

A specific *CNOT1* mutation results in a novel syndrome of pancreatic agenesis and holoprosencephaly through impaired pancreatic and neurological development

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We report a recurrent *CNOT1* *de novo* missense mutation, NM_016284.4:c.1603C>T, p.(Arg535Cys), resulting in a syndrome of pancreatic agenesis and abnormal forebrain development in three individuals and a similar phenotype in mice. *CNOT1* is a transcriptional repressor which has been suggested as being critical for maintaining embryonic stem cells in a pluripotent state. These findings suggest that *CNOT1* plays a critical role in pancreatic and neurological development and describe a novel genetic syndrome of pancreatic agenesis and holoprosencephaly.

Discovering genes with mutations causal of pancreatic agenesis is crucial to identifying factors needed for pancreatic development. To date pathogenic variants in six genes (*PTF1A* [MIM: 615935], *PDX1* [MIM: 260370], *GATA6* [MIM: 600001], *GATA4* [MIM: 600576], *HNF1B* [MIM: 137920], and *RFX6* [MIM: 615710]) have been reported to severely affect pancreatic development and result in pancreatic agenesis¹. Gene discovery in pancreatic agenesis has shown both similarities and marked differences between pancreatic development in human and mouse. In both species complete loss of function of *PTF1A*, *PDX1* or *RFX6* results in pancreatic agenesis. In contrast, whilst haploinsufficiency of *GATA6* is a common cause of pancreatic agenesis in humans², in mice *Gata6* knockout does not result in abnormal pancreatic development³. Knowledge of human pancreatic development is essential to guide progress of beta-cell replacement therapy for people with Type 1 diabetes.

We investigated an international cohort of 107 individuals diagnosed with pancreatic agenesis - defined by requiring both endocrine (insulin) and exocrine (pancreatic enzymes) replacement therapy within the first 6 months of life - and identified a mutation in a known gene in 98 of them (Table S1). To identify *de novo* mutations in the remaining nine subjects, exome sequencing was performed for the probands and both their unaffected parents when available (n=7) (Online Methods).

We identified a heterozygous missense mutation in *CNOT1* [MIM: 604917], NM_016284.4:c.1603C>T, p.(Arg535Cys), in three individuals with pancreatic agenesis. The variant had arisen *de novo* in two of them and was not present in the DNA sample from the 3rd individual's

father (maternal sample was not available for testing) (Figure 1A, Tables S2 and S3). We confirmed these results by Sanger sequencing (Online Methods, Figure S1). The p.(Arg535Cys) variant is absent from dbSNP138, DECIPHER and GnomAD and affects a residue which is highly conserved across species (up to *C. Elegans*) (Figure 1B). All three *in silico* prediction tools used (AlignGVGD, PolyPhen2 and SIFT accessed through AlamutVisual) predicted the variant to have a deleterious effect on protein function (Online Methods).

The three individuals who were heterozygous for the *CNOT1* p.(Arg535Cys) variant had strikingly similar clinical features (see Supplemental Note). In addition to pancreatic agenesis all three had definite (n=2) or possible holoprosencephaly (Figure 1A and Table S4), a disorder in which the prosencephalon (forebrain of the embryo) fails to develop into two hemispheres. P01 and P02 (who was previously reported by Hilbrands *et al*⁴) both had partial/semi-lobar holoprosencephaly, whilst P03 has dysmorphic features which could be consistent with holoprosencephaly (prominent central incisors and occiput, highly arched palate, and low-set ears) but brain MRI was declined by his parents and the diagnosis could not therefore be confirmed. All three individuals had very low birth weight (Z-score<-2), likely due to insulin deficiency in the last trimester of pregnancy, when insulin is the main foetal growth factor. Consistent with insulin deficiency in utero, the three case subjects all developed diabetes very early (2/3 diagnosed at 1 day and one at 13 weeks). P01 and P2 also had gallbladder agenesis, a clinical feature frequently associated with pancreatic agenesis.

