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Fgf signalling in the control of craniofacial and tracheal gland development

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The submucosal glands (SMGs) of the respiratory system are specialized structures essential for maintaining human airway homoeostasis. The significance of these glands is highlighted by their involvement in serious respiratory diseases, such as cystic fibrosis, where their phenotype and function are severely altered. Surprisingly, very little is known about SMG development and differentiation. To understand the molecular mechanisms involved we have investigated the development of nasal and tracheal SMGs in the *Fgf10* mutant mouse. *Fgf10* is expressed in the mesenchyme around the developing SMGs, and in heterozygous mice the tracheal glands have reduced branching. These mice also have an altered A/P distribution of the glands post-natally, a deficit that is not recovered in adults. In the nasal glands, some but not all glands were lost in the homozygous mutant, indicating that not all glands require *Fgf10* for initiation. Some of the glands present in the *Fgf10* homozygote were missing in the *Fgfr2b* mutant, suggesting compensation by another Fgf ligand. We aim to uncover the expression patterns of a number of *Fgfs* during early gland development and to study the functional consequence of loss of SMGs in *Fgf10* heterozygotes by assessing the ability of these mice to respond to respiratory challenges.

Chd7 is required in cardiomyocytes for the transcriptional control of heart development

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The importance of epigenetic regulation of gene expression during heart development is becoming increasingly apparent. CHD7 is a large nucleosome-remodelling protein, which shows ATP-dependent helicase activity. Haploinsufficiency for CHD7 is implicated in CHARGE syndrome, and patients with reported *CHD7* mutations present with a range of congenital cardiovascular defects. Mesodermal ablation of *Chd7* in mice, driven by *Mesp1-Cre*, results in a severe cardiovascular phenotype and embryonic lethality around E15.5–E16.5. Embryos show haemorrhage, oedema, structural heart defects and a low penetrance of great vessel defects. Interestingly, given this haemorrhagic phenotype, data collected on embryos lacking *Chd7* in the endothelial lineage did not result in haemorrhaging or any other major cardiovascular phenotype, while *Mef2c-Cre*-driven knockout in the second heart field produced a subset of the mesodermal cardiovascular phenotype. Early cardiomyocyte lineage-specific ablation is currently being investigated.

Microarray analysis and qRT-PCR has been used to identify genes that are dysregulated in the heart after mesodermal *Chd7* ablation. Key developmental signalling pathways are found to be disrupted, which can be linked with the cardiovascular defects observed. Alongside detailed characterization of temporal- and tissue-specific *Chd7* expression as the heart develops, this is leading towards insights into the role of CHD7 in cardiovascular development.

Map3k4 is a dose-dependent modifier of sensitivity to B6-Y^{POS} gonadal sex reversal

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In mammals the Y-linked gene *Sry* is a dominant male determinant, whose expression initiates the cascade of events that drive differentiation of the testis. In the mouse, *Sry* follows a distinctive spatiotemporal pattern and must be sufficiently expressed within an appropriate temporal window to enable activation of the male pathway. Deficiencies in a number of genes have been shown to disrupt the timely onset of *Sry* expression and lead to XY sex reversal, including the kinase MAP3K4. XY embryos lacking MAP3K4 exhibit delayed, insufficient expression of *Sry* and develop ovaries on the C57BL/6J (B6) background. While its loss results in failed testis determination, we have found that gain of MAP3K4 function using BAC transgenesis can rescue sex-reversal in B6 embryos carrying the Y chromosome from the *Mus poschiavinus* strain. Furthermore, loss of a copy of *Map3k4* results in more severe sex reversal in these embryos. Transcriptional profiling shows that delayed *Sry* expression underlies B6-Y^{POS} sex reversal and that overexpressing *Map3k4* partially restores the normal expression profile of *Sry*. We now aim to explore the epigenetic landscape of the *Sry*^{POS} locus in order to elucidate regulatory mechanisms contributing to B6-Y^{POS} sex reversal and its modification by transgenic expression of *Map3k4*.

