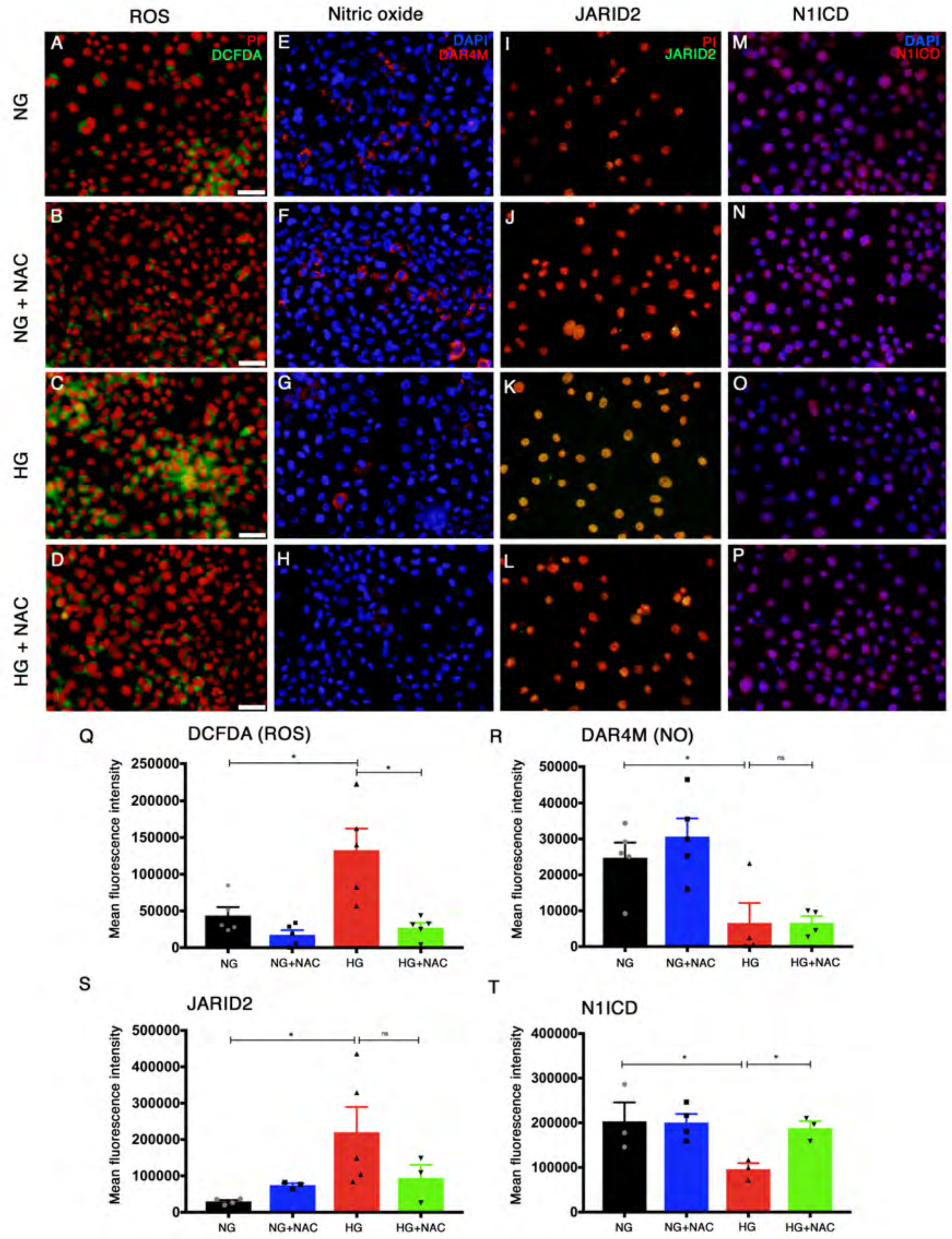
Supplemental Figure 3



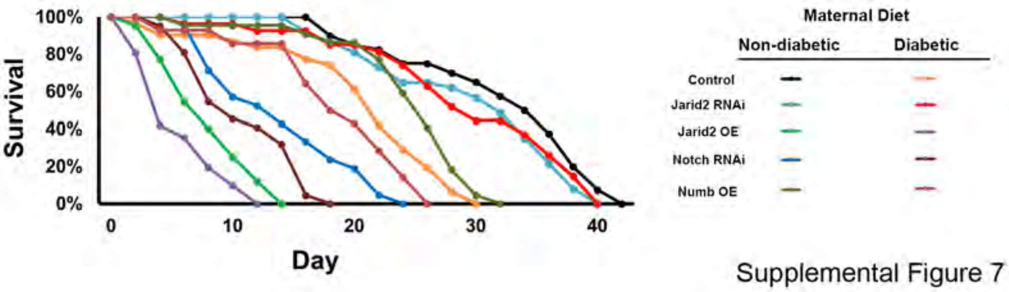
Supplemental Figure 4



Supplemental Figure 5

