

**Developmental inhibition of long intergenic non-coding RNA,
HOTAIRM1, impairs dopamine neuron differentiation and maturation.**

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SUPPLEMENTARY METHODS

Immunohistochemistry

Three dams underwent electroporation at E11 as described in the manuscript methods. At E13, 7 fetal brains were immediately dissected and immersion fixed in 4% paraformaldehyde overnight at 4 °C. Fixed brains were soaked in 30% sucrose solution prior to being embedded in Cryo-Embedding Compound (TissueTek, Emgrid Australia). Brain specimens were placed on a cryostat, and 20-µm coronal sections were cut through the entire mesencephalic region. One of every five sections were processed for immunohistochemistry. All sections were processed at the same time to exclude batch variation. The blocking and antibody buffer consisted of a phosphate-buffered saline solution with 0.5% Bovine serum albumin, 0.05% saponin, 0.1% Triton X-100 (Sigma-Aldrich, USA) and 0.05% sodium azide. Primary antibodies used for immunostaining were chicken anti-GFP (1:750, ab13970, Abcam, USA), sheep anti-TH (1:200, NB300, NOVUS biologicals, USA) and rabbit anti-Nurr1 (1:500, sc-990, Santa Cruz Biotechnology, USA). Fluorophore-conjugated secondary antibodies were donkey anti-chicken conjugated Alexa-488 (1:1000, CF488A, Biotum, USA), donkey anti-sheep conjugated Alexa-555 (1:1000, A-21436, Thermo Fisher Scientific, Australia) and donkey anti-rabbit conjugated Alexa-647 (1:1000, A-31573 Thermo Fisher Scientific, Australia). Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma-Aldrich, Australia).

Image acquisition and analysis

All images of immunostained brain sections were immediately visualised and imaged using a Nikon Plan Apo Lambda 60x/1.4 NA oil-immersion objective on a spinning disk confocal microscope (Diskovery; Andor Technology, UK) built around a Nikon Ti-E body (Nikon Corporation, Japan) and equipped with two Zyla 4.2 sCMOS cameras (Andor Technology) and controlled by Nikon NIS software. Images were captured with a ROI of 1500 x 1500

pixels and at a z-step size of 0.3 μm , providing a voxel size $0.093 \times 0.093 \times 0.3 \mu\text{m}^3$. All images were deconvolved with Huygens Professional version 18.04 (Scientific Volume Imaging, The Netherlands). A single 2D slice from the middle of the z-stack was used for image analysis and quantification in CellProfiler 3 [www.cellprofiler.org; 1]. All sections where eYFP was visible in the ventral midbrain were imaged and analysed (at least 2 per embryo). Positive eYFP counts from these sections were then adjusted to reflect the total proportion of cells in the ventral midbrain (rather than the specific sections imaged, as there were no eYFP+ cells observed on the remaining sections).

Table S1. Electroporation survival and numbers

Electroporation age	Embryos per litter (# dams)	Electroporated embryos per litter	Surviving embryos (%)
E11.5	13.7 ± 0.8 (10)	6.5 ± 0.3	64.5 ± 8.6

Table S2. Real Time-PCR primers

Gene	Species	Accession No.	Sequence (5'-3') Forward (F) and Reverse (R)
HPRT	Mus musculus	NM_013556	F: CAGTACAGCCCCAAAATGGT R: TTGCGCTCATCTTAGGCTTT
HOTAIRM1	Mus musculus	NR131181	F: GAGTCGAGACTGCCTTCTGC R: ACCCCCATTTCAGTGTGGT
TH	Mus musculus	NM_009377	F: CGCCGTCCAATGAACCTT R: CACTATGCCACCCCCAG
Lmx1a	Mus musculus	NM_033652	F: AACCAGCGAGCCAAGATGAA R: TGGGTGTTCTGTTGGTCCTGT
Nurr1	Mus musculus	NM_013613	F: GTGTTTCAGGCGCAGTATGG R: TGGCAGTAATTTTCAGTGTGGT
NgN2	Mus musculus	NM_009718	F: ATCTGGAGCCGCGTAGGAT R: CATCAGTACCTCCTCTTCCTCCTT
DAT	Mus musculus	NM_010020	F: CATTGCCACATCCTCCATGG R: CATTGCCACATCCTCCATGG
COMT	Mus musculus	NM_007744	F: GTCATCCTGATGGCCTCACT R: TACCGTCTGAGTCGTTGCTG
DLK1	Mus musculus	NM_010052	F: GGACAGGCCATCTGCTTCAC R: GCTCCTCGCCGCTGTTATAC
AHD2	Mus musculus	NM_001361504	F: GGAATACCGTGTTGTCAAGCC R: CCAGGGACAATGTTTACCACGC
Ki67	Mus musculus	NM_001081117	F: ATCATTGACCGCTCCTTTAGGT R: GCTCGCCTTGATGGTTCCT
MAOa	Mus musculus	NM_173740	F: GGGGGCTGTCATCAAGTGCAT R: TGGCAGGCATTGACCCATCTG
VMAT2	Mus musculus	NM_172523	F: GCAGTTGTGGTCCATGAG R: GCAGTTGTGGTCCATGAG