Integrated β-catenin, BMP, PTEN, and Notch signalling patterns the nephron

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The nephron is the single most important excretory tissue in mammals and its segments regulate water homoeostasis, blood filtration and metabolite excretion. Accordingly, when its function is disrupted complex pathological features are found. In spite of its importance little is known about how the nephron becomes patterned and segmented. We have identified the genetic mechanism that controls the patterning of the nephron. Our data show that the developing nephron is patterned by a gradient in the transcriptional activity of β-catenin that lies longitudinal to the proximal-to-distal axis of the nephron tubule. By modifying β-catenin activity we force cells within nephrons to adapt to the imposed β-catenin activity level, thereby causing spatial shifts in nephron-segments. The β-catenin signalling gradient interacts with the BMP pathway. BMP, through PTEN/PI3K/AKT signalling, antagonizes the β-catenin activity gradient and promotes segment-identities associated with low β-catenin activity. The β-catenin activity gradient also integrates with Notch function. Modulating β-catenin activity rescues segment-identities normally lost by inhibition of Notch. Similarly, co-suppression of Notch and PI3K restores the medial domain thus indicating that Notch promotes the medial domain by blocking PI3K activity to prevent excessive AKT and medial growth. Our data therefore identifies a comprehensive genetic network for nephron patterning.

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Development and characterization of a global Cre-inducible cell cycle reporter mouse

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Cell-cycle progression is central to embryonic development. Fucci (Fluorescent Ubiquitination-based cell cycle indicator) is a genetically encoded cell-cycle reporter system originally developed by the Miyawaki lab to visualize cell-cycle progression. The original probes were expressed ubiquitously and consisted of separate genetic constructs and mouse lines to label G1 and S/G2/M phases; consisting of fluorophores fused to the regulatory domains of hCdt1(30/120) and hGeminin(1/110), respectively. The relative abundances of these two probes are tightly regulated during cell-cycle progression, allowing one to define the cell-cycle status of a cell by its colour. We describe a modified version of the Fucci system (Fucci2a). Fucci2a fuses the Fucci probes using the self-cleaving *Thosea asigna* virus 2A peptide sequence (t2a) allowing the expression of the two probes from a single promoter. The single construct greatly simplifies the process of generating, maintaining and crossing mouse lines. It also provides the opportunity to incorporate other fluorescently tagged proteins into the construct, separated by a further self-cleaving peptide. We have characterized the behaviour of our Fucci2a constructs in stable cell lines and have made a Cre-inducible transgenic mouse line by targeting to the *ROSA26* locus.

Cellular mechanisms underlying neural tube defects in *Pax3* mutant mice

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Neural tube defects (NTDs), such as spina bifida and exencephaly, affect approximately 1 in 1000 pregnancies. They are caused by failure of neural tube closure during development, and can lead to lifelong disability or death. The *Splotch* mouse carries a mutation in the *Pax3* gene and is predisposed to NTDs. However, the mechanism underlying development of NTDs are not well understood. Three theories have been proposed for causes of spina bifida in the *Splotch* mouse – premature neuronal differentiation, excess apoptosis and reduced proliferation. I have been investigating these theories to find out if any of them could be the cause of the spina bifida in *Splotch* embryos. Although excess apoptosis and premature neuronal differentiation are not detected in *Splotch* mutant embryos, reduced proliferation is present and hence may be causative. Additionally, genetic crosses in mice identified a potential link between the Pax3 and canonical Wnt signalling pathways. These studies show that canonical Wnt signalling influences frequency of Pax3-related NTDs.

Identification and characterization of the motile ciliopathy genes *Heatr2* and *Zmynd10* using fly and mouse models

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Cilia are cellular organelles which perform sensory, motility and signalling functions crucial for development and homoeostasis. Their dysfunction often leads to ciliopathic diseases. While a significant portion of the cell's proteome is devoted to cilium formation/function, many cilia components and their roles remain unknown. Phylogenetic conservation of core ciliary genes and their regulation by key transcription factors, conserved across metazoans, has allowed the use of model organisms to identify new ciliary/ciliopathy candidate genes.

Broadly, all ciliary genes are transcriptionally controlled by RFXs; additional co-operation with FoxJ1 is required for motile cilia formation. Mammals have evolved further complexity for specialized motile cilia sub-types. Paring this complexity down to the relatively simpler model of *Drosophila* mechanosensory cilia led to the identification of novel ciliary genes *Heatr2* and *Zmynd10* controlled by an Rfx-Fox transcriptional code. Mechanosensory neurons being the only somatic cells in flies with cilia most akin to mammals, fly RNAi mutants for these genes displayed characteristic ciliopathy-like defects; these data will be presented. Mutations in these genes have recently been reported to cause a classic motile ciliopathy in humans called PCD. A novel

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PCD causing *HEATR2* mutation will be presented. Findings on the transcriptional control of these genes and expression studies in the mouse will also be presented.

