IDENTIFICATION AND ANALYSIS OF THE NOVEL GUMBY GENE AND ITS VERTEBRATE SPECIFIC ROLES IN THE MOUSE

by

ELENA RIVKIN

A thesis submitted in conformity with requirements for the degree of Doctor of Philosophy.

Graduate Department of Molecular Genetics

University of Toronto

© Copyright by Elena Rivkin, 2010
Identification and analysis of the novel gumby gene
and its vertebrate roles in the mouse

Elena Rivkin

Doctor of Philosophy

Department of Molecular Genetics

University of Toronto

2010

ABSTRACT

Forward genetic screens in the mouse are contributing significantly to our understanding of basic mammalian development and human disease. In one such screen, our laboratory has identified the novel mouse gumby mutant, which affects the development of the neural and vascular systems.

Here, I describe the characterization of the gumby mutant phenotype and the identification of its causative mutation in a novel, vertebrate-specific gene, which is one of the genes deleted in patients affected by Cri du Chat Syndrome that exhibit mental retardation and craniofacial deficits.

Expression and phenotypic analyses revealed a requirement for the gumby gene in the facial nerve axon guidance and angiogenesis. Lately, it has become evident that many common mechanisms and molecules operate during neural and vascular development. My results suggest that the gumby gene is an attractive candidate for regulating both processes and its analysis in the future may help us understand how the navigational mechanisms for both systems are intertwined.
My studies show that gumby is a cytoplasmic protein that is present in many embryonic and adult tissues. In yeast-two-hybrid assays gumby interacts with a member of the highly conserved Wnt pathway - Dishevelled 2 (Dvl2). In both $Dvl2^{-/-}$ and $gumby$ homozygotes, the level of the cardiac neural crest cell marker $Plexin2A$ is decreased. The three branches of the Wnt pathway have been shown to regulate a wide range of events during embryogenesis and adult homeostasis, and subsequently have been implicated in multiple human pathologies. Taken together my data suggest that gumby may be required for Wnt signaling in angiogenesis and/or facial nerve guidance. Given that Wnt signaling has been shown to play key roles in axon guidance, $gumby$ and its roles in Wnt signaling may also contribute to the mental retardation seen in patients with Cri du Chat Syndrome. Thus, further analyses of molecular and biologic roles of $gumby$ will provide important avenues for understanding the cell biology of human disease.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. Sabine Cordes for giving me the opportunity to work with her and for being a great mentor throughout this project. Without her guidance and persistent help, this dissertation would not be possible.

I am truly grateful for my committee members, Dr. C. C Hui and Dr. Joseph Culotti who have made their support and assistance available in a number of ways, and whose advice has shaped this project tremendously.

I would like to acknowledge my fellow lab members with whom I had the pleasure of working over the years. These include Stephanie Almeida, Michael Huynh, Dennis Kim, Teresa MacLean, Ryan Mui, Angela Sing, Kendra Sturgeon, and Joanna Yu. You have created a great research environment and I will remember and cherish you forever.

Lastly, I would like to offer my eternal gratitude to my family and friends who offered me support and encouragement during the completion of this project. I am especially grateful to my wonderful husband and our little creation Julia, who mean the world to me, and constantly inspire me to work harder and to aim higher.
# TABLE OF CONTENTS

Abstract ............................................................................................................................... ii
Acknowledgements ............................................................................................................ iv
Table of contents ................................................................................................................. v
List of tables ....................................................................................................................... ix
List of figures ...................................................................................................................... x
List of appendices ............................................................................................................. xii
Abbreviation key .............................................................................................................. xiii

## 1.0 INTRODUCTION ........................................................................................................ 1

1.1 Overview of work that has led to this project ............................................................... 1
   1.1.1 Forward genetic screen to identify cranial nerve mutations in the mouse ........... 1
1.2 Overview of vertebrate cranial nerves and their early development ................. 8
   1.2.1 The unique development of the facial cranial nerve ..................................... 12
      1.2.1.1 Basic neuroanatomical development of facial cranial nerve .......... 12
      1.2.1.2 Migration of facial branchiomotor neurons .................................... 13
      1.2.1.3 Axon guidance of facial cranial nerves ......................................... 15
1.3 Human cranial dysinnervation disorders ................................................................. 19
1.4 Cri du Chat Syndrome ............................................................................................. 21
1.5 Overview of Angiogenesis ...................................................................................... 22
   1.5.1 VEGF/VEGFR signaling in vascular development ........................................... 23
   1.5.2 Notch signaling in angiogenesis .................................................................... 25
   1.5.3 Wnt signaling in vascular development ........................................................ 27
      1.5.3.1 Short summary of Wnt pathways .................................................... 27
1.6 Common mechanisms of nerve and blood vessel development ..................... 35
   1.6.1 Pathways with roles in facial nerve axon guidance and angiogenesis .......... 37
   1.6.2 Pathways with roles in FBM migration and angiogenesis ............................... 40
      1.6.2.1 Neuropilins and VEGF ................................................................. 40
      1.6.2.2 Wnt signaling pathway ................................................................. 41
   1.6.3 Other axon guidance pathways with roles in cranial nerve development and angiogenesis ................................................................. 43
      1.6.3.1 Eph-Ephrins ................................................................................... 43
      1.6.3.2 Netrins and Unc5b ........................................................................ 45
      1.6.3.3 Slits and Robos ............................................................................ 46
1.7 Summary ..................................................................................................................... 47

## 2.0 MATERIALS AND METHODS ............................................................................. 49

2.1 Mouse Manipulations ............................................................................................... 49
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 Embryos harvesting</td>
<td>49</td>
</tr>
<tr>
<td>2.3 Synthesis of first strand cDNA</td>
<td>49</td>
</tr>
<tr>
<td>2.4 Mouse genotyping</td>
<td>49</td>
</tr>
<tr>
<td>2.4.1 Standard PCR assay</td>
<td>50</td>
</tr>
<tr>
<td>2.5 Genetic mapping of the <em>gumby</em> mutation</td>
<td>50</td>
</tr>
<tr>
<td>2.5.1 Initial mapping done by other members of our laboratory</td>
<td>50</td>
</tr>
<tr>
<td>2.5.2 Fine mapping of the <em>gumby</em> mutation</td>
<td>50</td>
</tr>
<tr>
<td>2.5.3 Sequencing the genes within <em>gumby</em> critical interval</td>
<td>52</td>
</tr>
<tr>
<td>2.5.4 Sequencing <em>Fam105b</em></td>
<td>52</td>
</tr>
<tr>
<td>2.5.5 Genotyping the T285A mutation using ARMS-PCR analysis</td>
<td>53</td>
</tr>
<tr>
<td>2.6 Cloning of <em>Fam105b</em></td>
<td>54</td>
</tr>
<tr>
<td>2.6.1 Synthesis of first strand cDNA</td>
<td>54</td>
</tr>
<tr>
<td>2.6.2 Cloning of Fam105b-pBluescript SKII</td>
<td>54</td>
</tr>
<tr>
<td>2.7 Histological Analysis</td>
<td>55</td>
</tr>
<tr>
<td>2.8 Northern Blot analysis</td>
<td>55</td>
</tr>
<tr>
<td>2.9 Apoptosis and Proliferation Assays</td>
<td>56</td>
</tr>
<tr>
<td>2.10 Whole-mount in situ hybridization (WISH)</td>
<td>56</td>
</tr>
<tr>
<td>2.10.1 <em>Fam105b</em> in situ probe synthesis</td>
<td>56</td>
</tr>
<tr>
<td>2.10.2 Whole mount in situ hybridization protocol</td>
<td>57</td>
</tr>
<tr>
<td>2.11 Generation of rabbit anti-<em>Fam105b</em> polyclonal antibody</td>
<td>59</td>
</tr>
<tr>
<td>2.11.1 Generation of GST-<em>Fam105b</em> fusion protein</td>
<td>59</td>
</tr>
<tr>
<td>2.11.2 Generation of MBP-<em>Fam105b</em> fusion protein</td>
<td>59</td>
</tr>
<tr>
<td>2.12 Western blot analysis</td>
<td>61</td>
</tr>
<tr>
<td>2.12.1 Preparation of cell and tissue lysates</td>
<td>61</td>
</tr>
<tr>
<td>2.12.2 Western blot</td>
<td>61</td>
</tr>
<tr>
<td>2.12.3 Immunoprecipitation</td>
<td>62</td>
</tr>
<tr>
<td>2.13 Testing <em>Fam105b</em> antibody specificity by preblocking with GST-<em>Fam105b</em></td>
<td>62</td>
</tr>
<tr>
<td>2.14 Immunohistochemistry</td>
<td>62</td>
</tr>
<tr>
<td>2.14.1 Tissue immunofluorescence</td>
<td>62</td>
</tr>
<tr>
<td>2.14.2 Cell immunofluorescence</td>
<td>63</td>
</tr>
<tr>
<td>2.14.3 Whole-mount PECAM-1 immunohistochemistry</td>
<td>64</td>
</tr>
<tr>
<td>2.14.4 Slide immunohistochemistry</td>
<td>65</td>
</tr>
<tr>
<td>2.15 BAC rescue analysis</td>
<td>65</td>
</tr>
<tr>
<td>2.15.1 Generation of BAC transgenic mice</td>
<td>65</td>
</tr>
<tr>
<td>2.15.2 Estimation of BAC copy number using sequencing</td>
<td>66</td>
</tr>
<tr>
<td>2.15.3 Quantitative PCR</td>
<td>67</td>
</tr>
<tr>
<td>2.15.4 Western blot analysis of BAC transgenics</td>
<td>67</td>
</tr>
<tr>
<td>2.15.5 Testing for BAC transgenic rescue</td>
<td>68</td>
</tr>
<tr>
<td>2.16 Fosmid rescue of the <em>gumby</em> phenotype</td>
<td>68</td>
</tr>
<tr>
<td>2.17 Designing a screening strategy to identify novel <em>gumby</em> mutations</td>
<td>69</td>
</tr>
<tr>
<td>2.18 <em>Fam105b</em> knockdown using siRNA technology</td>
<td>69</td>
</tr>
</tbody>
</table>

RESULTS ........................................................................................................................ 71

Chapter 3: Identification and characterization of the *gumby* candidate gene ....... 71
3.0 Overview .......................................................................................................................... 71
3.1 *Gumby* mutants have hypersprouting facial nerve, and die between E11.5-E12.5..... 71
3.2 Mapping the *gumby* mutation .......................................................................................... 74
   3.2.1 Refining *gumby* critical interval using meiotic recombination ................................. 74
   3.2.2 Analysis of *gumby* critical interval ........................................................................... 75
      3.2.2.1 *Gumby* critical interval is syntenic to human region deleted in Cri du Chat Syndrome .............................................................................................................. 75
      3.2.2.2 Evaluation of genes in *gumby* critical interval ..................................................... 78
3.3 Sequencing the genes in the critical interval .................................................................... 82
3.4 Literature analysis of the *Fam105b* gene ........................................................................ 82
3.5 Analysis of *Fam105b* expression and localization .......................................................... 86
   3.5.1 *Fam105b* is expressed in the embryo and adult mouse tissues ................................. 86
   3.5.2 Whole mount *in situ* hybridization analysis of *Fam105b* in mouse embryos ............. 87
   3.5.3 Generation and evaluation of *Fam105b* specific antibody ............................................. 91
   3.5.4 The *gumby* mutation does not affect expression levels of *Fam105b* protein ................ 93
   3.5.5 *Fam105b* is expressed in vascular endothelial.......................................................... 93
   3.5.6 *Fam105b* is expressed in the kidney tubules and intestinal epithelia ........................... 98
   3.5.7 *Fam105b* is expressed in placental endothelial and uNK cells ..................................... 102
   3.5.8 *Fam105b* is expressed in several neural tissues ......................................................... 105
   3.5.9 *gumby* mutation does not change the cellular localization of *Fam105b* ................. 108
3.6 Analysis of the *gumby* phenotype ................................................................................... 110
   3.6.1 Analysis of *Hoxb1*, *Phox2b*, and *Sox10* expression in *gumby* embryos ............... 110
   3.6.2 *Gumby* mutants likely die because of vascular deficits ............................................ 113
      3.6.2.1 Embryonic and extraembryonic morphology of *gumby* embryos at E10.5- E11.0 ......................................................................................................................... 113
      3.6.2.2 *gumby* embryos have normal levels of apoptosis or proliferation ..................... 117
      3.6.2.3 *gumby* embryos exhibit vascular defects in the head and in the trunk ...... 120
3.7 *Fam105b* interacts with *Dishevelled 2* ......................................................................... 122