GAD65	Mus musculus	NM_008078	F: AATGGTGTTTGATGGGAAGC R: AGGGTTTGAGATGACCATGC
YWHAZ	Mus musculus	NM_011740	F: TAGGTCATCGTGGAGGGTCG R: GAAGCATTGGGGATCAAGAACTT
TH	Homo sapiens	NM_199293	F: GCAGTTCTCGCAGGACATTG R: TGGATGCGTGAGGCATAGC
HPRT	Homo sapiens	NM_000194	F: TGACCAGTCAACAGGGGACA R: GCGACCTTGACCATCTTTGG
MAOa	Homo sapiens	NM_000240	F: GTGTCAGCCAAAGCATGGAG R: GCAGCAGATAGTCCTGAAATGC
VMAT2	Homo sapiens	NM_003054	F: CCGTACATCCTCATTGCTGC R: CCACCTCCCCATTTTGTGTG
NgN2	Homo sapiens	NM_024019	F:GCAAGCGTGGAAATTTAGGC R:GCAATCCTCCCTCCTGATT
<i>HOTAIRM1</i>	Homo sapiens	NR_038366	F:TAGTTATTGACCTGGAGACTGGTAGC R:TCAGTGCACAGGTTCAAGCC

All Primers were purchased from Sigma-Aldrich.

SUPPLEMENTARY RESULTS

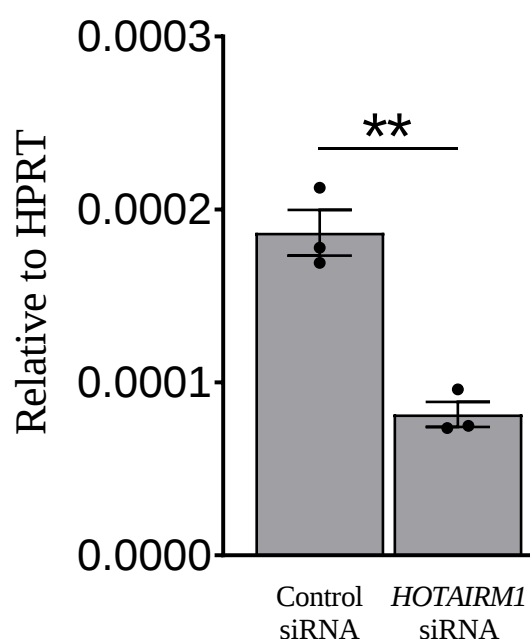


Figure S1. Expression levels of *HOTAIRM1* in SH-SY5Y cells after 24 h of transfection with 20 nM of control siRNA or *HOTAIRM1* siRNA. *HOTAIRM1* siRNA reduced *HOTAIRM1* levels by over 50% relative to control siRNA ($t_{1,4}=6.9$, $p<0.01$).