The DDD study⁵ has identified *de novo* *CNOT1* variants in three individuals with developmental delay (two missense, p.(Leu2323Phe) and p.(Arg623Trp), and a nonsense (p.(Gln33*)) variant) but none of them had holoprosencephaly or diabetes. Since our three case subjects were all heterozygous for the same novel missense *CNOT1* variant and none of the DDD participants with heterozygous *de novo* *CNOT1* variants had pancreatic or neurological structural malformations, we hypothesised that a mutation-specific mechanism rather than loss of function was responsible for the phenotype seen in our cases. We therefore generated a mouse line harbouring the *Cnot1*^{p.(Arg535Cys)} mutation using CRISPR (Online Methods).

Heterozygous mice were born at a lower than expected frequency (Table S5), but without an obvious phenotype, while homozygosity for the mutation was embryonically lethal. At E14.5, embryos were still alive and present at expected Mendelian ratios, and were therefore collected to assess their phenotype (Online Methods). Upon dissection, several gross morphological abnormalities were apparent in homozygotes, notably exencephaly, eye defects (mostly coloboma) and oedema (Figure 2A, 2B, Figure S2 and Table S6).

High resolution episcopic microscopy (HREM) highlighted a significant reduction in the size of the pancreas in homozygous embryos in addition to several other abnormalities (Figure 2C-G, Online Methods, DMDD website). The reduction in pancreatic size was found to be predominantly due to a smaller dorsal pancreas (Figure 2H, Figure S3). These results provide compelling evidence for the role of *Cnot1*^{p.(Arg535Cys)} in pancreatic development.

Expression analysis of pancreatic developmental factors on RNA extracted from pancreatic tissue in E14.5 wild-type, *Cnot1*^{p.(Arg535Cys)/p.(Arg535Cys)} and *Cnot1*^{p.(Arg535Cys)} embryos showed a significant increase of *Shh* expression in homozygous embryos, with decreased expression in *Pdx1*, *Ins*, *Hnf1b* and *Ptf1a* (Figure 3A). No difference in expression was detected for *Gata6* (Figure 3A) and *Rxra* (Figure S4).

The pancreatic and neurological phenotypes observed in *Cnot1*^{p.(Arg535Cys)/p.(Arg535Cys)} E14.5 mouse embryos are consistent with the pancreatic agenesis and holoprosencephaly observed in the three case subjects, confirming that the *de novo* *CNOT1* mutation is indeed the cause of their disease. Mice required a homozygous mutation in *Cnot1* to display a pancreatic and brain phenotype while a heterozygous *CNOT1* mutation resulted in the phenotype in three individuals in our cohort. This has been described with other pancreatic developmental genes (e.g *HNF1B*) and supports the hypothesis that the early stages of pancreatic development are not identical in mice and humans⁶.

The *CNOT1* protein has not previously been suggested to have a role in pancreatic development; it is known to act both as scaffold of the CCR4-NOT complex and as an independent factor. As such, it

mediates transcriptional repression⁷ and is expressed extremely early during embryonic development (E3.5 in the inner cell mass in mice⁸). *In vitro* studies have proposed that CNOT1 plays a critical role in maintaining human and mice embryonic stem cells in a pluripotent state by inhibiting primitive endoderm factors⁸. *CNOT1* expression peaks in undifferentiated human iPS cells compared to subsequent stages of *in vitro* differentiation towards pancreatic endocrine cells⁹, supporting its fundamental role in stem cells.