DNA damage repair protein Atmin controls ciliogenesis through modulation of intraflagellar transport

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Cilia are required for normal embryonic patterning and adult physiology; defects in their assembly cause disorders known as ciliopathies. *Atmin* (ATM interactor) is a transcriptional activator, previously implicated in DNA repair. We independently identified it as the gene mutated in the lethal ciliopathy gasping6 (*gpg6*). In *gpg6* one of Atmin's zinc fingers loses its function, resulting in defective ciliogenesis in multiple embryonic tissues and a ciliopathy-like phenotype. Transcriptional profiling has demonstrated altered expression of genes implicated in ciliogenesis. We have investigated their relationship with Atmin and our results points to an Atmin-dependent pathway impacting ciliogenesis, most likely though modulation of intraflagellar transport.

Investigating the molecular function of Tulp3 interacting protein Rgnef in Shh signalling

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Tulp3 has been identified among the genes required for neural tube development, and acts as a negative regulator of the Shh signalling pathway. In early embryonic development, Shh signalling is required for neural tube formation and dorsal ventral patterning. The role of Tulp3 is as part of a transportation complex that delivers specific G-coupled receptor proteins to the primary cilia. This current model is based on a range of discoveries that include associating Tulp3 with IFTA complex proteins and, more recently, primary cilia transportation of G-protein-coupled receptor Gpr161. In our Laboratory, we identified Rgnef (p190RhoGEF) as a Tulp3 interacting protein through yeast2-hybrid experiments. We further show that Rgnef is expressed ventrally in the neural tube of developing mouse embryos. Through cell-based Shh pathway activation assay, we show that siRNA knock-down of Rgnef expression suppresses pathway activation induced by agonist purmorphamine. Furthermore, overexpression of Rgnef results in significant pathway activation. In conclusion, these results demonstrate a role for Rgnef in Shh pathway activation.

High-throughput imaging and phenotyping of homozygous lethal mouse lines at MRC Harwell

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The international Mouse Phenotyping Consortium will generate 5000 conditional KO mouse lines by 2015 and make them available to the research community together with a broad phenotypic characterization and the expression profile of the targeted locus. MRC Harwell is responsible for producing, distributing and analysing 500 of these lines. We will present the pipeline of work focusing on the characterization of the embryonic lethal lines, including the identification of the window of lethality, gene expression at E12.5 and acquisition of morphological data by OPT and microCT. We will describe the first mutants analysed by this programme.

Tissue-specific vulnerability to chromosome segregation stress revealed by Caph2 mutant mice

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Chromosome segregation is a vital process, and its precise regulation is required to ensure that each daughter cell receives a correct chromosome complement. Mistakes in this process result in aneuploidy and tumour development. Our work shows that frequencies of aneuploid cells show profound variability between different stages of haematopoietic differentiation. In addition, we show that a mouse model with a hypomorphic mutation in a ubiquitously expressed subunit of Condensin II ($Caph2^{nes/nes}$) exhibits mitotic abnormalities in the T-cell and erythroid, but not B cell lineages. The mutation results in a developmental block specifically in the T-cell lineage – with the numbers of B cells and erythroid

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cells relatively unaffected. T-cell numbers are rescued in Caph2$^{nes/nes}$; P53$^{-/-}$ double-mutant animals. The most common cause of death in p53 mutant mice is thymic lymphoma, and interestingly the double-mutant mice succumb to thymic tumours significantly more rapidly than either Caph2$^{-/-}$ or p53$^{-/-}$ single mutants. From these data, we suggest that cells at different stages of haematopoietic development vary in their susceptibility to chromosome instability, which may explain why certain stages of differentiation are particularly vulnerable to transformation.

