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Constraints on tooth growth by the developing alveolar bone

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The growth of the tooth and alveolar bone is co-ordinated so that a studied distance always separates the two. We have called this distance, the tooth-bone interface (TBI). Lack of mineralization, a crucial feature of the TBI, creates the space for the developing tooth to grow and the soft tissues of the periodontium to develop. No studies have been done to understand the signals that maintain the bone-free TBI, or the influence of the TBI on tooth development. We have investigated the impact of the developing alveolar bone on the size and development of the mouse first molar (M1). We evaluated the growth and osteoclasts distribution of the M1 in explant cultures using two methods, isolation of the M1 from the surrounding alveolar bone, and enhancement of osteoclastogenesis through RANK-RANKL signalling after treatment with RANKL, an osteoclast activator. Both methods showed a significant increase in the size of M1. Our data indicate that alveolar bone and RANKL regulate tooth size without altering development and osteoclasts are indispensable in promoting the formation of the TBI. We intend to further investigate the interactions between the tooth and alveolar bone during development, looking at the roles of other genes involved in TBI formation.

Investigating the role of mitochondrial folate metabolism in neural tube defects

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Folate one-carbon metabolism (FOCM) is a network of reactions that provide one-carbon units for processes vital to cell function, such as de novo biosynthesis of purines and thymidylate for DNA replication and methionine for methylation. FOCM has long been associated with a common group of congenital disorders: neural tube defects (NTDs), in which the neural tube, the embryonic precursor of the brain and spinal cord, fails to close. Evidence from clinical trials and mice models have hinted at the potential roles that FOCM play during neurulation, but the multifactorial nature of NTDs and complexity of FOCM pathways has hampered elucidation of precise causal relationships between the two. We focused on the glycine cleavage system (GCS), a mitochondrial component of the FOCM mediated by four enzymes: *GLDC*, *AMT*, *GCSH* and *DLD*. Collectively, these enzymes cleave glycine to supply one-carbon units to FOCM reactions occurring in the cytoplasm. Deficiencies in the GCS result in non-ketotic hyperglycinemia, but recent evidence has emerged suggesting that they also predispose to neural tube defects. The aim of this project is to study the effects of GCS deficiency on folate metabolism and the developmental mechanisms underlying NTDs found in *Gldc* and *Amt* mice models.

Could the role of GADD45γ and MAP3K4 during testis determination be mediated by epigenetic regulation of *Sry* expression?

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In mammals, sex determination is controlled by the inheritance of the Y chromosome. The gonads form at around 10 days *post coitum* in mice and are bipotential until the expression of the Y-linked testis-determining gene, *Sry*. This expression initiates a genetic cascade leading to testis differentiation. We have shown that loss of either *Map3k4* or *Gadd45γ* causes male to female sex reversal in XY mouse embryos, due to a delay in *Sry* expression. We recently showed that those two molecules can act in the same pathway during testis differentiation and regulate the phosphorylation of p38 MAPK and of GATA4, a known regulator of *Sry*. To better understand the role of this pathway in the control of *Sry* expression, we are interested in epigenetic modifications at the *Sry* locus associated with its normal expression profile and whether these are disrupted in mutants. We showed that six CpGs at the *Sry* promoter are hypomethylated in testis in comparison with extra-gonadal tissues, but this hypomethylation is not affected by loss of *Gadd45γ* or *Map3k4*. Our present work consists of establishing a protocol for chromatin immunoprecipitation from small numbers of gonadal somatic cells that will allow us to study epigenetic modifications at *Sry*.