The increased expression of *Shh* in pancreatic tissue extracted from *Cnot1*^{p.(Arg535Cys)/p.(Arg535Cys)} embryos would be consistent with a model in which the *CNOT1* p.(Arg535Cys) mutation results in embryonic stem cells being maintained in an undifferentiated state through SHH-mediated inhibition of differentiation. SHH is a key developmental factor which is known to be crucial for pancreatic and brain development. Heterozygous loss of function mutations in *SHH* cause holoprosencephaly [MIM: 142945] and studies in both mouse and human embryos have shown that *SHH* expression needs to be repressed in the dorsal foregut endoderm for successful differentiation towards dorsal pancreas^{6,10}. A recent study has suggested that the transcription factors *Gata4* and *Gata6* (mutations in which are a cause of pancreatic agenesis) regulate pancreatic endoderm identity by directly inhibiting *Shh* in mice¹¹. It is therefore possible that the p.(Arg535Cys) variant results in *CNOT1* maintaining its inhibition activity on the GATA and other early differentiation factors and, as a consequence, SHH expression is not repressed (Figure 3B). Increased expression of *Shh* and decreased expression of *Pdx1*, *Ins*, *Hnf1b* and *Ptf1a* detected in RNA extracted from pancreatic tissue in the E14.5 *Cnot1*^{p.(Arg535Cys)/p.(Arg535Cys)} embryos would support this hypothesis. However, *Gata4* expression could not be assessed as the assay specificity was too low and *Gata6* expression was not found to be reduced. It is possible that *Gata6* activity is actually inhibited earlier during development and then re-activated by a different pathway (*Gata6* is needed for development of most endodermal-derived organs and heart) or could be inhibited by a different mechanism that does not result in reduced expression. The *CNOT1* p.(Arg535Cys) mutation also affects neurological development in both our cases and mouse embryos. It is possible that this mutation results in ectopic SHH expression during brain development. This would be consistent with previous reports of *Shh* ectopic expression impairing midline development¹². Another possibility is that the effect of the *CNOT1* mutation on SHH signalling differs between the brain and the pancreas, resulting in a reduced expression in the developing brain and increased expression during pancreatic development. Further experiments, ideally on younger embryos and human induced pluripotent stem cells, are needed in order to elucidate the mechanism by which the *CNOT1* p.(Arg535Cys) mutation results in impaired pancreatic and neurological development.

Our study identifies a spontaneous *CNOT1* p.(Arg535Cys) mutation as the genetic cause of a rare syndrome of pancreatic agenesis and holoprosencephaly, highlighting a previously unsuspected role of *CNOT1* as a key factor in both pancreatic and neurological development. This is the 5th gene causative of pancreatic agenesis described so far and the first pancreatic agenesis gene which is thought to be important for maintaining stem cells' pluripotency. These findings suggest a new mechanism by which impairment of the very early stages of development result in pancreatic agenesis and abnormal brain development.

Declaration of Interests

The authors declare no competing interests.

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Web Resources

<https://www.omim.org/>

<https://decipher.sanger.ac.uk/>

<https://gnomad.broadinstitute.org>

<https://dmdd.org.uk>

References

1. De Franco, E. & Ellard, S. Genome, Exome, and Targeted Next-Generation Sequencing in Neonatal Diabetes. *Pediatr Clin North Am* **62**, 1037-53 (2015).
2. Allen, H.L. *et al.* GATA6 haploinsufficiency causes pancreatic agenesis in humans. *Nat Genet* **44**, 20-22 (2011).
3. Morrissey, E.E. *et al.* GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev* **12**, 3579-90 (1998).
4. Hilbrands, R. *et al.* Pancreas and gallbladder agenesis in a newborn with semilobar holoprosencephaly, a case report. *BMC Med Genet* **18**, 57 (2017).
5. Firth, H.V. & Wright, C.F. The Deciphering Developmental Disorders (DDD) study. *Dev Med Child Neurol* **53**, 702-3 (2011).
6. Jennings, R.E. *et al.* Development of the human pancreas from foregut to endocrine commitment. *Diabetes* **62**, 3514-22 (2013).
7. Winkler, G.S., Mulder, K.W., Bardwell, V.J., Kalkhoven, E. & Timmers, H.T. Human Ccr4-Not complex is a ligand-dependent repressor of nuclear receptor-mediated transcription. *Embo j* **25**, 3089-99 (2006).
8. Zheng, X. *et al.* Cnot1, Cnot2, and Cnot3 maintain mouse and human ESC identity and inhibit extraembryonic differentiation. *Stem Cells* **30**, 910-22 (2012).
9. van de Bunt, M. *et al.* Insights into islet development and biology through characterization of a human iPSC-derived endocrine pancreas model. *Islets* **8**, 83-95 (2016).
10. Apelqvist, A., Ahlgren, U. & Edlund, H. Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. *Curr Biol* **7**, 801-4 (1997).
11. Xuan, S. & Sussel, L. GATA4 and GATA6 regulate pancreatic endoderm identity through inhibition of hedgehog signaling. *Development* **143**, 780-6 (2016).
12. Huang, X., Litingtung, Y. & Chiang, C. Ectopic sonic hedgehog signaling impairs telencephalic dorsal midline development: implication for human holoprosencephaly. *Hum Mol Genet* **16**, 1454-68 (2007).