Chapter 4: Functional confirmation of the *gumby* gene and other genetic analyses of *Fam105b* .......................................................................................................................... 126

4.0 Overview .......................................................................................................................... 126
4.1 Rescue analysis of *gumby* mutation ................................................................................ 127
   4.1.1 Attempting Fosmid rescue of *gumby* mutation .......................................................... 127
   4.1.2 BAC rescue of *gumby* mutation ................................................................................. 128
      4.1.2.1 BAC rescue of *gumby* lethality and vascular deficits ........................................... 131
      4.1.2.2 Analysis of *Fam105b* expression in BAC carriers .................................................. 133
4.2 Generation of *Fam105b* allelic series .......................................................................... 134
4.3 Attempting to knock down *Fam105b* using RNA interference technology ............. 137

5.0 DISCUSSION .................................................................................................................. 139

5.1 The vital role of ENU mutagenesis screen in identifying the *gumby* mutation ................. 139
5.2 The facial nerve abnormalities of *gumby* mutant embryos ........................................... 143
5.3 Possible roles of *Fam105b* in angiogenesis ................................................................. 144
5.5 Fam105b and the Wnt pathway ................................................................. 147
  5.5.1 The role of Wnt signaling in the migration of the facial nerve .......... 148
  5.5.2 The role of Wnt signaling in angiogenesis ........................................... 149
  5.5.3 The role of Wnt signaling in cardiogenesis ......................................... 150
  5.5.4 Wnt signaling in intestinal epithelium, kidney tubules, and liver ... 155
5.6 Fam105b levels may be controled posttransciptionally ......................... 157
5.7 Using the gumby mouse mutant to study human diseases ...................... 157
  5.7.1 The potential role of Fam105b in Cri du Chat Syndrome ................. 163
5.8 Future directions and concluding remarks ............................................. 167

Appendix A - Primers sequences ................................................................. 169

REFERENCES .......................................................................................... 172
LIST OF TABLES

**Table 1**: Overview of the genes that map to a region syntenic to human chromosome 5p band 15.2, where the majority of the CdCS symptoms have been mapped. Genes outlined in red are located in *gumby* critical interval. Outside of this region only known genes are shown…………………………………………………………………………………….79

**Table 2**: Mouse mutant phenotype and/or human disorders associated with genes that map to human chromosome 5p band 15.2 where the majority of CdCS symptoms have been mapped. Genes outlined in red are located within the *gumby* critical interval…….81
LIST OF FIGURES

Figure 1: Mutagenesis scheme for recovery of recessive neurodevelomental mutations... 3
Figure 2: Embryos homozygous for the gumby mutation have facial nerve abnormalities. ............................................................... 5
Figure 3: Mapping of ENU-induced mutations ................................................................. 7
Figure 4: Organization of cranial motor nerves and sensory ganglia in the mouse............ 9
Figure 5: The Wnt pathway .............................................................................................. 30
Figure 6: Nrarp coordinates Notch and Wnt signaling in endothelial cells...................... 34
Figure 7: Simplified model of vessel branching ............................................................... 35
Figure 8: Phenotype of gumby embryos at E10.5-E12.5.................................................. 73
Figure 9: Fine mapping of the gumby mutation ................................................................ 75
Figure 10: gumby critical interval is syntenic to a human region deleted in Cri du Chat Syndrome ................................................................. 77
Figure 11: T to A mutation in Fam105b changes a conserved tryptophan to an arginine 83
Figure 12: Genotyping gumby embryos ........................................................................ 86
Figure 13: Northern and Western blot analyses of Fam105b expression ......................... 87
Figure 14: Analysis of Fam105b expression by whole-mount in situ hybridization........ 89
Figure 15: Vibrotome sections of embryos processed for whole-mount in situ hybridization ................................................................................................... 90
Figure 16: Whole-mount in situ hybridization using the sense probe ......................... 90
Figure 17: Western blot analysis of Fam105b antibody .................................................. 92
Figure 18: Analysis of Fam105b protein levels in gumby mutants. ................................. 93
Figure 19: Fam105b co-localizes with endothelial marker PECAM-1 predominantly in neural tissues ................................................................. 96
Figure 20: Fam105b is expressed in a subset of endothelial cells ..................................... 98
Figure 21: Fam105b is expressed in the kidney, intestine and liver in embryos and adult mice ......................................................................................................... 99
Figure 22: Fam105b is expressed in the kidney tubules and in the intestinal villi........... 101
Figure 23: Fam105b expression in the placenta ................................................................. 105
Figure 24: *Fam105b* is expressed in dorsal root ganglia and sympathetic ganglia at E11.5.
.................................................................................................................................................. 106
Figure 25: *Fam105b* is expressed in the adult brain................................................................. 108
Figure 26: Fam105b is localized to the cell cytoplasm................................................................. 110
Figure 27: Expression analysis of *Hoxb1*, *Phoxb2*, and *Sox10* in *gumby* embryos........ 112
Figure 28: Yolk sac appears overtly normal in *gumby* mutants............................................... 114
Figure 29: Placenta appears normal in *gumby* mutants at E11.0.............................................. 115
Figure 30: H&E stained *gumby* embryos at E10.5 appear similar to normal littermates.
.................................................................................................................................................. 117
Figure 31: H&E stained gumby embryos at E11.5................................................................. 117
Figure 32: *gumby* embryos have normal levels of proliferation and apoptosis.............. 119
Figure 33: Branching of vessels in the head and the trunk is impaired in gumby embryos.
.................................................................................................................................................. 122
Figure 34: Expression analysis of *Pitx2* and *PlexinA2* in the migrating cardiac crest cells
in gumby embryos................................................................. 125
Figure 35: Schematic representation of BAC bMQ-396D3 ...................................................... 128
Figure 36: BAC rescue breeding strategy................................................................. 130
Figure 37: BAC rescues lethality and vascular abnormalities of *gumby* embryos........ 132
Figure 38: Analysis of *Fam105b* expression in BAC transgenic mice................................. 134
Figure 39: Generation of *Fam105b* allelic series................................................................. 137
LIST OF APPENDICES

Appendix A: List of primers used in this study.............................................................170

Appendix B: Generation of a transgenic mouse line expressing GFP-Cre protein from a

\textit{Hoxb4} neural enhancer...............................................................186
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ß-cat</td>
<td>ß-catenin</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AAA</td>
<td>aortic arch artery</td>
</tr>
<tr>
<td>AP</td>
<td>anteroposterior</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>ARMS</td>
<td>amplification refractory mutation system</td>
</tr>
<tr>
<td>AS</td>
<td>atrial septum</td>
</tr>
<tr>
<td>ASD</td>
<td>anterior septal defects</td>
</tr>
<tr>
<td>AV</td>
<td>anteriovenous</td>
</tr>
<tr>
<td>BA</td>
<td>branchial arch</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BM</td>
<td>branchial motoneuron</td>
</tr>
<tr>
<td>BP</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C3H</td>
<td>C3H/HeJ mouse strain</td>
</tr>
<tr>
<td>C57</td>
<td>C57Bl6/J mouse strain</td>
</tr>
<tr>
<td>CamK2</td>
<td>calcium-calmodulin-dependent kinase 2</td>
</tr>
<tr>
<td>CC</td>
<td>cardiac cushions</td>
</tr>
<tr>
<td>CCDDs</td>
<td>congenital cranial dysinnervation disorders</td>
</tr>
<tr>
<td>CdCS</td>
<td>Cri du Chat Syndrome</td>
</tr>
<tr>
<td>CHD</td>
<td>congenital heart diseases</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>CE</td>
<td>convergent extension</td>
</tr>
<tr>
<td>CGS</td>
<td>continuous gene syndrome</td>
</tr>
<tr>
<td>CNC</td>
<td>cardiac neural crest</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CT</td>
<td>chorda tympani</td>
</tr>
<tr>
<td>DAP</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Dig</td>
<td>digoxygenin</td>
</tr>
<tr>
<td>DII</td>
<td>delta like</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DORV</td>
<td>double outlet right ventricle</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Dvl</td>
<td>dishevelled</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENU</td>
<td>N-ethyl-N-nitrosurea</td>
</tr>
<tr>
<td>ESC</td>
<td>endothelial stalk cell</td>
</tr>
<tr>
<td>ETC</td>
<td>endothelial tip cell</td>
</tr>
<tr>
<td>F</td>
<td>forward</td>
</tr>
<tr>
<td>FBM</td>
<td>facial branchial nerve</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FHF</td>
<td>first heart field</td>
</tr>
<tr>
<td>fp</td>
<td>floor plate</td>
</tr>
<tr>
<td>FVM</td>
<td>facial visceral nerve</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Frz</td>
<td>frizzled</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde - 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>GSPN</td>
<td>greater superficial petrosal nerve</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin eosin</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>ISV</td>
<td>intersomitic vessels</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LA</td>
<td>left aorta</td>
</tr>
<tr>
<td>LEF</td>
<td>lymphoid enhancer factor</td>
</tr>
<tr>
<td>LRP</td>
<td>low-density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>Mb</td>
<td>midbrain</td>
</tr>
<tr>
<td>Mb</td>
<td>megabase</td>
</tr>
<tr>
<td>MBS</td>
<td>Mobius syndrome</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MIT</td>
<td>Massachusetts Institute of Technology</td>
</tr>
<tr>
<td>MR</td>
<td>mental retardation</td>
</tr>
<tr>
<td>NC</td>
<td>neural crest</td>
</tr>
<tr>
<td>Neuro2A</td>
<td>neuroblastoma 2A</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>Nrarp</td>
<td>notch-regulated ankyrin repeat protein</td>
</tr>
<tr>
<td>Nrp</td>
<td>neuropilin</td>
</tr>
<tr>
<td>OFT</td>
<td>outflow tract</td>
</tr>
<tr>
<td>O/N</td>
<td>overnight</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>planar cell polarity</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDA</td>
<td>persistent ductus arteriosus</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>Plxn</td>
<td>plexin</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PNVP</td>
<td>perineural vascular plexus</td>
</tr>
<tr>
<td>PTA</td>
<td>persistent truncus arteriosus</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>r</td>
<td>rhombomere</td>
</tr>
<tr>
<td>R</td>
<td>reverse</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RA</td>
<td>right aorta</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricle</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sec</td>
<td>seconds</td>
</tr>
<tr>
<td>Sema</td>
<td>semaphorin</td>
</tr>
</tbody>
</table>
SHF : second heart field
SMA : smooth muscle actin
SMS : Smith-Magenis syndrome
SNP : single nucleotide polymorphism
SSC : sodium chloride sodium citrate buffer
SSLP : simple sequence length polymorphism
SSN : superior salivatory nucleus
Taq : thermus aquaticus
TBS : tris buffered saline
TCF : T cell-specific transcription factor
TGA: transposition of the great arteries
uNK: uterine natural killer
VEGF : vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
VM : visceral motoneuron
VS : ventricular septum
VSD : ventricular septal defects
WISH : whole-mount in situ hybridization
WHS : Wolf-Hirschhorn syndrome
WT : wild-type
1.0 INTRODUCTION

1.1 Overview of work that has led to this project

Cranial nerves that innervate the vertebrate head, throat, and heart, arise in a stereotypical pattern in the embryonic hindbrain and act as sensitive indicators of general and region-specific neuronal development. In addition, cranial nerves are directly or indirectly affected in many human disorders, particularly craniofacial syndromes [1-3]. Although, analysis of mouse mutants has begun to identify molecules required for specification and pathfinding of cranial nerves (reviewed in [1]), at present, the molecular mechanisms that regulate cranial nerve development are mostly unknown. Most of our existing knowledge has been gained either by fortuitous observations of cranial nerve anomalies in knock-out or classical mouse mutants or by deliberate pairing of gene expression analyses with reverse genetics. In the latter approach, molecules expressed in neuronal subsets are identified and the consequences of their loss and gain of function analyzed in the mouse and chick. Most often, these reverse genetic approaches have focused on molecules with previously known neurobiological roles in invertebrates [1].

Using a forward genetic approach in the mouse, our laboratory has identified a series of mutations that affect cranial nerve development. One of these - the gumby mutation - is of particular interest with regards to the facial nerve (cranial nerve VII), which has been implicated in several human disorders. During the course of my PhD thesis, I have focused on the molecular, genetic and biological characterization of the gumby mutation.