Exploring the relationship between kidney pericytes and juxtaglomerular cells

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Pericytes, like renin-expressing juxtaglomerular (JG) cells, are vascular mural cells rooted within the vascular basement membrane of blood microvessels. They contribute to vascular development, stabilization, maturation and remodelling [1]. However, identifying these cells *in vivo* has proved challenging, since no single cell surface marker has been identified as being unique to pericytes [2]. Within the kidney, pericytes are closely associated with peritubular capillaries and glomerular endothelial cells [3]. Markers expressed in JG cells link them to arterial and interstitial pericytes both functionally and topologically [4]. Analysis of a microarray study comparing renin-expressing kidney cells with isolated whole kidney cells showed significant upregulation of pericyte markers in renin-expressing cells, in particular the transmembrane chondroitin sulphate proteoglycan NG2, which is expressed in nascent pericytes [5]. However, using FACS analysis on cells isolated from Ren-GFP transgenic mice we isolated distinct, non-overlapping populations of NG2-positive and renin (GFP)-positive cells. Furthermore, immunohistochemical studies of NG2 expression in adult RenGFP mouse kidneys showed no evidence of co-localization of NG2 and GFP suggesting that, in the adult, NG2 is not expressed in the same population of cells as renin. To resolve these findings we will characterize the ontogeny of NG2 expression, and will determine whether the recruitment of renin-producing cells following physiological and pharmacological stimulation leads to alteration of the expression of key markers including NG2.

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Comprehensive methylome analysis to the identification of new imprinted genes

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Imprinted genes (IGs) are characterized by parent-of-origin-specific expression triggered by genomic imprinting, an epigenetic mechanism that relies on DNA methylation to differentially mark the parental alleles. These genes are involved in key functions in mammals but, despite many previous investigations and recent reports suggesting > 1000 new candidates in brain, we do not know exactly how many genes are imprinted. To gain further insights, we are mapping parental-allele methylation differences in pre- and post-implantation embryos. Pre-implantation development is a key window to investigate, because

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the genome becomes largely demethylated, except for imprinted sequences. Therefore, first, we have profiled CpG island methylation in mouse fertilized blastocysts and parthenogenetic blastocysts by reduced representation bisulfite sequencing (RRBS) and compared these profiles with RRBS datasets from gametes. Second, in post-implantation embryos we have applied RRBS and sequencing of immunoprecipitated methylated DNA (MeDIP-seq) to tissues (brain and liver) from mouse hybrids to map parental-allele-specific methylation differences. Expression analyses of the same tissues are currently in progress (sequencing of mRNA libraries). Our results indicate that a limited number of sites differentially methylated in gametes retain allele-specific methylation throughout embryogenesis and suggest that few of the predicted new imprinted genes in brain are associated with differential methylation.

A search for sequence-specific signals in maternally imprinted CGIs

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Genomic imprinting refers to the differential epigenetic marking of the parental genomes in mammals and flowering plants. In the germline, both parental genomes are being epigenetically re-programmed causing DNA methylation differences at distinct loci called germline differentially methylated regions (gDMRs). The majority of CGIs are resistant to these changes and maintain their un-methylated state. During the early stages of embryonic development, the parental genomes undergo a second wave of epigenetic re-programming during which differential methylation is lost at most but not all gDMRs. The few gDMRs that endure are termed imprinting control regions (ICRs) since they ultimately cause the parent-of-origin-specific expression of the imprinted genes in the mammalian genome. There are seventeen ICRs known to originate in the maternal germline, that is, they become methylated during oogenesis, and this process requires *DNA methyltransferase 3-like* (*Dnmt3l*). However, the signals that are necessary to target the *de novo* DNA methylation mechanism to gDMRs in the oocyte and the signals that protect specifically ICRs from *post* fertilization re-programming, as well as the signals that protect the un-methylated state for the majority of CGIs are incompletely characterized. We precisely determined the genome coordinates of all known and some novel maternal ICRs in mouse using MeDIP-seq to generate and then compare the DNA methylation profiles of Dnmt3l-/+ embryos, wildtype embryos, sperm and C57/Bl6J × PWD/PhJ adult liver. As the coordinates of maternal gDMRs and the un-methylated CGIs, we used the 1062 oocyte-specifically methylated CpG islands (>=75% methylation) and ~7000 un-methylated CGIs (<=25% methylation) that were identified by Smallwood *et al*. (Nat Genet, 2011) using RRBS-seq. Using motif discovery tools like DREME and SCOPE we were able to identify a set of motifs that were significantly over-represented in ICRs/unmethylated CGIs. Having access to whole transcriptome data for mouse germ cells (Smallwood *et al*., Nat Genet, 2011 and Kobayashi *et al*., PLoS Genet. 2012) enables us to investigate the association between DNA methylation and gene activity 'transcription'. We are now classifying the CGIs according to their methylation state, transcription context and genomic location. Next, we will analyse the distinct sequence-features of each group.