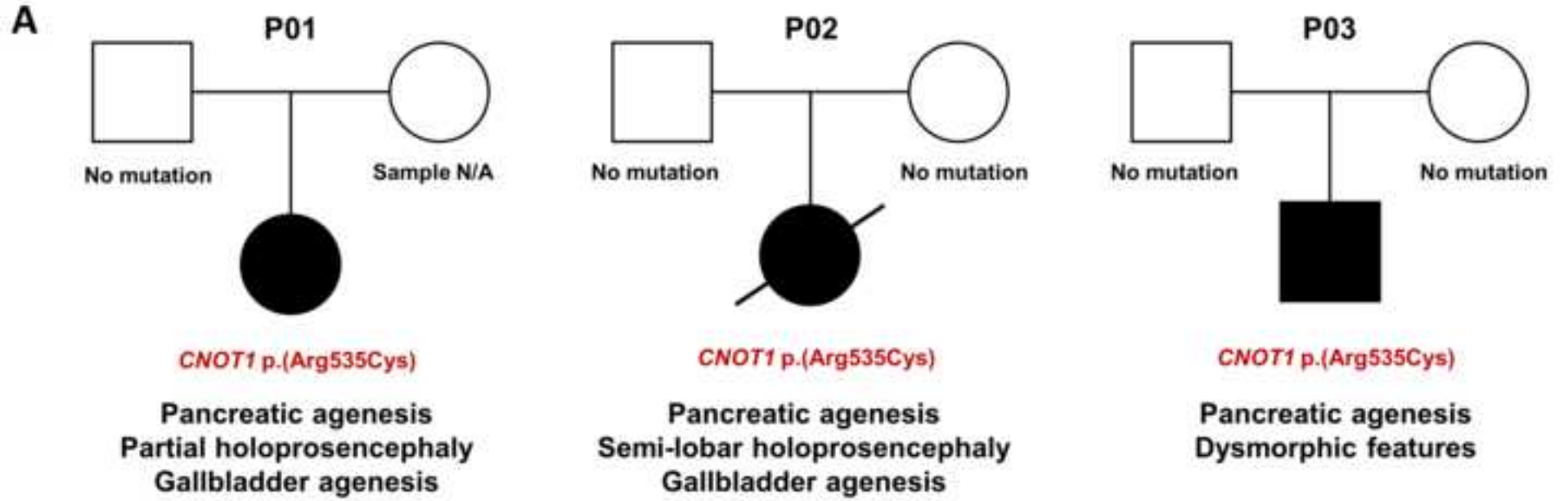
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Figure 1. Genetic and clinical findings in individuals with pancreatic agenesis. 1A: Partial pedigrees and clinical features of the three individuals with the heterozygous *CNOT1* p.(Arg535Cys) mutation. **1B:** Conservation of *CNOT1* residues 529 to 568 across 10 representative species. Residue p.Arg535 is highlighted in red. Residues identical to the human *CNOT1* protein are highlighted in blue, differences are highlighted in grey. **1C** Coronal brain MRI of P02 showing absence of the anterior interhemispheric fissure (red arrow), fusion of the frontal lobes (orange arrow), absence of frontal horns (green arrow), absence of the sylvian fissures (yellow arrow). Splenium of the corpus callosum is visible (blue arrow). **1D** Post mortem image of P02's liver (L), spleen (S), and duodenum (D). White arrow shows the orthotopic location of the pancreas, which is absent. Dashed arrow indicated site in which the absent gallbladder would be expected to be.