1.1.1 Forward genetic screen to identify cranial nerve mutations in the mouse

To identify molecules with previously unknown neurobiological roles or less incriminating expression patterns, a forward recessive genetic screen that systematically
recovers mutations affecting cranial nerve development in the mouse has been performed in our laboratory [4]. The potent mutagen N-ethyl-N-nitrosourea (ENU) and availability of the mouse genome sequence have made forward genetic screens and identification of the affected gene(s) highly feasible in the mouse [5]. Once treated with ENU, a single male mouse can produce 100-150 progeny (first generation, G1), each of which represents one mutagenized gamete. On average, the high efficiency of ENU mutagenesis results in a new mutation in any single locus in one of 500-1000 G1 animals. To screen for recessive mutations, each G1 male is used to create a three-generation pedigree, in which he is mated with three to six of his daughters (G2 females) (Figure 1). In our screen, we used ENU mutagenesis combined with direct immunohistochemical visualization method to identify recessive mutations that affect cranial nerve development. Briefly, twenty-five 8- to 10-week-old C57BL/6J (C57) male mice were treated with three weekly fractionated doses of 85 mg/kg of ENU. Ten weeks after their final ENU treatment, male mice had regained their fertility and were bred to normal C57 females. To generate pedigrees from 40 of the resulting G1 progeny males, each G1 male was bred to two normal C57 females and subsequently was bred with three to six of his resulting G2 daughters. G3 embryos were harvested at embryonic day (E) 10.5, and stained with 2H3 anti-neurofilament antibody (Hybridoma Bank). A G1 male was considered to carry a neurodevelopmental mutation if the identical phenotype was observed in multiple progeny from the same litter and in multiple progeny from other independent litters. To generate strains from G1 carrier males, the G1 male was bred to both C57 females for strain maintenance and to C3H/HeJ (C3H) females for genetic mapping studies.
Figure 1: Mutagenesis scheme for recovery of recessive neurodevelopmental mutations.

C57BL/6J male mice were treated with three doses of N-ethyl-N-nitrosourea (ENU), and upon recovery of fertility mated with normal C57BL/6J females, and the resulting offspring from this cross are referred to as generation (G) 1. Each G1 male is the product of an individual sperm and so has a unique set of ENU-induced mutations in a heterozygous manner. Third generation pedigrees were generated for 40 of the resulting G1 male progeny, which were first bred with normal C57Bl/6J mice, and subsequently mated with the resulting G2 daughters. E10.5 G3 embryos were harvested from five to
seven G2 daughters of each G1 male, and neurodevelopment was analyzed by immunohistochemistry with an anti-neurofilament antibody. A G1 male was designated as carrying a mutation if multiple embryos in multiple litters exhibited the same neuronal abnormality. (m) designates the ENU-induced mutation.

Immunohistochemical analysis was performed on E10.5 G3 embryos for several important developmental and technical reasons. First, the antibody penetrates the embryonic tissues well at this stage, and, thus, neurons and axonal processes can be detected in their stereotypic positions allowing early innervation processes to be observed. Second, in contrast to E8.5–E9.5 timed matings, the variation in E10.5 embryonic staging is minimal, and embryos can be harvested with ease. Third, embryos with more severe patterning defects often can survive up to E10.5, and thus mutations that affect overall neural patterning are still recoverable. Fourth, some genetic effects on axon migration may be missed unless mouse embryos are examined relatively early (E10.5–E12.5) in development, because early axon migration defects can in principle be corrected by remodeling and compensatory mechanisms by E15.5, as observed in Semaphorin 3A mouse mutants [4, 6]. In contrast to developmental gene expression-based assays, this immunohistochemical approach allows analysis of neuron development directly and makes no assumptions of what gene expression patterns may be the most important. Finally, processed embryos can be stored for over a year, thus preserving the experimental record [4].

In total after screening 40 pedigrees, our laboratory has recovered seven new neurodevelopmental mutations, with phenotypes ranging from single cranial nerve defects to global axon guidance deficits. Our lab was particularly interested in identifying new mutations that affected only one or a subset of cranial nerves, and thus decided to further pursue one such mutation, that we called gumby (referred to as 7-1 mutation in
Mar et al, 2005) (Figure 2). In normal embryos at E10.5, the facial nerve (cranial nerve VII) is well fasciculated and travels as one smooth stream towards its targets (Figure 2A,B). In contrast, in E10.5 embryos homozygous for the gumby mutation, the facial nerve consistently showed extra "sprouting" or "branching", although it followed its normal trajectories (Figure 2C,D).

Figure 2:Embryos homozygous for the gumby mutation have facial nerve abnormalities.
Whole-mount immunohistochemistry neurofilament stainings of E10.5 embryos are shown. The facial nerve (VII) of normal C57BL/6J E10.5 embryos (A,B) is well fasciculated and travels as one smooth stream toward its targets. In contrast, the facial nerve in embryos homozygous for the gumby mutation (gumby/gumby) (C,D) has small branches that jut off the nerve stem, giving it a rough "sprouty" appearance. Except for some minor sprouting, the apparent targeting of the vestibuloacoustic nerve (VIII) to the otic vesicle appears mostly normal. The arrows indicate the processes of the facial and vestibuloacoustic nerves that are affected in gumby mutants.

For the mapping of recessive mutations, the C57 progenitor male was bred to C3H females. Five of the resulting C57/C3H males were mated to several of their sisters, and progeny was tested to identify "carrier" males, which were heterozygous for the mutation. Resulting E10.5 embryos were scored for the mutant phenotype as described above. DNA
was generated from all embryos from litters containing two or more embryos with the expected mutant phenotype. Once a carrier male was confirmed, map position of mutations was determined by analysis of segregation of chromosome-specific markers on C3H inter-subspecific intercross panels by standard procedures [7]. Since the mutation arose on the C57 background, markers located near the mutation should show homozygosity for the C57-specific variant in mutant animals and be either homozygous for the C3H variant or heterozygous for the C3H and C57 variants in unaffected animals (Figure 3). For the *gumby* mutation, a total of 12 affected and 33 unaffected embryos were initially used in the genome-wide scan by genotyping with 70 MIT markers that show distinct C57 and C3H specific variations. This analysis localized *gumby* mutation on mouse chromosome 15 between MIT markers D15MIT130 at 20.7Mb and D15MIT138 at 39.8Mb (19.1Mb) [4].
In our breeding screen used to map ENU-induced mutations, a carrier C57BL/6J male outcrossed to a C3H/HeJ female to initiate the mapping process. The resulting heterozygous animals were then intercrossed to generate a large number of animals for mapping. At this stage, the offspring were phenotyped for the trait of interest, and affected and non-affected mice were identified. These mice were genotyped using simple sequence length polymorphisms (SSLPs) to determine recombination that has occurred.
during meiosis. These markers distinguish between C57BL/6 DNA and C3H/HeJ DNA, and so it is possible to determine linkage between a particular marker and the trait of interest. Here, the ENU-induced mutation is marked by an asterisk (*), indicating that the gene responsible for the phenotype is found at this location in the genome. Once sufficient animals have been tested, the chromosome location of the mutated gene was identified. In this case, the *gumby* mutation is linked to a chromosome 15 marker D15Mit130.

My analysis of the *gumby* mutation has led to the identification of a novel vertebrate protein Fam105b, and uncovered novel functions of this protein in embryonic development. My results revealed that in addition to guiding the facial nerve axons, *Fam105b* has a critical role in vascular remodeling. Second, *Fam105b* is one of the genes deleted in human Cri du Chat Syndrome, although its role in this disorder has not been characterized. Third, a yeast-two hybrid assay has identified an interaction between human Fam105b and Dishevelled 2 [8], a pivotal component of the highly conserved Wnt pathways. Thus, *Fam105b* might play a role in Wnt signaling, which will be the focus of future studies in our laboratory. This introductory chapter focuses on topics relevant to these findings and to the direction of future experiments.

1.2 Overview of vertebrate cranial nerves and their early development

In the adult, the hindbrain is composed of the medulla oblongata, the pons and the cerebellum. The primary role of the cerebellum is to coordinate movement. The medulla and the pons, together with the midbrain, control higher order behaviours such as respiration, circulation, and wakefulness. The hindbrain also contributes 8 of the 12 pairs of cranial nerves, numbers V through XII (Figure 4). Cranial nerves can have both motor and sensory components, and those derived from the hindbrain are responsible for a wide variety of behaviours, including taste, hearing, balance, mastication, facial expressions,
eye movements, and secretion of tears, saliva and mucous (reviewed in [1]). Early in development, the vertebrate hindbrain is transiently subdivided into seven to eight segments called rhombomeres (r1-r8), along its anteroposterior axis [9]. Rhombomeres are distinguished from each other by limited cell movements, differential cell adhesion and repulsion, and rhombomere-specific expression of various genes such as the Hox gene family of transcription factors [10, 11]. Later in development, the hindbrain neuroanatomy corresponds with this morphological segmentation such that each rhombomere contains a characteristic set of specific classes of neurons. Cranial nerves arise from specific rhombomeres, as shown in Figure 4, and can serve as markers for normal hindbrain development ([10] reviewed in [1, 12, 13]. In the mouse, axons from motor nuclei lying in r1-r3 form the trigeminal (V) nerve. The facioacoustic (VII/VIII) and glossopharyngeal (IX) nerves gather axons from motor nuclei in r4/r5 and r6 respectively.

Figure 4: Organization of cranial motor nerves and sensory ganglia in the mouse. (A) A schematic diagram shows the cranial motor nerves and sensory ganglia of the mouse. Cell bodies of the motor nerves are at different medio-lateral positions within the
hindbrain, while those of the sensory ganglia are located flanking the hindbrain. III-oculomotor nerve; IV-the trochlear nerve; V-the trigeminal nerve; VI-the abducens nerve; VII-the facial nerve; VIII-the vestibulocoustic nerve; IX-the glossopharyngeal nerve; X-the vagus nerve; XI-the spinal accessory nerve; and XII- the hypoglossal nerve. The IV, VI and XII nerves are somatic motor nerves and have ventrally exiting axons. Axons from the branchiomotor (V, VII, IX, X, XI) and visceromotor (III, VII, IX, X) nerve axons exit the neural tube dorsally in distinct exit points located in the even-numbered rhombomeres or in the case of the oculomotor nerve in the midbrain. Sensory ganglia and motor nerves use the same exit points. Mb-midbrain; fp-floorplate; sc-spinal cord; ov-otic vesicle. (Adapted from Lumsden and Keynes, 1989 [9]) (B) A sagittal view of an E10.5 mouse embryo stained with anti-neurofilament antibody shows the distinct appearance of each cranial ganglion.

Cranial motor neurons are classified into three types: somatomotor, branchiomotor, and visceromotor. Cranial somatomotor (SM) neurons are found in the midbrain and hindbrain, and innervate muscles that control eye and tongue movements.

Cranial branchiomotor (BM) neurons are located in the hindbrain and innervate muscles within the branchial arches and control jaw movements, facial expression, the larynx, and the pharynx [14]. Branchial arches (also called pharyngeal arches) are a series of bilateral paired outpouchings flanking the pharynx (five in mice and humans, BA1-4,6) that contribute to various structures of the jaw, face and neck. Each arch has a central mesodermal component, neural crest-derived cells and a cranial nerve. These are surrounded by layers of inner endoderm and outer ectoderm. The patterning of the branchial arches and the segmentation of hindbrain are also intimately linked, with migratory neural crest cells that make up the peripheral neurons and glia, bones, cartilages, connective tissues, aortic arches, and pigment cells of the branchial arches that are derived from specific rhombomeres. Thus, branchial arches 1, 2 and 3 are filled by neural crest cells migrating from rhombomeres 2, 4 and 6, respectively (reviewed in [15]).
The first arch (BA1) is innervated by the trigeminal cranial nerve (V) and gives rise to the muscles of mastication, anterior belly of the digastric muscle, the bones of the maxilla, the mandible, the incus and the malleus of the middle ear, and Meckel's cartilage. The second branchial arch (BA2), is innervated by the facial cranial nerve (VII) and gives rise to the muscles required for facial expression, the buccinator, the stapedius, posterior belly of the digastric, the bones of the stapes, styloid process, the hyoid and Reichert's cartilage. The third branchial arch (BA3) is innervated by the glossopharyngeal cranial nerve (IX) and gives rise to the stylopharyngeous muscles, and the hyoid bone. The fourth and the sixth branchial arches (BA4 and BA6) are innervated by the vagus cranial nerve (X) and give rise to the muscles of the palate and the larynx, respectively.

Cranial visceromotor (VM) neurons are located in the hindbrain and innervate parasympathetic nerves that innervate tear glands, sweat glands, and the smooth muscles and glands of the pulmonary, cardiovascular and gastrointestinal systems.