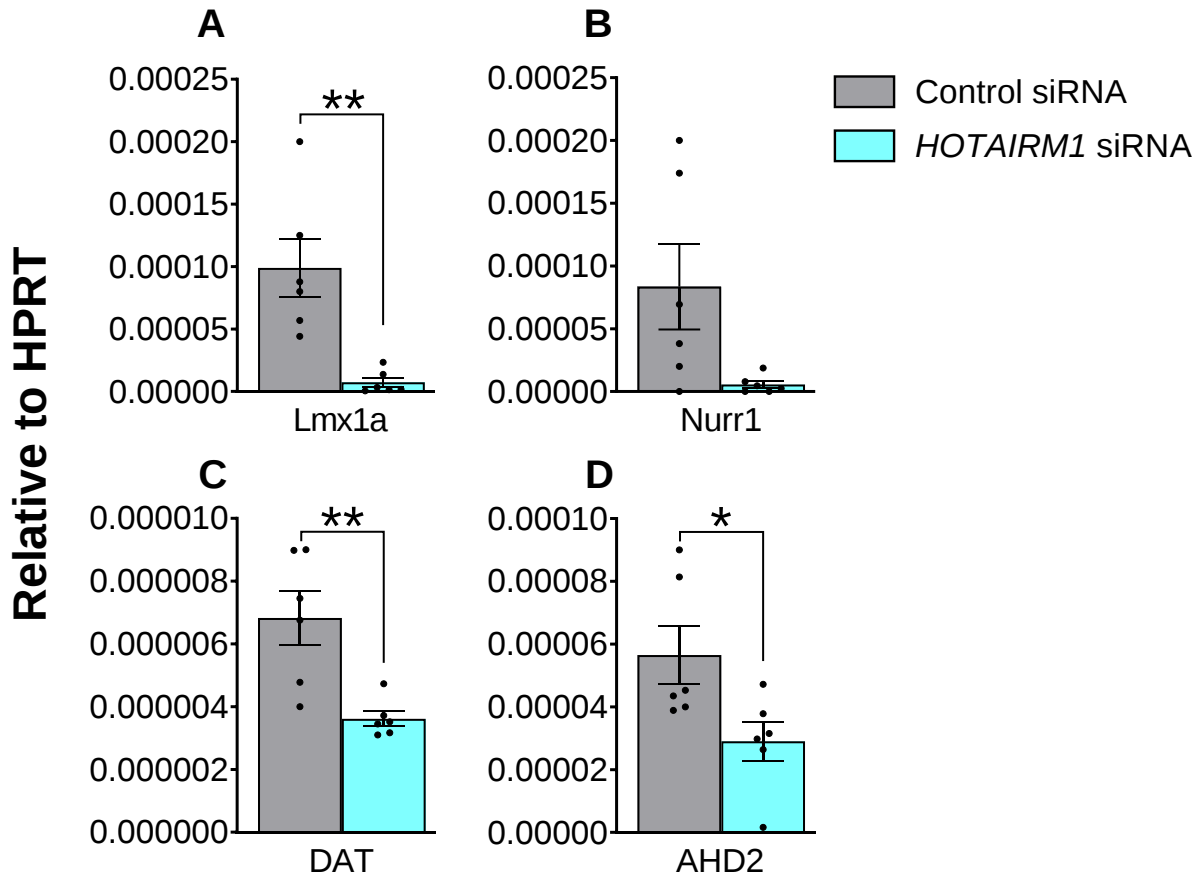


Figure S2. *In vitro* expression levels of Lmx1a (A), Nurr1 (B), DAT (C) and AHD2 (D) after *HOTAIRM1* siRNA transfection and 48 h differentiation in SHSY5Y cells. Levels were extremely low for all targets (requiring over 35 cycles of PCR). However, *HOTAIRM1* attenuation did significantly decrease Lmx1a ($F_{1,10}=14.6$, $p<0.01$), DAT ($F_{1,10}=4.9$, $p<0.01$) and AHD2 ($F_{1,10}=6.3$, $p<0.05$) levels. siRNA, small interfering RNA; *HOTAIRM1*, HOX-antisense intergenic RNA myeloid 1; *Lmx1a*, LIM homeodomain gene 1a; *Nurr1*, nuclear receptor related 1 protein; *DAT*, dopamine transporter; *AHD2*, aldehyde dehydrogenase-1a1. Data are expressed as Mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

Table S3. In vivo E13 raw PCR data ($^{\wedge}$ dCT)

	Wild-type (n=7-8)		Control siRNA (n=6-7)		<i>HOTAIRM1</i> siRNA (n=7-9)	
	Mean	SEM	Mean	SEM	Mean	SEM
<i>HOTAIRM1</i>	0.009038	0.001658	0.007765	0.000489	0.006300	0.000602
TH	0.170731	0.021572	0.179574	0.024847	0.179280	0.012391
<i>Lmx1a</i>	0.218766	0.015728	0.199240	0.019078	0.216141	0.007426
<i>Ngn2</i>	0.068547	0.002837	0.072080	0.004636	0.073056	0.004613
<i>Nurr1</i>	1.995351	0.179458	1.810895	0.151794	1.809064	0.087033
COMT	0.448983	0.021077	0.441362	0.027647	0.459967	0.019471
GAD65	1.814011	0.048398	1.706021	0.104366	1.732414	0.063425
AHD2	0.005878	0.001043	0.009950	0.002562	0.003542	0.000501
DLK1	1.524257	0.126563	1.292695	0.056949	1.361457	0.070035
Ki67	0.155954	0.011839	0.162774	0.011133	0.215717	0.020321