Global identification of active transcription start sites in germ cells and correlation with CpG methylation

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Gametogenesis in mammals is characterized by epigenetic reprogramming. This involves DNA methylation erasure in primordial germ cells, followed by methylation establishment, which in the female germline takes place after birth during oocyte growth. In oocytes, it was previously observed that methylated CpG islands (CGIs) often localize within active transcription units, as in the case of the differentially methylated regions of imprinted genes. Moreover, it was demonstrated that methylation in oocytes is not limited to CGIs, but extends over whole gene bodies, appearing to divide the genome into hypermethylated domains over transcription units and hypomethylated domains devoid of transcription. This suggests that transcription is a key determinant of the DNA methylation landscape in oocytes. In many cases, when annotated transcription start sites (TSSs) are methylated, upstream unannotated oocyte-specific TSSs could be identified, providing transcription across the corresponding CGI. Therefore, standard genome annotation is not suitable for genome-wide correlation of oocyte transcriptome and methylation. Using deep RNA-sequencing, we mapped the oocyte transcriptome and rigorously tested the correlation with methylated domains. Our data also demonstrate, with greater precision that previously, that methylated CGIs are mostly intragenic, while unmethylated CGIs are intergenic or associated with active TSSs. Oocyte transcriptome annotation together with genome-wide TSS mapping allowed us to identify TSSs active in oocytes. This will be used to investigate the timing of induction of TSSs during oocyte growth and correlation with initiation of *de novo* methylation of specific CGIs *in vivo* in size selected oocytes, as well as in cultures of growing oocytes.

Investigating the source and function of maternal Delta-like homologue 1 (DLK1) in pregnancy

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Delta-like homologue 1 (*Dlk1*) is a paternally expressed imprinted gene whose product is a Notch family protein present in both membrane-bound and soluble forms. DLK1 has been implicated in the development of a wide variety of embryonic tissues, as well as in post-natal roles such as adipogenesis. *Dlk1* is expressed at high levels in numerous tissues in the embryo and young neonate; additionally, in these animals soluble DLK1 is present at high concentrations in the blood plasma. In contrast, *Dlk1* expression is low and highly restricted in the adult animal and plasma DLK1 levels are also low. The exception to this is pregnancy, when maternal plasma DLK1 dramatically increases, peaking at term at concentrations approximately eightfold that of non-pregnant adults. This project investigates the source of this additional plasma DLK1 and its effects upon maternal metabolism and resource partitioning during pregnancy.

Genome-wide analysis of proteins that bind to DNA and regulate gene expression

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Chromatin immunoprecipitation and next-generation sequencing are being used to interrogate the coincident and allele-specific binding of the proteins CTCF, Cohesin, ATRX and MeCP2 genomewide in the murine brain, a tissue that expresses a significant proportion of imprinted transcripts. Identifying regions co-binding these proteins will help generate a model to understand how these proteins influence

gene expression, particularly at imprinted loci. Using these tools we aim to understand more about the proteins that participate in the genomic landscape around imprinted loci and drive this unusual mode of gene regulation. These loci will act as models for studying DNA-binding proteins and their roles in transcription.

At the level of the individual locus, mechanisms of gene regulation are being investigated using imprinted retrogenes as a model. Retrogenes are transcriptionally active intronless genes located within an intron of a 'host' gene. The role of epigenetic factors influencing gene transcription are being investigated at the *Mcts2/H13* locus. Expression of an intronic retrogene can cause premature termination of a 'host' transcript from the same allele. Our hypothesis is that this premature termination is caused by transcription of the retrogene interfering with host gene transcription. These studies will provide a mechanistic component to our whole genome analyses.

Paternally expressed, imprinted insulin-like growth factor-2 in chorionic villi correlates significantly with birth weight

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Fetal growth involves highly complex molecular pathways. IGF2 is a key paternally expressed growth hormone that is critical for *in utero* growth in mice. Its role in human fetal growth has remained ambiguous, as it has only been studied in term tissues. Conversely the maternally expressed growth suppressor, *PHLDA2*, has a significant negative correlation between its term placental expression and birth weight.

The aim of this study is to address the role in early gestation of expression of *IGF1, IGF2*, their receptors *IGF1R* and *IGF2R*, and *PHLDA2* on term birth weight. Real-time quantitative PCR was used to investigate mRNA expression of the aforementioned genes in chorionic villus samples (n=260). Expression was correlated with term birth weight using statistical package R including correction for several confounding factors.