Cranial motor nerves gather axons from motor neurons from one region within the hindbrain and innervate a specific target region. By contrast, some cranial ganglia gather axons from sensory neurons, and from autonomic parasympathetic and sympathetic neurons, to coordinate their initial migration towards a larger target area. Cranial sensory ganglia are generated from ectodermal placodes, neural crest cells, or both [16]. The cell bodies of sensory neurons are located outside the neural tube [1, 14]. There are large and reproducible variations in the relative numbers and positions of placode-derived and neural crest-derived neurons in specific cranial ganglia. For instance, whereas the trigeminal ganglion contains many neural crest-derived neurons, the vestibuloacoustic ganglion contains none and the facial ganglion contains very few. The mechanisms that
lead to these variations are not well understood [17]. The specification of sensory cranial ganglia requires neural-specific transcription factors *neurogenins (neurogenin 1 and 2)* [18, 19]. The patterning and diversification of cranial sensory ganglia are accomplished in part by the complementary utilization of the two *neurogenins: neurogenin 1* is required for the specification of proximal subset of sensory neurons derived from neural crest cells, as well as the trigeminal and the otic placodes. These include the trigeminal ganglion (V), a small portion of the proximal facial (VII) ganglion, the vestibuloacoustic (VIII) ganglion, and the proximal parts of the glossopharyngeal (VI) and vagal (X) ganglia [19]. In contrast, neurons originating from the distal epibranchial placodes, namely neurons of VII (geniculate), IX and X ganglia, depend on *neurogenin 2* [18]. These distal sensory neurons also depend on the paired homeodomain proteins *Phox2a* and *Phox2b* for their differentiation and survival [20, 21].

1.2.1 The unique development of the facial cranial nerve

1.2.1.1 Basic neuroanatomical development of facial cranial nerve

The facial nerve, which grants our faces their expressions, has unique and important features that allow insights into more complex neuronal cell migration and axon guidance, processes important throughout neurodevelopment. Both its cell migration and axon guidance appear to be governed by the major signaling pathways that also regulate other developmental processes such as neural crest migration and angiogenesis. Thus, the distinctive appearance of cranial nerves and ganglia in general, and the unique migration of the facial nerve, has made it a particularly attractive model for studying the mechanisms that control vertebrate neural migration and axon guidance, as well as more general processes during embryo development.
The cranial facial nerve (VII) contains axons from BM, VM and sensory neurons. Facial branchiomotor (FBM) neurons are born in r4, whereas facial visceromotor (FVM) are born in r5. Axons of FBM and FVM exit the hindbrain through a shared point in r4, their axons pass through the geniculate ganglion and then segregate again to innervate specific targets in the head and neck. FBM, which constitute the largest portion of the facial nerve, innervate the muscles of the second branchial arch (BA2) which control facial and jaw movement, crucial for facial expression, speaking, and eating. Preganglionic facial visceromotor (FVM) neurons innervate the parasympathetic submandibular ganglion as the chorda tympani (CT), and the parasympathetic pterygopalatine ganglion as the greater superficial petrosal nerve (GSPN). The efferent fibers of CT provide secretomotor innervation to two salivary glands, the submandibular and sublingual glands and to the vessels of the tongue. The efferent GSPN forms part of a chain of nerves that innervates the lacrimal glands and the mucose glands of the nose, palate and pharynx. The CT facial branch also carries special sensory taste fibers from the anterior two-thirds of the tongue. The special sensory component of GSPN provides skin sensation carried from the skin in and around the earlobe [17, 22, 23]. Rhombomere 4 also contains a populations of vestibuloacoustic neurons (cranial nerve VIII), which are efferent to the hair cell of the inner ear; a subset of these neurons (contralateral vestibuloacoustic neurons) translocate their cells bodies across the midline (reviewed in [1]).

1.2.1.2 Migration of facial branchiomotor neurons

Although we are now beginning to understand the genetic control of hindbrain motoneuron specification, little is known about the molecular mechanisms that control their subsequent development. A characteristic of several cranial neurons is their complex
and stereotypical migration in the embryonic hindbrain to form their final motor nuclei. For instance, the facial visceromotor neurons (FVM) are born close to the floorplate in r5 between E9.0 and E11.0, and between E11.5 and E14.5 they migrate dorsally within r5 to form the superior salivatory nucleus (SSN) [24]. A more extensive migration is observed in FBM neurons. FBM neurons are born in r4 between E9.0 and E11.0, and between E10.5 and E14.0, their cell bodies migrate tangentially along the ventral midline, reaching first r5 and then r6. In r6, these neurons begin a radial migration where they finally settle to form the facial motor nucleus [22, 25]. Given this extensive translocation of these neurons, the mechanisms of branchiomotor migration have been predominantly explored in FBM neurons. The analyses of several loss-of-function mutations have provided some information on the molecular mechanisms controlling FBM neuronal specification and migration (reviewed in [14]). Together, several observations suggest that the tangential migration involves a dynamic cross-talk between migrating neurons and the environment through which the cell body travels. Evidence for the role of environmental factors in the initiation of caudal migration has been provided by homotopic transplantation experiments between chick and mouse tissues [26]. When chick r5 was replaced with mouse r5 or r6, chick FBM neurons, which normally do not migrate, underwent caudal migration, indicating that r5 or r6 regions in mice emit guidance cues to initiate FBM neuron migration, although the identity of these cues and cell-intrinsic factors that enable FBM neurons to respond remain largely unknown. Several mouse mutants have been identified exhibiting defects in the caudal migration of FBM neurons. For instance, FBM neurons require transcription factor Hoxb1, and the transcription factor Phox2b, for their survival and caudal migration out of r4. *Hoxb1* is
expressed exclusively in r4 and in postmitotic FBM neurons throughout migration; *Phoxb2* is expressed in all BM and VM precursors [21, 26-28]. Transcription factors *Gata2* and *Gata3*, which are expressed in progenitors of FBM neurons, are required for FBM differentiation and caudal migration out of r4 [29]. Another transcription factor, *Ebf1*, is expressed in differentiating FBM neurons in r4 and throughout the migratory process and is involved in controlling the response of FBM neurons to their local environment during migration [24]. In addition, previous work has shown that FBM neurons regulate the expression of cell-surface receptors and adhesion molecules in a rhombomere-specific fashion leading to changes in adhesion properties, and in the selection of the appropriate migratory pathway [24]. Thus, the cell surface antigen *Tag-1* is expressed in FBM neurons throughout their migration in r4 and r5, but not in r6. By contrast, the neurotrophin *glial-cell-derived neurotrophic factor (GDNF)-receptor subunit Ret* and *netrin 1 receptor Unc5c* are only expressed during the migration in r5 and r6, while adhesion molecule *cadherin8* and *netrin 1 receptor neogenin* are only expressed during radial migration in r6 ([24, 30] reviewed by [31]). The functional significance of *cadherin8*, and *neogenin* in the radial migration, and the roles of *Ret, Unc5c* in the caudal migration remain to be explored. Other molecular pathways have been implicated in the FBM caudal migration including Wnt planar cell polarity (PCP) [32-35] and *VEGF/Nrp1* [36] signaling pathways discussed in more detail below.

### 1.2.1.3 Axon guidance of facial cranial nerves

As they leave the hindbrain, the axons of VM and BM neurons converge on shared dorsal exit points, whereas the axons of most somatic motor neurons exit ventrally [37]. Several genes and molecular pathways have been implicated in guiding the cranial motor axons
to their final targets (reviewed in [1, 12] and references therein) however, our understanding of the genetic hierarchies important for guiding individual cranial axons is still very incomplete. In general, cranial axon guidance can be divided into two parts: migration in the neural tube and migration in the periphery. Migration in the neural tube is governed by anteroposterior and dorsoventral cues within the hindbrain that guide axons to defined exit points, while migration outside the neural tube is governed by cues from secondary targets, the presence of survival factors and, of course, cell-intrinsic programmes of cranial nerves that dictate their responses to local guidance cues [1]. The initial phase of migration involves repulsion of all subtypes of cranial motor axons by the floor plate, which produces repulsive signals ensuring that motor axons and cell bodies do not cross the midline, and that BM/VM axons grow dorsally. Based on in vivo and in vitro experiments, candidates for BM and VM repulsion are the axon guidance molecules netrin 1, the Slits and Semaphorin3A [38-40].

In addition to these mechanisms, the compartmental nature of rhombomeres has a key role in axon guidance within the rhombomeres. Rhombomeres are formed and maintained partially as a consequence of the ephrin-Eph tyrosine kinase signaling pathway, which has also been implicated in axon repulsion (reviewed in [41]). Each rhombomere expresses a unique set of Eph receptors and ephrin ligands. In addition, ephrin ligands are expressed in specific branchial arches, whereas EphA receptors are expressed in a subset of branchiomotor neurons, suggesting that ephrins may play a role as branchial arch cues that control cranial motor pathfinding in the periphery [41, 42]. For instance, it has been shown in chick embryos that ephrin-Eph signaling plays a role in trigeminal motor axon topographic mapping. Specifically, r2 and r3-derived trigeminal motor neurons express
high and low levels of EphA receptors, respectively, and project to distinct target muscles which express ephrin-A5 in opposite patterns. Ephrin-A5 causes growth cone collapse of both r2 and r3-derived trigeminal motor axons in vitro and overexpression of ephrin A5 and/or their target Eph receptors in r3 trigeminal motor neurons, has led to aberrant axon branching pattern in vivo, suggesting that ephrins play a repulsive role for trigeminal nerves inside their muscle targets [43]. However, in the mouse, no single mutation in any Eph receptor or ephrin ligand has resulted in aberrant axon migration of cranial nerves, possibly due to some functional redundancy among ephrins and their receptors. Thus the complete role of ephrins in cranial axon guidance has not been fully elucidated.

Cranial motor axon behaviour in the periphery depends on a balance of positive and negative influences. However, these remain largely unknown and to this day only a few region-specific or nerve-specific cues have been identified. Explants and genetic experiments have indicated that for the branchiomotor neurons, some attractive and growth-promoting cues, such as hepatocyte growth factor (HGF) are provided by the branchial arches [44], and for the visceromotor neurons, their intermediate or final target ganglia. For instance the sphenopalatine ganglion is essential for the pathfinding of GSPN axons from the facial ganglion to the target area [45, 46]. In addition, following the rostrocaudal inversion of an odd-numbered rhombomere, trigeminal BM axons that project to incorrect target muscles are eliminated, further supporting a specific recognition between BM neurons and their targets [47]. Many vertebrate axons travel significant distances. Studies done in explant cultures indicate that survival factors, such as neurotrophic factors, act to promote axon outgrowth from the hindbrain. In addition, it is apparent from genetic analyses that sensory ganglia can at times promote the survival
of motor neurons and *vice versa* [48]. For instance, it was shown that *Hoxb1* positive, r4-derived neural crest cells that preferentially give rise to Schwann cells, might provide the guidance cues and/or survival factors that are needed for the projection and maintenance of the FBM nerve. An intriguing piece of evidence in favour of this idea is that a conditional deletion of *Hoxb1* in the neural crest results in a phenotype similar to that of *Hoxb1*-null mice. Specifically, in both models, in a significant proportion of animals the facial nerve fails to branch and migrate correctly and dies leading to facial paralysis. Thus, FBM axons might require an interaction with Schwann cells for their guidance and survival, possibly through the production by the Schwann cell of neurotrophic factors. This hypothesis is further supported by the prolonged survival of misspecified FBM neurons in *Hoxb1*-mutant mice that are also mutant for *Bax* - a gene that is required in cell death pathways that are associated with neurotrophic factor deprivation [12, 49].

Finally, it has been shown that the transmembrane proteins *neuropilins* (*Nrp1* and *Nrp2*) bind to secreted class 3 *Semaphorins* (*Sema3*) to regulate the guidance of cranial nerves in the mouse. Both *neuropilins* are expressed by cranial neurons, while several *Sema3* proteins are expressed in regions surrounding their paths, and in the branchial arch neural crest cells. In mice, the loss of these signals results in severe defasciculation of the facial, trigeminal, glossohypopharyngeal and vagus cranial nerves, consistent with their general role as repellent cues that channel growing axons into fascicles [50, 51]. *Neuropilins* convey *Semaphorin* signals by selectively recruiting a member of the A-type *Plexin* (*PlxnA*) family of receptors. In guiding the facial nerve, Nrp2 signals through *Sema3F* and *PlxnA3* and the disruption of this signal results in the defasciculation of the FBM nerve. Nrp1 signals through *Sema3A* and *PlxnA4*, and the disruption of this signal results in the
defasciculation of the FBM nerve and the FVM GSPN branch. Thus, 
\textit{Nrp1/Sema3a/PlxnA4} and \textit{Nrp2/Sema3F/PlxnA3} synergize to pattern the facial nerve, 
whereby both are required in branchiomotor neurons, but only \textit{Nrp1/Sema3A/PlxnA4} is 
required in visceromotor neurons [52].