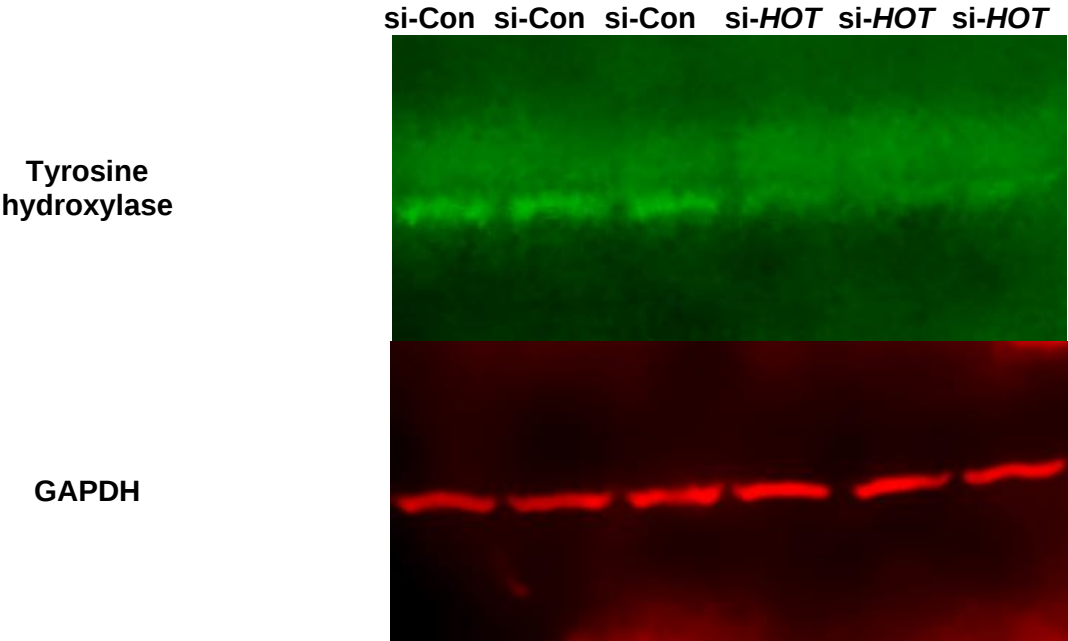
siRNA, small interfering RNA; *HOTAIRM1*, HOX-antisense intergenic RNA myeloid 1; *TH*, tyrosine hydroxylase; *Lmx1a*, LIM homeodomain gene 1a; *Ngn2*, neurogenin 2; *Nurr1*, nuclear receptor related 1 protein; *COMT*, Catechol-O-Methyltransferase; *GAD65*, Glutamate decarboxylase 65; *AHD2*, aldehyde dehydrogenase-1a1; *DLK1*, Delta Like Non-Canonical Notch Ligand 1.

Table S4. In vivo E17 raw PCR data ($^{\wedge}$ dCT)

	Control siRNA (n=9)		<i>HOTAIRM1</i> siRNA (n=6)	
	Mean	SEM	Mean	SEM
TH	0.076048	0.002810	0.059451	0.004944
<i>Imx1a</i>	0.063052	0.004347	0.048026	0.002282
<i>Ngn2</i>	0.000777	0.000054	0.000849	0.000103
<i>Nurr1</i>	0.331911	0.014314	0.262107	0.017453
DAT	0.059102	0.003167	0.040410	0.003987
VMAT2	0.138197	0.002727	0.114264	0.007866
MAOa	0.581047	0.012495	0.568935	0.008390
GAD65	0.890737	0.012945	0.815939	0.039680
COMT	0.240018	0.006924	0.232185	0.006513
DLK1	0.192695	0.009065	0.177225	0.007809
AHD2	0.129625	0.006915	0.093531	0.011899
Ki67	0.049776	0.003538	0.061484	0.007711

siRNA, small interfering RNA; *HOTAIRM1*, HOX-antisense intergenic RNA myeloid 1; *TH*, tyrosine hydroxylase; *Lmx1a*, LIM homeodomain gene 1a; *Ngn2*, neurogenin 2; *Nurr1*, nuclear receptor related 1 protein; *DAT*, dopamine transporter; *VMAT2*, vesicular monoamine transporter 2; *MAOa*, monoamine oxidase A; *GAD65*, Glutamate decarboxylase 65; *COMT*, Catechol-O-Methyltransferase; *DLK1*, Delta Like Non-Canonical Notch Ligand 1; *AHD2*, aldehyde dehydrogenase-1a1.

Figure S3. Representative blots for tyrosine hydroxylase protein levels



si-Con, control small interfering RNA; *si-HOT*, HOX-antisense intergenic RNA myeloid 1 targeted small interfering RNA; *TH*, tyrosine hydroxylase; *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase.