Transcript levels of *IGF2* and *IGF2R* revealed a significant positive correlation with birth weight (0·009 and 0·04, respectively). No effect was observed for *IGF1, IGF1R* or *PHLDA2* and birth weight. Critically, small for gestational age (SGA) neonates had significantly lower *IGF2* levels than appropriate for neonates ($p=3{\cdot}6\times10^{-7}$).

Our findings show that *IGF2* mRNA levels at 12 weeks gestation could provide a useful predictor of future fetal growth to term, potentially predicting SGA babies. This research reveals an imprinted, parentally driven rheostat for *in utero* growth.

The role of DNA sequence signals in the epigenetic reprogramming of CpG islands during oogenesis and early embryogenesis

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The reprogramming of epigenetic marks is a genome-wide process and yet CpG islands (among a few other sequence classes) escape this overall trend. Nevertheless, not all CpG islands respond similarly; while the majority of them resist the global *de novo* DNA methylation establishment, around 1000 CpG islands acquire methylation in oocyte (most of them remain un-methylated in sperm therefore, they called germ line differentially methylated regions [gDMRs]). Additionally, about 25 gDMRs maintain this acquired germ line methylation during pre-implantation development and in the somatic cells (permanent gDMRs) while the rest lose their methylation after fertilization. Emerging sequencing technology contributes to the understanding of the molecular mechanisms for some of these modifications; yet the role of the DNA sequence remains to be determined. Here, we investigated the role of the DNA sequence of CpG islands in attracting the specific protein complexes for either establishing or maintaining the DNA methylation at specific oocyte-methylated CpG islands and protecting the default un-methylated state of the majority of CpG islands during oogenesis and pre-implantation embryos. The results of association studies shows high correlation between CpG islands gene-body methylation and transcription initiated at upstream promoters in oocytes, supporting previous observations. Although *de novo* motif finding has successfully identified TGCCGC (the recognition site of Zfp57/Kap1 protein complex), it did not uncover any similarly significant motif at permanent gDMRs, suggesting the absence

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of another DNA-interacting protein complex that may maintain the DNA methylation at these regions post fertilization. Further analysis on this motif indicates that its density, in addition to its coverage, is indispensable discriminative feature that distinguishes permanent gDMRs from other CpG islands. Furthermore, the absence of discriminative motifs and the lack of 8–10 bp CpG spacing and global periodicity at either oocyte-methylated CpG islands (including permanent gDMRs) or their shores relative to oocyte un-methylated CpG islands support a sequence-independent mechanism for *de novo* methylation establishment during oogenesis. Finally, a CG-rich, independent of CpG density motif that is significantly similar to E2F1 recognition site is overrepresented at oocyte un-methylated relative to methylated CpG islands. Results from logistic linear regression showed that this motif acts independently of transcriptional activation at un-methylated CpG islands, proposing a role in protecting them from *de novo* methylation.

Cyp26b1 null embryos display a 22q11 deletion syndrome-like cardiovascular phenotype which is modified by the presence of an additional *Tbx1*$^{+/-}$ allele

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Control of retinoic acid (RA) homoeostasis/dosage is required for normal development and the Cyp26 enzymes are known to metabolize RA to less active forms. *Cyp26b1* null mice display cardiovascular and thymic phenotypes at E15.5 which are reminiscent of 22q11-deletion syndrome phenotypes. These include small/ectopic thymus and abnormal development of the outflow tract (OFT), ventricular septum and aortic arch remodelling. Developmental anomalies of the pharyngeal arch arteries, neural crest and OFT detected at E10.5 may underlie these later phenotypes.

Animal models and non-deleted patient data suggest haploinsufficiency of *TBX1* is a major underlying cause of 22q11DS and *Tbx1* mutant embryos recapitulate the majority of the 22q11DS phenotype. *Cyp26b1* expression is down-regulated/altered in *Tbx1* mutant mice both by RT-PCR and *in situ* hybridization. As described above the cardiovascular phenotype of both *Tbx1* and *Cyp26b1* mutant mice is very similar. To determine if *Tbx1* and *Cyp26b1* might be acting within the same genetic pathway crosses between *Tbx1*$^{mcm/+}$ and *Cyp26b1*$^{+/-}$ mice were performed and analysed at E15.5. The results suggest that a *Tbx*$^{+/-}$*1* allelle in a *Cyp26b1* null context can modify the aortic arch phenotype observed.