1.3 Human cranial dysinnervation disorders

In combination, these and other yet unknown patterning mechanisms ensure that the 
axons of hindbrain motor neurons are wired appropriately to perform their adult 
functions. Disruptions of these mechanisms have profound ramifications on human 
health. The identification of genetic hierarchies, signaling cascades, and pathfinding 
strategies of the cranial nerves are therefore critical for understanding human disorders 
such as congenital facial nerve palsy, Möbius syndrome, and other human congenital 
cranial dysinnervation disorders (CCDDs) (reviewed in [2]). Human CCDDs result from 
aberrant innervations of the ocular and facial musculature, and generally arise from 
abnormal development of one or multiple cranial nerve nuclei or their axonal 
connections. The CCDDs encompass Duane syndrome, congenital fibrosis of the 
extraocular muscles (CFEOM), Möbius syndrome, Horizontal Gaze Palsy with 
Progressive Scoliosis (HGPPS), congenital facial paralysis, as well as several others [2].

While some only have abnormalities of ocular or facial innervation, others have 
additional systemic malformations that can be attributed to the underlying genetic defect. 
The occurrence of vascular malformations in some patients with CCDDs suggests that 
normal innervation is necessary for the development of a normal vasculature, or that the 
development of some nerves and blood vessels depends on common underlying genetic 
processes. In some cases, such as Möbius syndrome, an association with autism has been
proposed, suggesting that disorders of cranial motor neuron development might have far-reaching significance for understanding human disorders of brain wiring [53].

Given the developmental origins of cranial nerves, a cranial nerve anomaly could be caused by developmental perturbation at a number of key steps. For instance, disruption of hindbrain development or patterning could lead to misspecification or complete absence of a given cranial nerve(s). Alternatively, disruptions of axon guidance pathways could also impact nerve development, innervation or survival. The former is observed in *Hoxa1*<sup>−/−</sup> mouse mutants in which r4 is markedly reduced, and r5 is almost absent. Subsequently, the cell bodies and nerves of the facial and the abducens cranial nerves are either considerably reduced or absent, and variable defects in cranial sensory ganglia, and the otic pit (that gives rise to the inner ears) are present in E18.5 *Hoxa1*<sup>−/−</sup> fetuses [54].

Not surprisingly then, human HOXA1 syndromes, which include Athabaskan Brainstem Dysgenesis Syndrome (ABDS), Bosley-Salih-Alorainy Syndrome (BSAS) and Navaho Brainstem Syndrome result in similar phenotypes that include horizontal gaze abnormalities, sensorineural deafness, facial weakness, vascular malformations of the internal carotid arteries and cardiac outflow tract, mental retardation and autism spectrum disorder [55].

Other disorders with facial nerve deficits include Möbius syndrome (MBS) and congenital facial paralysis. MBS is a rare congenital disorder mainly characterized by paralysis of the facial and/or abducens cranial nerves, although occasionally it is accompanied by the paralysis of other cranial nerves. The disorder is frequently accompanied by craniofacial and limb malformations, and defects of the skeletal muscles. Congenital facial paralysis results from improper development of the facial nucleus.
and/or cranial nerve without a defect in other areas in the brainstem, or defects in facial muscles [56]. It is believed that there are genetic causes for both disorders but they have not yet been identified.

### 1.4 Cri du Chat Syndrome

In addition to CCDDs, many other genetic syndromes are characterized by distinct craniofacial dysmorphologies [3]. This is also true of many human deletion syndromes (also referred to as segmental aneuploidy syndromes or contiguous gene syndromes (CGSs)), such as Cri du Chat (OMIM 123450), William-Beuren syndrome (OMIM 194050) and DiGeorge syndromes (OMIM 188400). CGSs are caused by gene dosage imbalance of one or more critical genes within the aneuploid segment. In most cases, the mechanisms underlying dosage sensitivity in these disorders are largely unknown [57], and whether one gene, several contiguous genes, or position effects due to rearrangement contribute to the phenotype, remains a question pertinent to most known deletion syndromes. One of the complicating issues in elucidating the genes responsible for CGSs is that although mouse models of deletion disorders are technically feasible, especially in cases where a high degree of synteny and linkage conservation exists between human and mouse chromosomes, the analysis of these mouse mutants is often hindered by different sensitivity to dosage of a particular gene product across species [57].

Cri du Chat Syndrome (CdCS), which is relevant for our understanding of the gumby mutation and vice versa, is a rare disease with an incidence of 1:25,000-1:50,000 live births, resulting from a deletion of variable size occurring on the short arm of chromosome 5 (5p). The main clinical features are a high-pitched monochromatic cry, microcephaly (small heads), broad nasal bridge, epicanthal folds, micrognathia (small
jaw), and severe psychomotor and mental retardation. Other malformations may also be present: cardiac, neurological and renal abnormalities, preauricular tags, syndactyly, hypospadias and cryptorchidism [58-61]. Several independent groups have developed a cytogenetic and phenotypic map of 5p deletions for CdCS, although the results from these studies are not completely in agreement. Other than the high-pitched cry, which is mapped to a 15.3 [critical region 1 (CRI)], all other symptoms have been mapped to band 15.2 (CRII). The existence of two separate critical regions, and the lack of credible CdCS cases without the deletion, make it likely that the haploinsufficiency for a single gene may not explain the spectrum of phenotypes associated with CdCS disorder. Therefore, in the future, systematic characterization of genes that map to CdCS critical interval may prove valuable to our understanding of CdCS pathology.

1.5 Overview of Angiogenesis

In addition to our initial observation that *gumby* mutants exhibit facial nerve defects, thus implicating the *gumby* gene in axon guidance, subsequent analyses indicate that the *gumby* gene also plays an important role in angiogenesis. Recently, many studies have highlighted that many mechanisms and signaling pathways that control axon guidance also regulate angiogenesis (reviewed in [62, 63]).

The vertebrate vasculature is developed by two processes. In vasculogenesis, angioblasts adhere and differentiate into endothelial tubes that are first organized into a primitive vascular plexus and then into larger blood vessels including the dorsal aorta, cardinal veins and heart primordia (reviewed in [64]). In contrast, angiogenesis involves the sprouting of new vessels from pre-existing vessels or splitting of larger vessels to form the final microcapillary network (reviewed in [65]). These patterns become precisely
adapted to organ anatomy and physiology; therefore, they differ extensively between organs [65, 66]. In the adult, blood vessels acquire a quiescent, non-angiogenic state but retain considerable growth potential that is activated during wound healing as well as in certain physiological processes [67]. Angiogenesis is critical to ensure the supply of oxygen and nutrients to tissues throughout the body. This process is especially important for the central nervous system (CNS) as the neural tissue is extremely sensitive to hypoxia and ischemia [68]. Defects in vessel formation and remodeling lead to a wide range of pathological states including tumour progression, diabetic retinopathy, rheumatoid arthritis, and psoriasis (reviewed in [69]).

Angiogenesis involves a series of complex and coordinated events. Following stimulation by pro-angiogenic factors, endothelial cells (EC) degrade their extracellular matrix, and undergo proliferation, chemotactic migration, remodeling of their extracellular matrix and ultimately network formation and stabilization to generate new vasculature [65]. Several well-characterized molecular pathways have been implicated in vascular patterning including the VEGF, Notch, Eph/ephrin and Wnt pathways and likely many novel factors will be discovered in the future that participate in this process.

1.5.1 VEGF/VEGFR signaling in vascular development

It has been well established that members of the vascular endothelial growth factor (VEGF) family are major regulators of vasculogenesis and angiogenesis in embryonic development, adult life and many pathological conditions (reviewed in [65]). In mammals, the VEGF family consists of five family members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PIGF), which are secreted glycoproteins that form either disulphide-linked or non-covalent homodimers (reviewed in [70]). VEGF
family members signal by binding to a group of high affinity receptors, which include three cell surface proteins of the receptor tyrosine kinase family: VEGFR1 (flt-1), VEGFR2 (flk-1), and VEGFR3 (flt-4); and neuropilins. The most compelling evidence for the importance of VEGF signaling pathways in embryogenesis comes from the studies of mice deficient in components of the VEGF pathways. These studies have demonstrated an absolute requirement for correct VEGF levels in the embryo, and demonstrated that all VEGF receptors are crucial for vascular development during embryogenesis [70, 71].

VEGF signaling modulates a range of endothelial cell behaviours, ranging from their initial patterning in the embryo, to their recruitment during wound healing, or tumour angiogenesis, to their maintenance in normal tissues. During vertebrate development, vascular sprouting is induced by local production of VEGF-A, the most potent known pro-angiogenic factor. VEGF-A activates its receptor VEGFR2 and its co-receptor Nrp1. VEGF-A acts in a paracrine manner, as it is produced by the endoderm or ectoderm, whereas its receptors are expressed by mesoderm-derived angioblasts and later endothelial cells. VEGF/VEGFR2/Nrp signaling controls several processes in endothelial cells, such as cell migration, proliferation and survival. However, it is not yet clear how these are coordinately regulated to result in more complex morphogenetic events, such as tubular sprouting, fusion and network formation. Recently, it has been shown that VEGF-A in combination with other signaling molecules controls angiogenic sprouting in the postnatal mouse retina by guiding filopodia extension from specialized endothelial cell situated at the tips of the vascular sprouts ([72, 73] reviewed in [74]. These endothelial tip cells (ETCs) are highly polarized cells, present at the forefront of navigating blood
vessels that use filopodia to guide a sprouting vessel towards an angiogenic stimulus like VEGF. Endothelial stalk cells (ESCs) trail behind and follow the tip cell, proliferate to form an elongating stalk, and create a lumen [73]. The combined effect of migration by tip cells and proliferation by stalk cells results in vascular growth. Interestingly, although VEGFR2 is expressed in both ETCs and ESCs, it induces different biological effects in these cells in response to VEGF-A. In tip cells, VEGFR2 is abundant on filopodia, and induces migration. In stalk cells, which are exposed to lower levels of VEGF-A, it induces proliferation [73]. Genetic studies suggest that tip-cell migration depends on a gradient of VEGF-A, whereas stalk-cell proliferation is regulated by VEGF-A concentration [72, 73, 75]. Once the branch is formed and perfused, and re-oxygenation lowers VEGF-A, maintenance of low VEGF-A levels secures quiescence of endothelial cells of mature vessels [76].

1.5.2 Notch signaling in angiogenesis

Another major pathway involved in angiogenic sprouting is the Notch pathway (reviewed in [67, 77]). The Notch pathway is an evolutionarily conserved signaling pathway with roles in invertebrates as well as in almost every vertebrate organ and tissue. In mammals, four Notch receptors (Notch1-Notch4) interact with five ligands, namely Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged1, and Jagged2. Both Notch receptors and ligands are transmembrane proteins and Notch signaling is a form of cell-cell communication, helping a group of similar cells to integrate contextual information and to regulate cell fate determination. One mechanism, referred to as lateral inhibition, involves presentation of Notch ligand to an adjacent Notch receptor cell, resulting in activation of the Notch pathway in one cell and suppression in the adjacent cells. This
locks the cells into distinct cellular fates; one promoted by Notch signal activation and the other is dependent on lack of Notch signaling [78] (Figure 6A). So far, based on studies in mouse and zebrafish, receptors Notch1 and Notch4, and ligands Dll1, Dll4, Jagged1 as well as several downstream components have been implicated in various steps of vascular morphogenesis (reviewed in [67, 77, 79-82] and references therein).

One of the primary functions of the Notch pathway during vascular development is to establish arterial versus venous fate in endothelial cells. Until fairly recently, it was believed that the primary factor that regulates the differentiation of arteries and veins was blood flow. However, emerging evidence suggests that the arteriovenous (AV) identity of endothelial cells is established prior to the initiation of blood flow. Artery-specific expression has been reported for several Notch receptors and ligands, and disruption of Notch signaling leads to a loss of artery-specific defects in mouse and zebrafish, suggesting that Notch plays a role in promoting arterial fate [67].