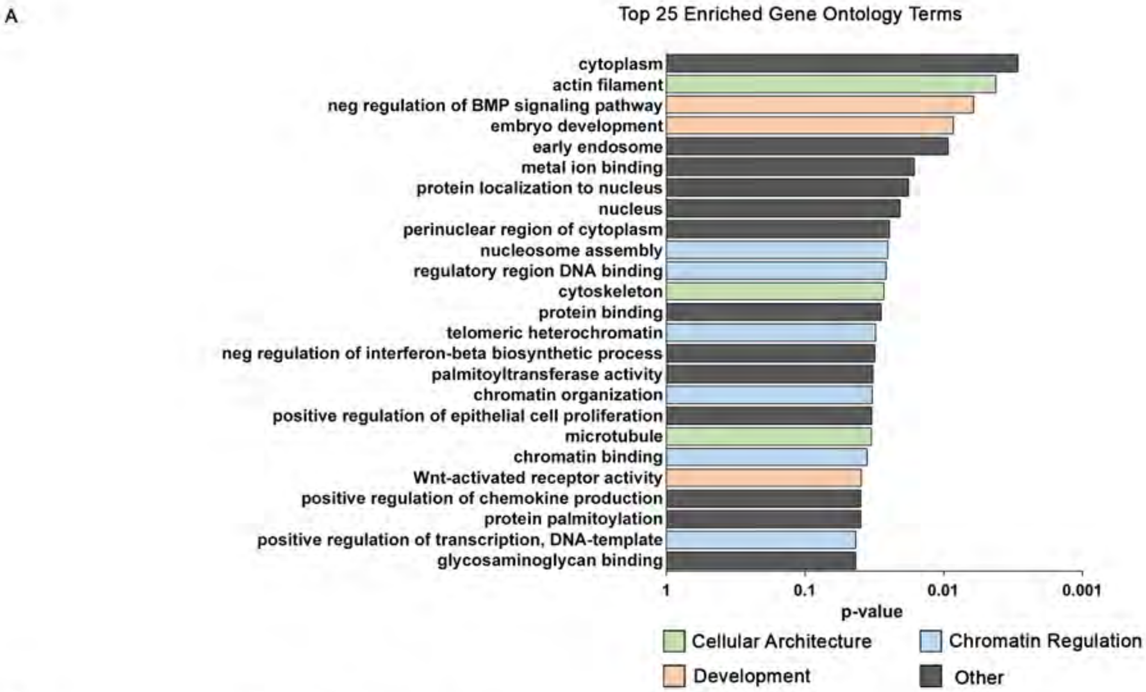


Supplemental Figure 6



Supplemental Figure 7

A



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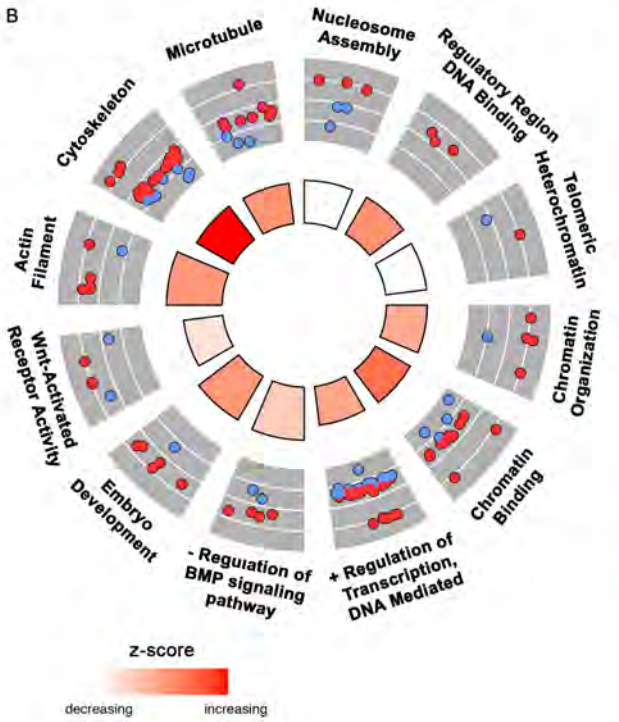