Figure 2. Neurological and pancreatic abnormalities in mouse embryos homozygous for the *Cnot1* p.(Arg535Cys) mutation. 2A Table listing the gross external phenotypes observed in E14.5 embryos. Numbers do not add to total as many embryos displayed multiple phenotypes. Significance by Fisher's exact test, assuming an additive model. Exencephaly: $p=3.2 \times 10^{-9}$, Spina bifida: $p=0.027$, Eye defect: $p=5.5 \times 10^{-8}$, Oedema: $p=2.6 \times 10^{-7}$, Midline defect: ns **2B** Images showing representative E14.5 embryos: top shows wildtype embryo, bottom shows embryo homozygous for the *CNOT1* p.(Arg535Cys) mutation with exencephaly and coloboma. **2C and 2E:** Coronally sectioned, semi-transparent 3D volume models of stage matched E14.5 embryos with superimposed models of the pancreas of a homozygous (C) and wildtype (E) embryos. **2D.** Overlay of extracted surface models of the pancreas of a homozygous (blue, magenta), and control (orange, green). **2F, 2G.** Coronally sectioned solid 3D volume rendered model of the abdomen of the embryos shown in 2C & 2E with superimposed pancreas. dp, dorsal pancreas; vp, ventral pancreas; li, liver lobes; s, stomach; sp, spleen; k, kidney; g, gonad; bd, bile duct. Scale bars: 1000 μ m in C, E-G; 500 μ m in D. **2H:** Graph showing the volume of the dorsal pancreas of E14.5 embryos in μm^3 . Blue squares show wildtypes, green circles are heterozygotes and red triangles are homozygotes. Data analysed using ANOVA with TukeyHSD posthoc test, effect of genotype $p=8.85 \times 10^{-8}$; post-hoc WT-Hom, $p < 10^{-10}$; Het-Hom, $p=1.36 \times 10^{-4}$, WT-Het, ns.

Figure 3. Expression data and possible mechanism involving *CNOT1* in pancreatic development 3A Graphs showing relative expression of genes in the pancreas of E14.5 embryos. Bars show mean +/- SE. Data analysed using ANOVA with TukeyHSD posthoc test. Results of posthoc tests shown on graphs, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *Shh*; effect of genotype $p=0.0107$. *Pdx1*; effect of genotype $p=0.0189$. *Ins*; effect of genotype $p=7.03 \times 10^{-6}$. *Hnf1b*; effect of genotype $p=0.0294$. *Ptf1a*; effect of genotype $p=0.00781$. *Gata6*; effect of genotype $p=ns$. $n=4-12$ animals per genotype. **3B** Schematic representation of the proposed role for *CNOT1* in pancreatic development.

Figure 1



B

| | p. | 535 | 568 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--------------|----|-----|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human | G | Q | S | P | S | I | R | Q | L | I | M | H | A | M | A | R | W | Y | M | R | G | E | Q | Y | D | W | A | K | L | S | R | I | L |
| Rhesus | G | Q | S | P | S | I | R | Q | L | I | M | H | A | M | A | R | W | Y | M | R | G | E | Q | Y | D | W | A | K | L | S | R | I | L |
| Mouse | G | Q | S | P | S | I | R | Q | L | I | M | H | A | M | A | R | W | Y | M | R | G | E | Q | Y | D | W | A | K | L | S | R | I | L |
| Dog | G | Q | S | P | S | I | R | Q | L | I | M | H | A | M | A | R | W | Y | M | R | G | E | Q | Y | D | W | A | K | L | S | R | I | L |
| Elephant | G | Q | S | P | S | I | R | Q | L | I | M | H | A | M | A | R | W | Y | M | R | G | E | Q | Y | D | W | A | K | L | S | R | I | L |
| Chicken | G | Q | S | P | S | I | R | Q | L | I | M | H | A | M | A | R | W | Y | M | R | G | E | Q | Y | D | W | A | K | L | S | R | I | L |
| X Tropicalis | G | Q | S | P | S | I | R | Q | L | I | M | H | A | M | A | R | W | Y | M | R | G | E | Q | Y | D | W | A | K | L | S | R | I | L |
| Zebrafish | G | Q | S | P | S | I | R | Q | L | I | M | H | A | M | A | R | W | Y | M | R | G | E | Q | Y | D | W | A | K | L | S | R | I | L |
| Lamprey | G | Q | S | P | T | I | R | Q | L | I | M | H | A | M | A | R | W | Y | M | R | G | E | Q | Y | D | W | T | R | L | S | R | I | L |
| C Elegans | S | L | S | K | H | M | R | Q | H | V | I | Y | C | L | T | S | M | H | A | A | | | | | D | S | S | Q | L | A | K | I | L |