2i inhibition of Erk1/2 and Gsk3β drives genome-wide demethylation to the epigenetic ground state of pluripotency

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Genome-wide erasure of DNA methylation takes place in primordial germ cells (PGCs) and early embryos but the signalling mechanisms that induce reprogramming are unknown. Here we show that inhibition of Erk1/2 and Gsk3β signalling in mouse ES cells by small molecule inhibitors (PD0325901 and CHIR99021 = 2i) induces genome-wide demethylation on a scale similar to that in PGCs, with only major satellites, IAPs and imprinted genes relatively resistant to erasure. Demethylation involves oxidation of 5mC in part by Tet1 together with disengagement of the de novo methyltransferases (Dnmt3a, Dnmt3b) as well as the co-activator Dnmt3L and we identify a cis-acting regulatory region in Dnmt3b

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that is highly responsive to signalling. Notably, this epigenetic and transcriptional ground state of pluripotency resembles closely that of inner cell mass (ICM) cells of the blastocyst. These insights provide a novel framework for understanding how signalling pathways regulate epigenetic reprogramming.

Assisting research into human embryonic and fetal development

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The Human Developmental Biology Resource (HDBR) is a unique resource funded by the MRC and Wellcome Trust. It provides human embryonic and fetal tissue for gene expression studies related to congenital disease, including both birth defects and inherited metabolic disorders. Use of the material should particularly illuminate developmental gene expression underlying aspects of functioning that characterize humans as opposed to lower animals (e.g. higher brain function, language). This research is essential if we are to introduce new methods for prevention of congenital defects and develop an improved understanding of 'what makes us human'.

The HDBR has Tissue Bank ethics approval for the collection, storage and distribution of material between 4 and 22 weeks of gestation. A significant proportion of the HDBR material is karyotyped and chromosomally normal material is provided for research but karyotypically abnormal material can also be provided on request. The HDBR's material can be used to generate cell lines, stem cells, protein, RNA and DNA. The HDBR can also provide cDNA from embryonic tissue for gene expression analysis. In addition, paraffin wax and frozen sections of embryos and early fetuses are available for in situ hybridization and immunohistochemistry.

Further details can be found at www.hdbr.org.

The chromatin-remodelling factor CHD7 controls multiple developmental programmes during development of the cerebellum

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Chd7 (Chromodomain helicase DNA-binding factor 7) is a chromatin remodelling factor that regulates gene expression by interacting with gene regulatory elements. Human CHD7 mutations are associated with CHARGE syndrome, a genetic disorder with an incidence of 1/10 000. A characteristic brain defect associated with CHARGE syndrome is hypoplasia of the cerebellar vermis.

We produced mouse models to study the effects of Chd7 deficiency on cerebellar development. We found that the cerebellar vermis was lost in Chd7;Fgf8 double heterozygote, indicating that Chd7 interacts with Fgf8 during development. Chd7 regulates the expression of critical genes that control the formation of the mid-hindbrain boundary, such as Otx2 and Gbx2, and is required for the maintenance of Fgf8 expression and signalling. As a result, the isthmus organizer and rhombomere 1 are lost by E9.5 in Chd7 null embryos.

Chd7 is strongly expressed in the cerebellar granule cell precursors (GCps) at birth, suggesting additional roles for Chd7 during cerebellar development. We observed significant cerebellar hypoplasia when deleting Chd7 from GCps specifically. We found that GCps proliferation index was reduced in the mutant cerebella, and the differentiation is enhanced. Our findings provide a mechanistic explanation for the cerebellar hypoplasia associated with CHARGE syndrome.