The role of the Notch pathways in regulating embryonic vascular development is highly intertwined with the VEGF-A pathway. Studies in mammalian cell culture have shown that VEGF-A administration induces Notch1 and Dll4 expression in human arterial endothelial cells, but not in venous endothelial cells. More recent studies have also demonstrated cross-talk between the VEGF and Notch signaling in controlling vascular sprouting. The emerging picture suggests that the VEGF pathway acts as a potent upstream activating stimulus for angiogenesis, whereas both VEGF and Notch pathway help guide cell fate decisions that appropriately shape this activation. The Notch pathway is involved in a feedback loop with VEGF: VEGF/VEGFR2 induces Dll4 expression, while Dll4/Notch signaling downregulates VEGFR2, thus dampening VEGF activity [83-
The existing model suggests that when cells are hypoxic, they upregulate VEGF-A, and a VEGF-A gradient, is formed. Endothelial cells at the vascular front are exposed to the highest VEGF-A level and therefore produce more Dll4 than their neighbours. As VEGF upregulates Dll4, it signals in a contact-dependent manner to neighbouring endothelial stalk cells activating Notch1, which in turn downregulates VEGFR2. High VEGFR2 signaling in the tip cell allows the cell to extend its filopodia and migrate forward, while the VEGF migratory response is dampened in the stalk cell. The tip cell suppresses the same response in stalk cell, thus gaining a competitive advantage to acquire tip-cell motility [88]. Notch further modifies this morphogenetic response by regulating VEGFR1, VEGFR3 and Nrp1, and promotes stabilization of the stalk by recruiting supporting pericytes [88, 89].

1.5.3 Wnt signaling in vascular development

1.5.3.1 Short summary of Wnt pathways

Wnts and their downstream effectors were originally discovered in Drosophila and subsequently shown to be conserved in all metazoans. Wnt signaling controls a broad spectrum of events during development, including cell-fate specification, proliferation, survival, migration and cell adhesion (reviewed in [90]). Not surprisingly, misregulation of the Wnt pathway also leads to a variety of abnormalities and degenerative diseases including cancer, retinal angiogenesis defects and many others (reviewed in [90-92]). Genetic and biochemical data have identified >50 proteins directly involved in transducing Wnt signals (The Wnt Homepage, http://www.stanford.edu/rnusse/wntwindow.html). There are at least three signaling pathways involved in the Wnt signal transduction process: the canonical or $\beta$-catenin
dependent, and two non-canonical branches: the planar cell polarity (PCP) pathway and the Ca^{2+} pathway. All these pathways are activated by the binding of extracellular Wnt to Frizzled transmembrane receptors. However, canonical Wnt signaling eventually causes the activation of $\beta$-catenin complexes, whereas non-canonical Wnt signal transduction uses a multitude of different downstream effectors instead.

In the canonical pathway (Figure 5A,B), in the absence of Wnt, $\beta$-catenin levels are kept low by a protein complex consisting of Axin, adenomatous polyposis coli protein (APC) and glycogen synthase kinase 3B (GSK3B). Here, $\beta$-catenin is phosphorylated and targeted for ubiquitination and proteasomal degradation. Binding of Wnt secreted ligands to cell-surface Frizzled (Frz) receptors, and low-density lipoprotein receptor-related protein (Lrp) coreceptors, activates cytoplasmic Dishevelled (Dvl) which in turn inhibits Axin/GSK3B/APC mediated phosphorylation of $\beta$-catenin, thus preventing its degradation. Consequently, $\beta$-catenin accumulates, enters the nucleus and interacts with members of the T cell-specific transcription factor /lymphoid enhancer factor (TCF/LEF) family of transcription factors to induce transcription of downstream target genes (Figure 5B). Through the activation of specific target genes, the canonical Wnt pathway tightly regulates cell proliferation, differentiation, and survival, and is critical for embryonic patterning [90-92].

In contrast, the PCP pathway (Figure 5C) is $\beta$-catenin independent but also uses Frizzled receptors and Dishevelleds. This pathway involves the core PCP molecules Vangl/2, Celsr1, Prickle, and Diego, which modulate the activity of the pathway by forming a multiprotein complex with Frizzled and Dishevelled. Downstream effectors of the PCP pathways include Rho-like GTPases, and Jun kinase (JNK) which activate downstream
signaling cascades that lead to modification of the actin cytoskeleton and also direct effects on transcription (reviewed in [93, 94]). Best studied in Drosophila eye and the bristles of the wing and thorax, planar cell polarity is distinct from and perpendicular to apical-basal polarity. In vertebrates, the best studied processes requiring proper PCP signaling include skin development and body hair orientation, polarization of the sensory epithelium in the inner ear, and convergent extension (CE) movements during gastrulation.

The third, poorly characterized, Wnt-Ca\(^{2+}\) pathway (Figure 5D), involve Wnt signaling through Frizzled receptors and Dishevelleds, leading to the release of calcium from intracellular storage sites in a process likely mediated through heterotrimeric G-proteins. This pathway activates several downstream targets including phospholipase C (PLC), calcium-calmodulin-dependent kinase 2 (CamK2) and protein kinase C (PKC). The Wnt-Ca\(^{2+}\) pathway is important for cell adhesion and cell movements during gastrulation (reviewed in [95, 96]).
Figure 5: The Wnt pathway

(A,B) Canonical Wnt signaling pathway controls gene expression by stabilizing β-catenin (β-Cat). Frizzled (Frz) proteins are 7 transmembrane proteins that together with the low density lipoprotein receptor-related protein 5 and 6 (Lrp5/6) function as Wnt receptors. In the absence of Wnt signaling (A), β-catenin is phosphorylated by Glycogen synthase kinase 3β (GSK3β), that functions in a multiprotein complex with Axin and adenomatous polyposis coli (Apc), leading to its proteosomal degradation. (B) Binding of Wnts to their receptors results in Dishevelled (Dvl) activation and subsequent suppression of GSK3β activity, thus protecting β-catenin from degradation. Accumulated β-catenin translocates to the nucleus, and interacts with members of the T cell-specific transcription factor (TCF) and lymphoid enhancer factor (LEF) family of transcription regulators to modulate gene expression of downstream target genes.

(C) For planar cell polarity (PCP) signaling,
Wnt signaling is transduced through Frizzled independent of LRP5/6. Dishevelled is at the branch point of canonical and non-canonical pathways. At the biochemical level, the events leading to non-canonical Wnt signal transduction have not been fully characterized. Signaling involves a multiprotein complex that associates at the cell membrane. This complex involves the core protein Frizzled, Dishevelled, Prickle (Pk), Vangl/Strabisum (Vangl), Celsr/Flamingo (Celsr) and Diego. Recruitment of Dishevelled to the complex activates downstream signaling via downstream effectors Rho-like GTPases Rho and Rac, and kinases JNK and ROCK. PCP results in cytoskeletal rearrangements resulting in changes in cell adhesion, migration, and polarity, and gene transcription. (D) For the Wnt/Ca2+ pathway, Wnt signaling via Frizzled mediates activation of heterotrimeric G-proteins, which engage Dishevelled, calcium-calmodulin kinase 2 (CamK2) and protein kinase C (PKC).

Research is still ongoing as to the complete roles of the two non-canonical signaling branches in regulating different biological phenomena. The two forms of intracellular Wnt signaling, canonical and non-canonical, conduct signaling cross-talk. For example, non-canonical Wnt signaling can antagonize Wnt/β-catenin signaling, a phenomenon that may occur through several mechanisms [97, 98].

The mouse genome contains 19 Wnt-encoded ligands, 10 Frizzled receptor genes, 2 Lrp coreceptors and 3 Dishevelleds (Dvl1-3) (http://wwwstanford.edu/~rnusse/wntwindow.html). Wnt molecules have been grouped as canonical (Wnt1, Wnt3, Wnt3a, Wnt7a, Wnt7b, Wnt8) and non-canonical (Wnt5a, Wnt4, Wnt11) pathway activators, however, the ability of the two groups to trigger canonical or non-canonical signaling cascades is not absolute [84]. In addition to Wnts themselves, the events that occur in Wnt-responsive cells depend on the ability of Dvls proteins that function in all three Wnt pathways, to interpret distinct types of stimuli and transmit them to the right pathway. Dvls in all organisms are comprised of three highly conserved domains: an amino-terminal DIX domain, a central PDZ domain, and a carboxy-terminal DEP domain. The three conserved domains function uniquely in each.
Wnt pathway. Canonical Wnt signaling utilized the DIX and PDZ domains of Dvl; the PCP and Ca^{2+} pathways utilize PDZ and DEP domains. In addition to distinct Dvl domains, specificity of the signal is in part controlled by signal-dependent phosphorylation of Dvl serine and threonine sites, and by various Dishevelled associated proteins (DAPs), although these processes are still poorly understood (reviewed in [99]).

Wnt signaling pathways are widely known for their role in a myriad of processes during development. Nonetheless, the essential contribution of the pathways to vascular morphogenesis has been revealed only recently. Addition of Wnt1 or expression of constitutively active β-catenin induced proliferation and survival of cultured endothelial cells (EC) [100]. Mutations in the Wnt receptor Frizzled 4 are associated with human angiogenic vitreoretinopathy, and the knockout of either Frizzled 5 or Wnt2 is lethal in mice due to defects in yolk sac and placental angiogenesis [101-103]. Endothelial-specific β-catenin mutant mice die by E12.5 due to mild patterning defects in the large vessels of the vitalline, umbilical cord and the head. In addition, at E11.5 the vascular plexus in non-neural tissues is overtly normal, but there are major vascular defects in the CNS vasculature, including decreased vascular density, a loss of capillary beds in the forebrain and the spinal cord (but not the hindbrain), and thickened perineural vascular plexus (PNVP) [68]. Similar vascular malformations are observed in animals systemically injected with a Wnt signaling inhibitor - Frizzled 8-Fc, and in Wnt7b, and Wnt7b/7a mutant mice suggesting that the defects in β-catenin mutants are due to impaired Wnt signaling, and not other functions of β-catenin. There is considerable evidence that although ECs display many common features, they also exhibit remarkable
heterogeneity reflected by vessel size-specific, organ-specific and even age-specific differences [66]. ECs in different organs are required to perform different organ-appropriate functions. Brain ECs, for instance interact with astrocytic feet to produce the blood-brain-barrier (BBB) by forming a continuous endothelium with complex tight junctions and highly regulated polarized endocytosis and transcytosis [66]. Studies suggest that Wnt/β-catenin regulates EC expression of the BBB-specific transporter \textit{Glut-1}. Therefore, these data further support the functional diversity of embryonic ECs and indicate that canonical Wnt/β-catenin signaling is required for the formation of CNS blood vessels, but not for the formation of blood vessels in non-neural tissues [68].

In the mouse retina and zebrafish, Dll4/Notch signaling induces expression of another gene called \textit{notch-regulated ankyrin repeat protein} (\textit{Nrarp}). \textit{Nrarp} is expressed in endothelial stalk cells at branch points, and it differentially modulates Notch- and Wnt-signaling activity to balance stalk cell proliferation and maintain vessel stability during vessel formation. Specifically, \textit{in vitro} experiments have shown that \textit{Nrarp} forms a ternary complex with Notch intracellular domain (NICD) [104], and its overexpression in zebrafish and \textit{Xenopus} embryos blocks Notch activity by inhibiting NICD-mediated transcription by means of a completely unknown mechanism [104, 105]. In addition to counteracting Notch signaling, \textit{Nrarp} positively regulates Wnt signaling by preventing ubiquitination of Lef1, thus stabilizing this transcriptional factor, a pivotal component in the Wnt signaling cascade [86, 105]. By inhibiting Notch, \textit{Nrarp} induces stalk cell proliferation, and by inducing Wnt signaling, \textit{Nrarp} promotes proliferation and vessel stability [86]. Thus, \textit{Nrarp} could be an important element integrating Notch and Wnt
signaling in endothelial stalk cells to control vascular sprouting and regression [86] (Figure 6B).