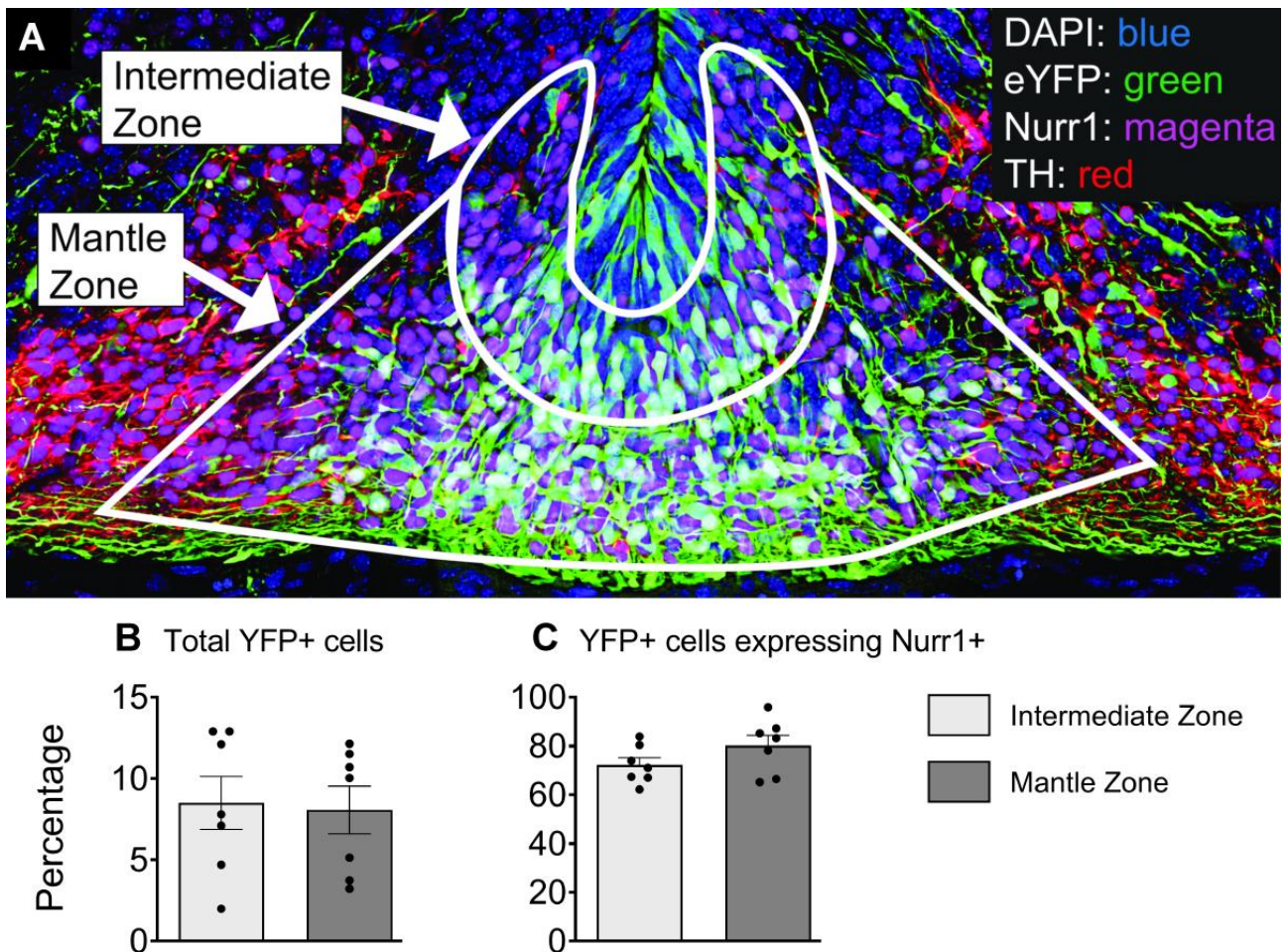


Figure S4. Proportion of cells tagged at E13 with eYFP after *in utero* electroporation at E11. The number of eYFP+ cells and Nurr1+ cells were quantified in the intermediate zone and mantle zone of E13 embryos (**A**). Levels of transfected cells were similar between subregions with approximately 8% of all cells in the ventral midbrain expressing eYFP (**B**). The majority of eYFP transfected cells were also Nurr1+ (**C**) suggesting most transfected cells were immature dopamine cells. *eYFP*, enhanced yellow fluorescent protein; *Nurr1*, nuclear receptor related 1 protein. Data are expressed as Mean ± SEM.

SUPPLEMENTARY REFERENCES

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