CNOT1 exon 14

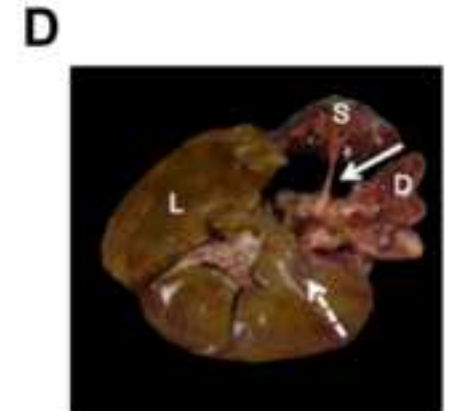
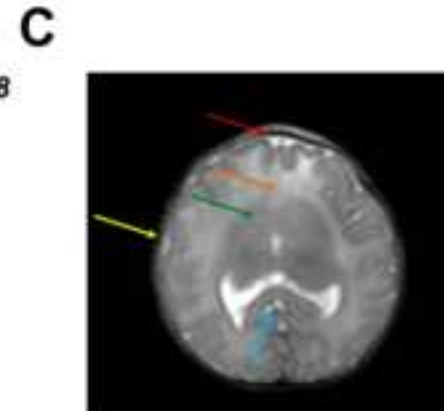


Figure 2

A

| Genotype | Wildtypes | Heterozygotes | Homozygotes |
|----------------------------|-----------|---------------|-------------|
| Phenotype | Count (%) | Count (%) | Count (%) |
| Exencephaly | 0 (0%) | 0 (0%) | 12 (35%) |
| Spina bifida | 0 (0%) | 1 (1%) | 3 (9%) |
| Eye defect | 0 (0%) | 3 (4%) | 13 (38%) |
| Oedema | 2 (4%) | 11 (15%) | 19 (56%) |
| Failure of midline fusion | 0 (0%) | 3 (4%) | 1 (3%) |
| Other phenotypes | 8 (18%) | 6 (8%) | 7 (21%) |
| Normal external appearance | 35 (78%) | 57 (77%) | 8 (24%) |
| TOTALS | 45 | 74 | 34 |