Figure 6: Nrarp coordinates Notch and Wnt signaling in endothelial cells. (A) Endothelial fate specification is achieved by molecular mechanisms involving VEGF and Notch signaling pathways. Hypoxic cells upregulate VEGF, leading to formation of a gradient. The cell exposed to the highest level of VEGF becomes the tip cell (green). VEGF-induced VEGFR2 activation leads to upregulation of Notch ligand Dll4 by the tip cell. Dll4 activates Notch in the neighbouring cell, which in turn downregulates VEGFR2 (as well as VEGFR1/R3 and Nrps), and therefore dampens VEGF signaling. This latter cell will adapt a stalk phenotype (yellow) because the VEGF-induced migratory response is dampened. Tip cells have a high expression of VEGFR2, Nrps and Dll4, whereas levels of these molecules are decreased in the stalk cells. (B) Nrarp coordinates Notch and Wnt signaling in endothelial stalk cells. Nrarp is induced in stalk cells by Notch signaling. Nrarp in turn counteracts Notch signaling by destabilizing Notch intracellular domain (NICD) and induces Wnt signaling by stabilizing Lef1 transcription factor. Together this promotes vessel proliferation and stabilization. Adapted from Carmeliet et al, 2009 [88].
In response to hypoxia, cells produce VEGF, leading to formation of a gradient (dark green circle). Endothelial cell exposed to the highest VEGF concentration is selected to become the tip cell (light green). The tip cell leads the sprout at the forefront and invades the surrounding tissue by extending numerous filopodia. The sprout elongates via proliferation of endothelial stalk cells (blue). The new branch connects with another branch via tip-cell fusion. Maturation and stabilization of the new vascular plexus relies on the recruitment of peri-vascular cells (pink). In the established vasculature, ECs adapt a quiescent endothelial phenotype (grey). 

Adapted from Carmeliet et al, 2009 [88].

1.6 Common mechanisms of nerve and blood vessels development

Today emerging evidence suggests that blood vessels and nerves share common guidance signals and mechanisms, and illustrates that the navigational mechanisms of both systems are intertwined (reviewed in [62, 63, 106, 107]). Both form a complex and precisely branched network, reaching the most distant cells in the organism. Both consist of two cell types, endothelial/peri-vascular cells, and neurons/glia, respectively. The formation of both systems is highly dynamic and subject to intense remodeling throughout development. Neurons send out a cable-like axon that migrates over often considerable
distances to reach its targets. The task of leading the axon is performed by the growth cone: a motile, sensory structure at the axon tip. Using filopodial extensions and retractions, the growth cone continually explores the spatial environment to accurately select a correct trajectory among the maze of possible routes. Unlike axons, vasculature arises mainly by local movements of endothelial cells (EC). In angiogenesis, vessels sprout off side branches through delamination and migration of endothelial cells, rather than formation of a cellular extension as in the case of axons. Nevertheless, specialized endothelial tip cells (ETC), discussed above, form at the tips of growing vessels resembling axonal growth cones, and share similarities with axonal growth cones [73]. Like growth cones, they extend and retract numerous filopodia to explore their environment, responding to gradients of growth factors, such as VEGF. In peripheral tissues, axons and vessels often take advantage of one another to follow the same path. In some cases, vessels produce signals, such as artemin and neurotrophin 3, that attract axons to track alongside the pioneer vessel [108, 109]. Conversely, nerves and glial cells may also produce signals such as VEGF to guide blood vessels [110].

The idea that vascular and neuronal branching patterns might be established coordinately is further supported by the observation that many of the same guidance cues and receptors control axon guidance and vascular morphogenesis, thus possibly explaining how axons and vessels align, by responding to common cues [62]. Guidance cues can be attractive or repellant, and can operate either at close range or over a distance. Axons and vessels are often guided through tissue corridors by attractive cues made by cells along the corridors, and by repulsive signals that prevent them from entering surrounding tissues [62, 111].
Here I will discuss the molecules that have been implicated in cranial axon guidance and vascular patterning, with particular focus on those involved in facial nerve development.

1.6.1 Pathways with roles in facial nerve axon guidance and angiogenesis

Semaphorins, Neuropilins and Plexins

Semaphorins are a large family of signaling proteins, both secreted and membrane-bound. They primarily act as short-range inhibitory cues that deflect axons away from inappropriate regions, or guide them through repulsive corridors, but evidence suggests that Semaphorins are capable of long range diffusion, and some may also function as an attractive cues for certain axons. Furthermore, Semaphorins have been implicated in a wide range of activities, from cell migration and morphogenesis to angiogenesis and tumour progression [112]. Semaphorins signal through multimeric receptor complexes: membrane-bound Semaphorins (class 4-7) bind Plexins, whereas secreted class 3 Semaphorins (Sema3A-3G) bind Neuropilins, which function as non-signaling co-receptors with Plexins. An exception to this is the secreted Sema3E which binds PlexinD1 (PlxnD1) directly [113]. Semaphorins regulate the formation of neuronal networks in many regions of the CNS, including the hippocampus, cerebral cortex, olfactory system, cranial and spinal nerves, as well as regulate the migration of neural crest and neuronal progenitor cells [62, 111, 114, 115]. Genetic studies have implicated Semaphorins in a wide variety of neural wiring processes. Insufficient repulsion by Semaphorins results in axon projection defects such as defasciculation, overshooting, aberrant trajectories, and misrouting [50, 52, 115, 116].
Sema3A has been suggested to be one of the repellent cues to prevent BM/VM/SM axons and cell bodies from crossing the midline. This is primarily based on the observation that Sema3A repels these axons in collagen gel co-cultures [40]. It is currently unclear whether they operate in a similar manner in vivo, as Sema3A is not expressed by the hindbrain floor plate, and no midline defects have been reported in Sema3A mutant mice. Nevertheless, the analysis of mutant mice deficient for Semaphorins or their receptors illustrates that Sema3/Nrp/Plexin signaling is required for the development of the facial, trigeminal, glossopharyngeal, vagus, trochlear and oculomotor nerves [50, 51, 117]. Mice lacking Sema3A or its receptor Nrp1 show defasciculation of the facial, trigeminal, glossopharyngeal and vagus cranial nerves [50, 51], while mice lacking Sema3F or its receptor Nrp2 show partial defasciculation of the facial and ophthalmic trigeminal nerves, severe defasciculation of the oculomotor nerve, and misprojected trochlear nerve [115, 117, 118]. Interestingly, in guiding the facial nerve, it has been shown that different Sema/Nrp/Plexin combinations are required for the FBM and FVM motor axon guidance (discussed previously), suggesting that Sema3F/Nrp2/PlxnA3 and Sema3A/Nrp1/PlxnA4 pathways co-operate in a non-redundant fashion during facial nerve development [52].

Numerous data suggest that Semaphorins and their receptors also regulate cardiovascular development. Endothelial cells (EC) express both Neuropilins and various Plexin receptors [36, 119]. Sema3A knockout mice show cardiac defects characterized by a right ventricular hypertrophy and a dilated right atrium [120]. Sema3C knockout mice exhibit improper septation of the cardiac outflow tract and interruption of the aortic arch [120, 121]. Nrp1 knockout mice show dilation of the right atrium and hypertrophy of the right ventricle. PlexinD1 is expressed in ECs [122], and in mice and zebrafish, mutations of
*PlxnD1* (zebrafish *out-of-bound* (*obd*) mutation) results in the failure of intersomitic vessels (ISVs) to select the appropriate branching site along the dorsal aorta. This leads to vessels extending ectopically throughout somites, and the loss of the normal segmented pattern [119, 123]. The mouse mutant phenotype of *PlxnD1* reflects loss of repulsion by *Sema3E*, as *Sema3E* mouse mutant embryos exhibit a highly similar vascular phenotype [123]. *Sema3E* binds *PlxnD1* directly rather than via a Neuropilin [124], and these observations argue in favour of a direct role of Semaphorins in the development of the vascular system, that is independent of Neuropilins.

In contrast to class 3 Semaphorins, little is known about the function of the mammalian membrane-bound Semaphorins. However, the analysis of membrane-bound *Sema5A* has uncovered roles in axon guidance [125, 126] as well as vascular patterning [127], suggesting that the involvement in cardiovascular and nervous system development is not restricted to the Sema3 proteins. *Sema5A*, which has been implicated in Cri du Chat syndrome (discussed below), is widely expressed in several mesodermal tissues and in a subset of neurons [125-127]. Previous work has shown that *Sema5A* is bifunctional axon guidance cue for neurons with both attractive and repulsive abilities [125, 126]. The bifunctional response is mediated via its two domains: the thrombospondin repeat domain (TSR) mediates an attractive response, and the sema domain mediates a repulsive response [125, 126]. Using rat organotypic explants it was shown that *Sema5A* guides diencephalic neurons (fasciculus retroflexus) to extend between prosomere (diencephalon rostral-caudal compartments) 1 and 2, without crossing into either prosomere [126]. In the zebrafish, *Sema5A*, which is expressed in myotomes at the time of motoneuron extension, guides Caudal Primary (CaP) motor axons extension in the ventral myotome.
[125]. In Sema5A homozygous mutant mice, which die between E11.5 and E12.5, the complexity of cranial vessels is decreased [127], suggesting that Sema5A also plays a role in the remodeling of the vascular system. PlexinB3 receptor was identified as a functional Sema5A receptor in COS cells [128], but PlexinB3 was not detected in mice at the time at which Sema5A functions to pattern the vasculature [127], suggesting that other Sema5A functional receptors might be present, possibly PlexinA3 [125].

1.6.2 Pathways with roles in FBM migration and angiogenesis

1.6.2.1 Neuropilins and VEGF

Both Nrp1 and Nrp2 are expressed in cranial motor neurons including the facial nerve, and Nrp2 is strongly expressed in the muscle precursors of all branchial arches [114]. Neuropilins are essential for the patterning of the facial nerve in the mouse, where they bind Sema3 receptors to guide the facial branchiomotor neuronal axons in the second branchial arch (discussed above). In addition Nrp1 binds VEGF-A isoform VEGF164 to control the rostra-caudal migration of FBM somata within the hindbrain [36]. Consequently, in Nrp1 and VEGF164 mouse mutants, the caudal migration of FBM somata is impaired [36]. Apart from their role in axon guidance, Neuropilins have also been implicated in vascular patterning, although this probably reflects their role in modulating VEGF164 rather than Semaphorin activity. Nrp2 is expressed in venous endothelial cells and lymph vessels, and reduction in lymphatic vessels is observed in Nrp2 mutant mice [129, 130]. Nrp1 is expressed widely in developing vasculature, preferentially in arterial endothelial cells [129], and Nrp1 mutant mice die at E12.5-E13.5 and in addition to neuronal and heart defects described above, also have impaired neural vascularization, improper
development of aortic arches and dorsal aorta, and a partial disorganization in the yolk sac vasculature [131]. Although no discernable abnormal endothelial phenotype has been reported for Nrp-2 mutant mice, double Nrp mutant mice die at E8.5 and exhibit severe vascular abnormalities in the yolk sac and embryo proper, and impaired embryo development [116]. These results suggest that both Nrp1 and Nrp2 are needed for normal vasculogenesis and angiogenesis in the developing yolk sac and embryo. The two genes must provide some as yet undetermined nonoverlapping functions in blood vessel formation so that both are needed.

Numerous studies established that VEGF is a key vascular regulator. Although it remains to be established whether VEGF is a relevant axon guidance cue, growing evidence suggests that VEGF might play a role within the nervous system ([62, 132] and references therein). Both VEGF-A and VEGFR2 are expressed in the neurons and their precursors [133], and in vitro assay using undifferentiated rat neural progenitors demonstrated that VEGF-A signaling promotes neuronal survival, proliferation, and directional migration [134]. In addition, in vivo studies have demonstrated that VEGF164 is required for the caudal migration of FBM somata within the hindbrain, likely by attracting Nrp1-expressing FBM neurons [36].