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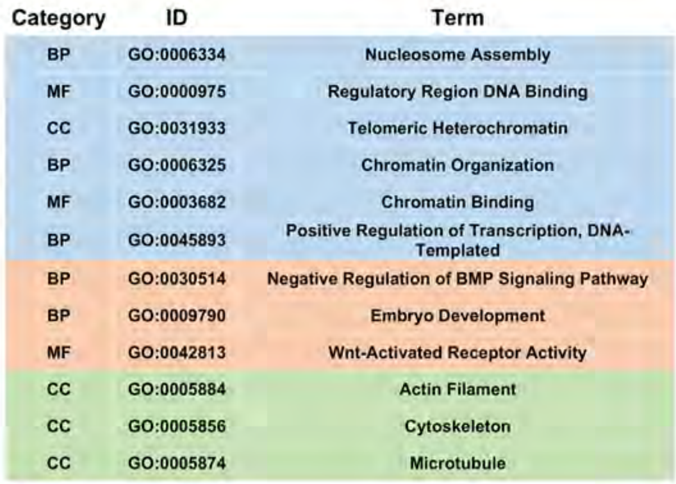
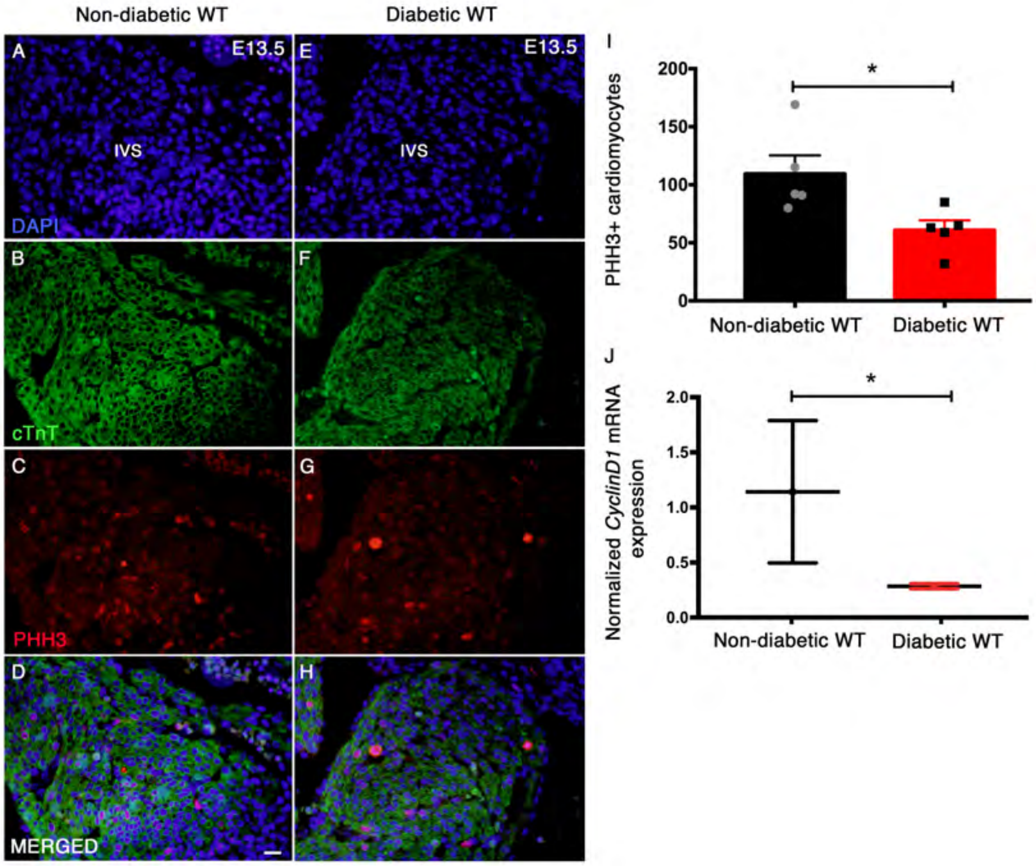


Category	ID	Term
BP	GO:0006334	Nucleosome Assembly
MF	GO:0000975	Regulatory Region DNA Binding
CC	GO:0031933	Telomeric Heterochromatin
BP	GO:0006325	Chromatin Organization
MF	GO:0003682	Chromatin Binding
BP	GO:0045893	Positive Regulation of Transcription, DNA-Templated
BP	GO:0030514	Negative Regulation of BMP Signaling Pathway
BP	GO:0009790	Embryo Development
MF	GO:0042813	Wnt-Activated Receptor Activity
CC	GO:0005884	Actin Filament
CC	GO:0005856	Cytoskeleton
CC	GO:0005874	Microtubule

🟦 Chromatin Regulation
🟧 Development
🟩 Cellular Architecture

Supplemental Figure 8



Supplemental Figure 9