Cohesins involved in Cornelia de Lange syndrome aetiology are ubiquitously but differentially expressed in human tissues

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Cornelia de Lange Syndrome (CdLS) is a severe developmental disorder that is believed to be caused by

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alterations within three genes (*NIPBL, SMC1A, HDAC8*) in the majority of cases. All these genes encode proteins of the 'cohesin complex', which display in cells two temporally separated major roles: one controlling the cell cycle ('canonical') and the other involved in regulating gene-expression ('non-canonical'). CdLS is characterized by malformations affecting multiple systems, with a common feature of severe mental retardation. In this study we examined the expression levels of *NIPBL*, *SMC1A* and *HDAC8* in different human organs and tissues. All three genes were found to be highly expressed in haematopoietic tissues and cerebellum. Cohesins' robust expression in hyper-proliferative haematopoietic organs could be related to both their canonical role and also to their transcriptional control (non-canonical) of stem cells and/or regulation of haematopoietic factors such as *RUNX1*. High levels of expression in the cerebellum are consistent with results of our recent study showing that cephalic structures development, especially at the hindbrain level, is impaired in an *in vivo* model (*D. rerio*) of *nipblb* haploinsufficiency. These results suggest that CdLS intellectual disabilities may result from altered cerebellum morphogenesis and development.

Cilia, flow and polycystins: how the embryo determines left from right

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The left-right asymmetry of the internal organs of vertebrates is determined during embryogenesis by a biophysical mechanism; asymmetric fluid flow. In the mouse embryo, this leftward flow of liquid is generated by posteriorly tilted motile cilia in the cavity of the ventral node, a pit-like structure at the anterior tip of the primitive streak. Downstream of flow, asymmetries in gene expression are induced; the *Nodal* gene, a master regulator of left-right determination, becomes activated throughout the left lateral plate mesoderm but remains absent from the right side. However, a major unanswered question is how the embryo senses the fluid flow to elicit gene expression asymmetries. In the talk, I will discuss two polycystin proteins, Pkd1l1 and Pkd2, that are required for left-right patterning. I provide evidence that Pkd1l1 is a strong candidate to be the elusive sensor of flow, showing that it acts downstream of flow but upstream of early gene expression asymmetries. Furthermore, our genetic experiments have revealed a novel pathway linking the generation and sensation of flow to the establishment of asymmetric gene expression. Finally, I discuss a small extracellular domain of Pkd1l1 that is essential for function and might be central to the mechanism of flow sensation.

Fuz mutant mice reveal shared mechanisms between ciliopathies and fibroblast growth factor-related syndromes

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Ciliopathies are a broad class of human disorders, with craniofacial dysmorphology as a common but poorly understood feature. Among the hallmarks of ciliopathies is high-arched palate, a condition that impairs speech and degrades quality of life. We present here the ciliopathic *Fuzzy* mutant mouse as the first animal model of high-arched palate. Using mouse and frog, we show that this defect arises not, as commonly suggested, from midface hypoplasia, but rather from increased migration of neural crest into the first branchial arch, resulting in maxillary hyperplasia. High-arched palate is also common in fibroblast growth factor (FGF) hyperactivation syndromes, and we find that craniofacial *Fgf8* gene expression is significantly expanded in *Fuz* mutant mice. Moreover, genetic reduction of *Fgf8* levels in *Fuz* mutant mice ameliorates the maxillary phenotypes. Thus, FGF over-expression underlies the palatal defects in this ciliopathy model. Our findings reveal a surprising cause for a common craniofacial anomaly and identify a novel aetiological link between two classes of human disease: FGF-hyperactivation syndromes and ciliopathies.

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Flutter: a novel mouse mutant with hyperkinetic cilia

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Hyperkinetic cilia are a hallmark of primary ciliary dyskinesia (PCD). Mutations in human axonemal dynein heavy chain 11 (*DNAH11*) often underlie this disease. Mouse *Dnahc11*^{iv} mutants have immotile cilia, organ situs abnormalities and PCD-like symptoms. Motile cilia in the embryonic node drive leftward fluid flow, sensation of which activates the left-sided Nodal cascade. We have identified flutter, a hypermorphic *Dnahc11* allele displaying abnormal organ situs. Nodal cilia proved hyperkinetic, with increased cilia beat frequency (CBF). Cilia rotation analysis in flutter reveals reduced rotational amplitude and heterogeneity of motion. Expression of the left-side determining gene *Pitx2*, normally restricted to the left lateral plate mesoderm (LPM), was expressed in the left LPM, right LPM or bilaterally in flutter mutants, reflecting aberrant activation of the Nodal cascade. At E13.5, flutter embryos display incidence of *situs inversus totalis* and left pulmonary isomerism. These results identify flutter as a novel model for hyperkinetic cilia defects in left-right patterning; work to assess PCD phenotypes is ongoing.