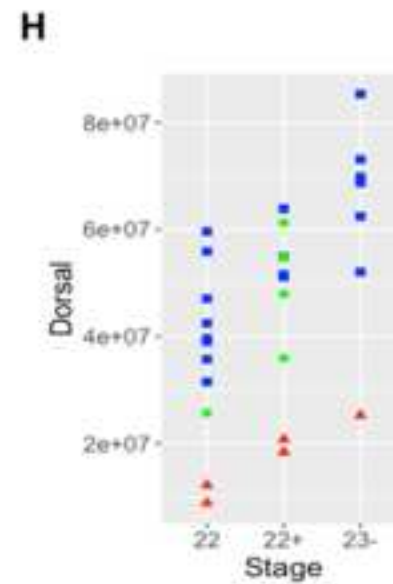
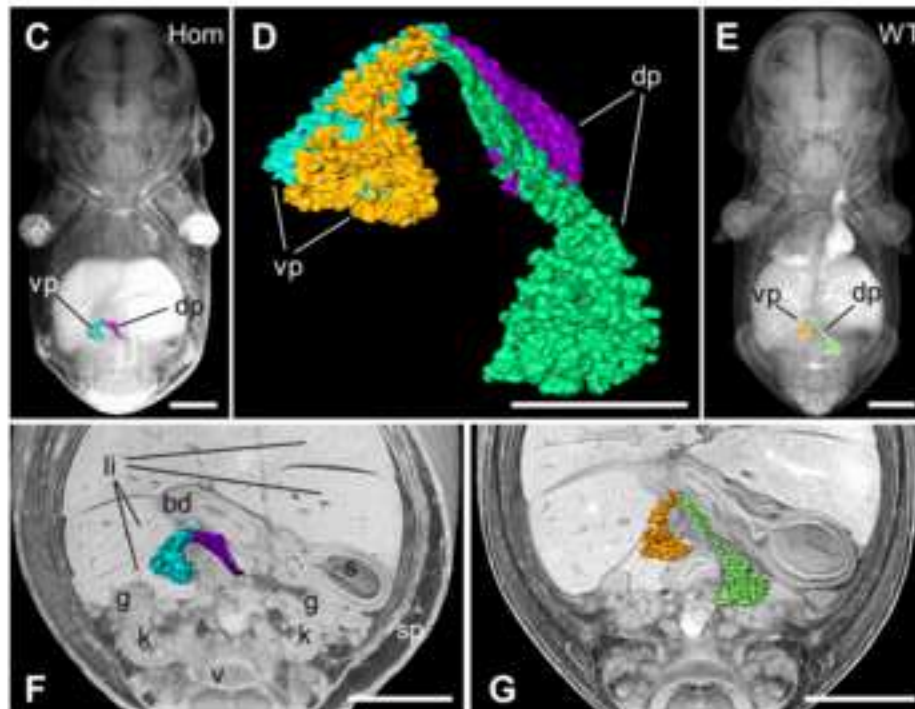
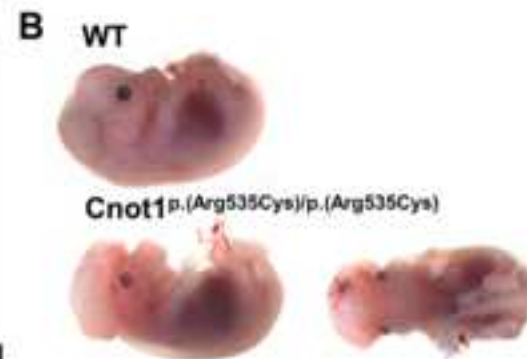
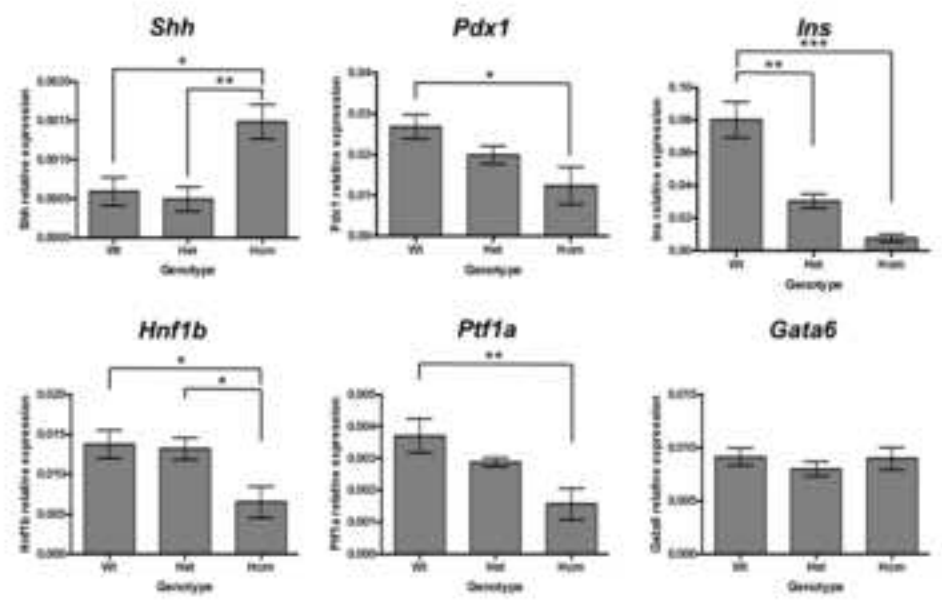


Figure 3

A



B