The MAPK signalling pathway is required for polarization of the primitive endoderm in embryoid bodies

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Our work utilizes embryoid bodies formed from mouse embryonic stem cells as a model system. The outer-most cell layer of an embryoid body is an epithelial cell type comparable with the primitive endoderm. To investigate the relationship between cell polarity and fate of the primitive endoderm in an embryoid body, we have utilized a pharmacological small-molecule inhibitor approach. A role for the MAPK signalling cascade in the development of the primitive endoderm has previously been shown both in embryoid bodies and *in vivo*. Upon pharmacological inhibition of both the FGFR receptor and MAPK signalling cascade, as expected we observed a loss of primitive endoderm cell fate markers in the cells of the outer layer of the embryoid body. Interestingly, we also observed a disruption in polarization of the epithelial layer, demonstrated by mislocalization of an apical polarity complex protein, tight junction and adherens junction proteins as well as a disruption of the basement membrane. We believe that this work demonstrates an important role for the MAPK signalling cascade in the polarization of the primitive endoderm in embryoid bodies.

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Nipbl expression analysis in the developing mouse embryo

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NIPBL – Nipped B like – is one of the human components of the cohesin complex, which is a highly conserved protein complex with two main functions: (1) to control sister chromatid cohesion during S phase and (2) to regulate gene expression. Mutations in *NIPBL* account for 50% of the cases in Cornelia de Lange Syndrome (CdLS), a congenital birth defect affecting almost any organ, with an estimated incidence of 1:10000 births and with variable phenotype severity. It has been reported that *NIPBL* is expressed in the fetal kidney, the foetal liver, in the cephalic neural tissues, the limb buds and branchial arches. However, a detailed expression analysis of *Nipbl* during embryogenesis that will help explain the genotype/phenotype of this congenital malformation has not been reported. The aim of this project was to characterize in detail *Nipbl* gene expression in mouse embryos at different stages of development. Using *in situ* hybridization on paraffin sections of different stage mouse embryos we found strong expression in the developing heart, in the lung epithelium, in the gut, and in the developing cerebellum. These previously unreported sites of expression correlate to CdLS affected organs and complement our recent study on *Danio rerio* embryos.

Discovery of novel causative genes in familial cleft lip with or without cleft palate

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Cleft lip and palate (CLP) is well known to be among the most common birth defects, but we still have difficulty to explain why the majority of cases occur. In general, sporadic cases with no family history may be more related to environmental risks, while the presence of one or more affected relatives in the same family will suggest genetic factors as the main contributor. The aim of this study is to identify new CLP genes using next generation sequencing (exome sequencing) methodologies. We have collected an extensive set of DNA samples from multigeneration CLP families and we have chosen to investigate those where there are four or more affected members. We analysed exome data both within and between families to identify unique and shared rare variants. Further characterization involves segregation analysis and replication screening in large patients' cohorts. The identification of new genes responsible for CLP will be vital to improve our understanding of normal and abnormal lip and palate development and we hope to be able to make use of this information to develop accurate diagnosis and future risk assessment as well as the potential to develop novel treatment or preventative therapies.

The autism- and schizophrenia-associated gene, *GNB1L*, regulates WNT-signalling

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GNB1L is located on chromosome 22q11.2, within the 22q11 Deletion Syndrome (22q11DS) critical region. 22q11DS is characterized by abnormal development of pharyngeal arch derivatives and is associated with increased risk for a range of behavioural disorders, including autism and schizophrenia. *GNB1L* has been implicated in both autism and schizophrenia by a number of genetic studies, independent of 22q11DS. Mice hemizygous for *Gnb1l* display neuro-behavioural deficits. We have therefore sought to discover the function of GNB1L in order to determine its possible role in autism and schizophrenia.

GNB1L consists of six WD40 repeats, a loosely conserved motif that mediates protein–protein interaction. A yeast two-hybrid screen identified interaction of GNB1L with HIPK1. *Hipk1* has been previously shown to be involved in neural development and to act as a modulator of Wnt-signalling. We show that GNB1L acts as a negative regulator of Wnt-signalling and likely acts in the same pathway as HIPK1. Mutations in the WNT-signalling pathway have been previously linked with both autism and schizophrenia. Our results suggest that this pathway is dysregulated in 22q11DS and may contribute to the behavioural disorders seen in these patients.