Causes of otitis media in a new mouse model

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Otitis media (OM), the inflammation of the middle ear (ME) is the most common disease and cause for surgery in infants. Chronic forms can be accompanied by excessive effusion (OME), often leading to conductive hearing loss (CHL). Though the pathogenesis is multifactorial, there is evidence for genetic factors predisposing to OM.

In this study we report that adult heterozygous Df1 (Df1/+)-knock out mice modelling DiGeorge syndrome have a significant mono- or bilateral hearing impairment and a very high incidence of OME. The severity of OME (thickness of mucosa, infiltration of inflammatory cells) was observed to correlate directly with the level of CHL. MicroCT analysis of Df1/+ mice revealed that auditory bullas (AB) are smaller in these mice and may impact on correct function of the ME such as clearance and cavitation. We also found morphological changes of the ME epithelium after the onset of OME by using SEM and histological methods. Df1/+ mice displayed increased goblet cell density and formation of mucus glands as well as shortened and sparsely distributed cilia at the orifice of the Eustachian tube. We also analysed gene expression of genes deleted in Df1/+ mice such as Tbx1 and Septin5 in pre- and postnatal stages.

Multiple congenital melanocytic naevi and neurocutaneous melanosis are caused by mosaicism for *NRAS* codon 61 mutations, leading to an increased risk of melanoma in affected tissues

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Congenital melanocytic naevi (CMN) can be associated with neurological abnormalities, an increased risk of melanoma, and characteristic facies. Mutations in *NRAS*, *BRAF* and *Tp5*, and polymorphisms in *MC1R* have been described in individual CMN samples, however, their role in the pathogenesis of multiple CMN within the same subject and development of associated features has not

been clear. We hypothesized that a single post-zygotic mutation in *NRAS* could be responsible for multiple CMN in the same individual, as well as for melanocytic and non-melanocytic central nervous system (CNS) lesions. Fifty-five samples from 15 patients with multiple CMN were sequenced after selective amplification of putative mutant alleles using site-directed mutagenesis and enzymatic digestion of the wild-type allele. Oncogenic missense mutations in codon 61 of *NRAS* were found in affected neurological and cutaneous tissues of 12/15 patients, but absent from unaffected tissues and blood, consistent with *NRAS* mutation mosaicism. In ten patients the mutation was consistently c.181C > A, p.Q61K, and in two c.182A > G, p.Q61R. All 11 non-melanocytic and melanocytic CNS samples from five patients were mutation positive, despite *NRAS* rarely reported as mutated in CNS tumours. Homozygosity was associated with onset of melanoma in two cases, implying a multi-step progression to malignancy. These results suggest that single post-zygotic *NRAS* mutations are responsible for multiple CMN and associated neurological lesions.

A novel *RAB18* mouse model of Warburg Micro syndrome

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Warburg Micro syndrome is a heterogeneous autosomal recessive disorder associated with ocular (congenital cataracts, optic atrophy, microphthalmia, microcornea, atonic pupils and cortical blindness) and neurological abnormalities (postnatal microcephaly, severe global developmental delay, progressive spastic paraplegia, frontal polymicrogyria and peripheral neuropathy) and hypothalamic hypogonadism. Previous studies have identified causative loss-of-function mutations within *RAB3GAP1*, *RAB3GAP2* and *RAB18* resulting in clinically indistinguishable conditions. *RAB3GAP1* and *RAB3GAP2* encode the catalytic and non-catalytic subunits of the RAB3 GTPase activating protein with specificity for the RAB3 family. RAB3 functions in modulating the regulated exocytosis of hormones and neurotransmitters. The role of *RAB18* in trafficking has not as yet been determined.

Little is known about the pathology underlying Warburg Micro syndrome, so to enable a deeper understanding of the disease we have generated a novel *RAB18* null mutant mouse model from embryonic stem cells and begun its characterization. *RAB18* mutant mice present with congenital nuclear cataracts, atonic pupils and progressive hindlimb muscle weakness, recapitulating the Warburg Micro syndrome phenotype. We have examined in detail the nature of the peripheral neuropathy and identified specific abnormalities in the nerves and at the neuromuscular junction of mutant mice.

DIRAS3 imprinting regulates expression and alternative splicing of a long non-coding RNA, *GNG12-AS1* gene

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The *DIRAS3* is a maternally imprinted gene that belongs to the RAS superfamily of GTPases. *DIRAS3* expression is down-regulated in several cancers due to loss of heterozygosity and DNA hypermethylation at the promoter. A novel lncRNA *GNG12-AS1* is present at the *DIRAS3* locus. We found that the *GNG12-AS1* gene is a processed nuclear RNA polymerase II transcript that initiates from a single transcription start site and has several splice variants. In normal cell lines with imprinted *DIRAS3* expression, the *GNG12-AS1* is biallelically expressed, with a small minority of splice variants being monoallelic. In cancer cell lines with loss of *DIRAS3* imprinting, *GNG12-AS1* expression becomes monoallelic. Transcription of *GNG12-AS1* attenuates transcription of the active *DIRAS3* gene, while high levels of *DIRAS3* transcription reciprocally inhibit *GNG12-AS1* expression. Imprinted expression of *DIRAS3* is positively regulated by CTCF binding at a differentially methylated region upstream of its promoter. *GNG12-AS1* transcription levels do not seem to be directly related to CTCF binding but allele specific splicing and the allelic expression seems to be regulated by CTCF-cohesin. The *GNG12-AS1*/*DIRAS3* locus is the first example of imprinted co-transcriptional splicing and a potential model system for the study of long-range effects of CTCF-cohesin on splicing and allele exclusion.

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A trans-homologue interaction between miR-127 and *Rtl1*: roles in mouse muscle development

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miR-127 is an imprinted microRNA located in the *Dlk1-Dio3* domain on mouse chromosome 12, and is part of a cluster of microRNAs expressed exclusively from the maternally inherited chromosome. A protein-coding gene, *Rtl1*, is expressed from the same locus on the paternally inherited chromosome homologue in the antisense direction. The result is a trans-homologue interaction between the two chromosomes, whereby the maternal miR-127, and its neighbouring miRNAs, down-regulate the paternal *Rtl1* mRNA through RNAi as a result of their perfect complementarity.

The trans-homologue interaction between miR-127 and *Rtl1* is unusual, eutherian-specific and its evolution is not fully understood. miR-127 is known to regulate *Rtl1*, but it is possible that *Rtl1* in turn regulates miR-127 through titration of the microRNA. There is also evidence linking the expression of miR-127 and *Rtl1* to the transcription of imprinted *Dlk1*.

Using a mouse miR-127 knockout model we have found a role for miR-127 and *Rtl1* in mouse muscle, where they appear to be developmental regulators of respiratory rate linked to exercise adaptation and fibre type. The trans-homologue interaction is an example of a dosage control mechanism in embryonic development that has important implications for metabolism in adult life.

A novel role for imprinting in heart development

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Dopa Decarboxylase (Ddc) is an enzyme that plays a fundamental role in the biosynthesis of catecholamine neurotransmitters and serotonin. A short form transcriptional variant of *Ddc* called *Ddc_exon1a*, which originates from an alternative promoter at exon 1a, is highly expressed in the developing myocardium of the prenatal heart and is progressively silenced during postnatal development. *Ddc_exon1a* is epigenetically regulated via genomic imprinting in mouse heart with expression coming only from the paternally inherited allele. It is the only known example of a gene imprinted specifically in the heart. Using mice harbouring a paternally inherited deletion of the *Ddc* gene, we have examined the role of Ddc in heart development. Using microarray and episcopic fluorescence image capture technology (EFIC) we demonstrate that Ddc plays a role in regulating cellular proliferation and morphogenesis of the developing myocardium.