1.6.2.2 Wnt signaling pathway

The first direct demonstration that Wnt proteins have a role in axon guidance was obtained in studies of commissural neurons in the Drosophila CNS. In organisms with a bilateral nervous system, it is essential that there is communication between the two halves. This link is provided by axon tracts known as commissures which cross the midline at defined points. During Drosophila development each body segment comprises
an anterior and a posterior commissural tract that crosses the midline and joins one of the two lateral longitudinal tracts that extend the length of the embryo. Axons choose between the two major subdivisions of the crossing pathways - the anterior or posterior. \( DWnt5 \) is expressed in the midline, and its loss causes misrouting of anterior commissural axon tracts. After commissural axons have reached and crossed the midline, they make a sharp anterior turn toward the brain (anteroposterior (AP) guidance). An independent study seeking guidance cues along the anteroposterior axis of the vertebrate CNS identified Wnt proteins as attractive cues for AP guidance of spinal cord commissural axons after midline crossing. The Wnt receptor \( Frizzled 3 \), was implicated in mediating the attractive guidance function of Wnt, as \( Frizzled 3 \) mutants display defects in AP guidance [135, 136]. In addition, the inactivation of \( Frizzled 3 \) in mice also results in other axonal abnormalities within the forebrain, suggesting that in addition to guiding commissural axons at the spinal cord midline, Wnt-Frizzled signaling might also play a much broader role in axonal development. The axon guidance signaling pathway downstream of \( Frizzled 3 \) has not been investigated but, interestingly, the pathfinding of commissural axons is normal in \( Lrp6 \) mutant embryos. This, combined with the observation that no direct connections to actin cytoskeleton have been found in the canonical pathway, suggests that the canonical Wnt signaling pathways is likely not required for Wnt-mediated commissural axon guidance [135, 136]. It is more attractive to consider a possible role for the PCP pathway because cellular polarity, and directed cell movement created by the function of this pathway, are analogous to the directed migration of axon growth cones. In addition, several recent experiments provided \textit{in vitro} and \textit{in vivo} evidence implicating PCP signaling in FBM motor neuron migration in the
hindbrain [34, 35]. As mentioned earlier, in mouse and zebrafish, FBM neurons are born in r4 and subsequently migrate caudally through r5 and r6, where they form the facial motor nucleus [22]. In mouse and zebrafish, many PCP components are expressed either in FBM cells (including Wnt11, Frizzled 7, Prickle1, Vangl1/2, and Dvl3) or throughout the hindbrain (Wnt7a and Wnt5a), at the time of FBM migration [31, 34]. In addition, inhibition of JNK, ROCK kinases, and ablation of Frizzled 3, Celsr2, Scribble, and Vangl2 in mice or zebrafish severely perturbs rostracaudal migration of FBM neurons, providing direct evidence for the role of the PCP pathway in the migration of these neurons [34, 35, 137]. The effect of the PCP signaling cascade on FBM migration is likely mediated primarily through Wnt5a, although other as yet unidentified Wnts might also be involved [34].

1.6.3 Other axon guidance pathways with roles in cranial nerve development and angiogenesis

1.6.3.1 Eph-Ephrins

Another important family of guidance molecules are the ephrins. The Eph/ephrin system functions in cell-to-cell rather than long range communications, because Eph receptors and all known ephrin ligands are attached to the plasma membrane. In mammals, 13 Eph receptors are categorized into A (EphA1-8) and B (EphB1-4 and Eph6) subfamilies. The eight ephrin ligands comprise ephrinA1-5 which are tethered to the membrane via a glycosyl-phosphatidyl-inositol (GPI) anchor, and ephrinB1-3, which contain transmembrane and cytoplasmic regions. EphrinA ligands bind EphA receptors, and ephrinB ligands bind EphB receptors. Eph/ephrin interaction at the cell surface triggers bidirectional signals both in receptor- and ligand-expressing cells, referred to as the
forward and reverse signaling respectively. The ephrin/Eph signaling pathway is believed to provide repulsive guidance cues for growing axons, as well as for migrating neural crest cells during embryonic development (reviewed in [41]). Although their roles in cranial nerve development is not well characterized, Ephrins may play several roles in the development of hindbrain axons (reviewed in [1, 14, 42]), which have been discussed earlier.

Expression analysis and loss-of-function studies in mice indicate that ephrin-Eph signaling is involved in vascular patterning. First, repulsive ephrin-Eph signals provide short-range guidance cues for vessels to navigate through tissue boundaries. For instance, ephrinB2 repels EphB3/EphB4 expressing ISVs from entering the somites [138, 139]. Targeted inactivation of the mouse ephrinB2 or EphB4 results in early lethality due to vascular defects in both the yolk sac and embryo proper [138, 140]. The receptor EphB4 and ligand ephrinB2 are expressed in developing veins and arteries, respectively, and are critical for their specification and maintenance [138, 139]. At least two more Eph receptors - EphB2, EphB3 and one ligand- ephrinB1 were found to be expressed in or adjacent to vascular endothelial cells, and were shown to be required for the remodeling of the embryonic vasculature. Furthermore using an in vitro sprouting assay that recapitulates some aspects of sprouting angiogenesis in vivo [65], it was shown that ephrinB1 and ephrinB2 have stimulatory influences on capillary sprouting with comparable potency to known angiogenic factors such as VEGF-A [138], suggesting that their in vivo role might be to promote capillary sprouting. Another ephrin ligand, ephrin-A1 is expressed at sites of vascular development in the mouse, including all the major
vessels of the head, in the ISVs and the branchial arch arteries [141], although its mutant phenotype has not yet been examined.

1.6.3.2 Netrins and Unc5b

Netrins were identified as midline-derived chemoattractants that guide commissural axons to the midline. As for many guidance cues, responsiveness to netrins is dynamically regulated during axon migration; for example, to avoid commissural axon stalling at the midline, where netrin levels are maximal, responses of the axons to netrins thus must be silenced. Netrins act through receptors of two distinct families: attraction is mediated by deleted in colorectal carcinoma (DCC) family of receptors, and repulsion is mediated by the Unc-5 family of receptors either alone or in combination with DCC receptors (reviewed in [62, 111]). Four mammalian homologues of Unc5 (Unc5a-d) have been identified, and their role as receptors for repulsive signals has been shown in many different systems [111, 142]. Multiple data from in vitro experiments and expression analysis in the zebrafish, rat and chick suggest that netrin-mediated signaling may play a role in the repulsion of cranial BM and VM neurons axons from the midline [1, 14]. Netrin 1 repels BM/VM axons in collagen gel co-cultures [38]. Netrin1 is expressed in the hindbrain floor plate at the time of axon extension, and Unc5a and Unc5c are expressed in cranial motor neurons [1, 30]. In netrin 1 [143] and Unc5c [144] mouse mutants, the trochlear (cranial nerve IV) motor neuron cell bodies enter the floor plate, suggesting a loss of repulsion from the midline [38]. Unc5c is expressed in FBM after E12.0 during their migration in r5 and r6 and after E13.5, in FBM nucleus in r6 [24, 30], although its functional significance in FBM migration has not been established.
In additional to their role in axon guidance, a recent study shows that netrin1 and Unc5b also regulate blood vessel guidance [145]. In the mouse embryo, Unc5b is expressed throughout the vasculature, and its expression is highest in vascular tip cells. Unc5b mutant mice die around E12.5 likely due to vascular abnormalities leading to heart failure [145]. In Unc5b mutant mouse embryos, and in netrin1a and Unc5b morphant zebrafish, vessels in the head, in the nervous system and ISVs appear thinner, have increased capillary branching and tip cell filopodia extensions. These experiments suggest a model where in zebrafish Netrin-1a, which is expressed in the floor plate and the somites, acts via its receptor Unc5b to negatively regulate capillary branching in the developing vascular system. In mice, the identity for the ligand Uncb5 remains to be determined, as vascular defects have not yet been reported in netrin-1 mutants.

1.6.3.2 Slits and Robos

Slits are large secreted proteins that signal through the Roundabout (Robo) family of receptors. Slits, like netrins are multifunctional, acting as repellents for some axons, and as stimulators of branching and elongation for other axons. In vertebrates, the best understood functions of Slit proteins are in the midline guidance of commissural axons in the spinal cord and in guiding ipsilateral and contralateral axons through the optic chiasm by providing a repulsive corridor [62, 111]. Slit/Robo signaling has also been suggested to repel BM/VM cranial motor axons from the midline. Cranial motor neurons express the receptors Robo1 and Robo2, while Slits are highly expressed in the hindbrain floor plate at the time of axon extension [39]. Attenuation of Slit-Robo signaling either using dominant-negative approaches in chicks or in mutant mice leads to BM and VM axon navigation defects. Specifically, the axons BM and VM neurons (but not SM) project
aberrantly into or across the ventral midline, and fail to project dorsally or to reach their exit points, suggesting an \textit{in vivo} role for the Slit proteins in motoneuron repulsion [39]. Recently, a vascular-specific \textit{Robo} homologue, \textit{Robo4}, has been identified [87, 146]. \textit{Robo4} is expressed primarily in endothelial cells, and its expression is enriched in endothelial stalk cells [147]. \textit{In vitro}, \textit{Robo4} bound Slit2 in one study [146], although another study failed to detect such binding [87], and thus it is unclear whether \textit{Robo4} acts via Slits. \textit{In vitro}, Slit2 was able to inhibit the migration, and tube formation, but not the proliferation of endothelial cells [147]. \textit{Robo4} knockdown study in zebrafish showed that some \textit{Robo4} expressing ISVs failed to sprout from the aorta or were arrested midway through their dorsal migration path [148]. However, in a \textit{Robo4}^{AP/AP} null mouse mutant (generated by knock-in of alkaline phosphotase), no similar angiogenic deficits were detected [147]. Further studies should help clarify the extent to which Slit-Robo signaling is involved in angiogenesis.

1.7 Summary
At this point in time, while some signaling pathways that play major roles in facial nerve development and angiogenesis have been identified, we know very little about how these signals are transduced, coordinated and integrated. Thus, identifying novel intracellular molecules with key roles in these developmental processes may help define these molecular signals in greater detail and may offer potential therapeutic strategies. For instance, the evidence that blocking guidance molecules such as Slits and Robos impairs tumour angiogenesis in an animal model provides a first glimpse of this therapeutic potential [149] and other similar discoveries are likely to follow. In addition, the cross-
talk between endothelial and neuronal cells appears to regulate multiple aspects of the development of both systems and promises many exciting discoveries in the future.
2.0 MATERIALS AND METHODS

2.1 Mouse Manipulations

Mice used in these studies were housed in standard vented cages in conformity with the Canadian Council of Animal Care (CCAC) recommendations.

2.2 Embryos harvesting

Upon crossing with male mice, females were examined in the morning for the presence of a vaginal plug. The midday following the presence of a vaginal plug was considered embryonic (E) stage E0.5. At the desired stage, the pregnant females were killed by CO₂ asphyxiation and embryos were dissected in cold phosphate-buffered saline (PBS), pH 7.4, and fixed with 4% paraformaldehyde/PBS (PFA) at 4°C overnight (O/N).

2.3 Synthesis of first strand cDNA.

Whole embryo RNA was prepared using Trizol™ reagent according to manufacturer’s instructions (Invitrogen). First strand cDNA was synthesized from RNA using random primers and the Superscript™ First-Strand Synthesis System (Invitrogen) according to manufacturer’s instructions (1.5μg of RNA used in 20μl RT reaction; 2μl of cDNA was used for subsequent PCR reactions).

2.4 Mouse genotyping

Yolk sacs (or ear punches) were collected for genotyping and digested in 200μl of lysis buffer (40mg/ml proteinase K (Invitrogen) in 10mM Tris pH 8.3, 50mM KCl, 1.5mM MgCl₂, 0.45% Nonidet P40, 0.45% Tween-20) at 63°C O/N. Genomic DNA was purified by standard chloroform extraction/ethanol precipitation method, and dissolved in 50μl of dH₂O.
**2.4.1 Standard PCR assay**

The following PCR conditions were used in all cases unless otherwise stated.

For 50µl reaction: 5µl of 10x Invitrogen MgCl₂(-) PCR Buffer, 2µl of 50mM MgCl₂, 0.4µl of 25mM dNTPs, 2µl of 10pmol each primer, ~50ng of genomic DNA or 2µl of total cDNA (derived from E10.5-E11.5 embryos), 0.25µl of *Taq* DNA polymerase (Invitrogen), dH₂O. PCR reaction was incubated for 3 min at 95°C, followed by 30-35 cycles of 30 sec denaturation (95°C), 30 sec annealing (55°C-60°C), 1 min extension (72°C), and an additional 7 min extension at 72°C at the end. For SNP genotyping using Restriction Fragment Length Polymorphism (RFLP), 17µl of PCR product was mixed with 2µl of appropriate buffer and 1µl of restriction enzyme, and digested for 1 h at 37°C.

**2.5 Genetic mapping of the gumby mutation**

**2.5.1 Initial mapping done by other members of our laboratory**

The genetic map position of the mutations identified in this screen was determined by analysis of segregation of chromosome-specific markers on C3H/HeJ inter-subspecific intercross panels by standard procedures. Using this method, the mutation was mapped to chromosome 15 between MIT markers D15MIT130 at 20.7Mb and D15MIT138 at 39.8Mb. Linkage was confirmed by additional analyses of an additional 20 intercross progeny. Further mapping has moved the distal boundary to D15Mit111 32.2Mb (11.5Mb region).

**2.5.2 Fine mapping of the *gumby* mutation**

Polymorphisms at D15Mit130 and D15Mit111 were used thereafter to identify heterozygous carriers and homozygous mutants. For the initial screen all SSLP MIT markers chosen for mapping give PCR products 100-250 bp long, and the C57 and C3H
alleles can be distinguished reliably on ethidium-stained agarose gels because they differ in size by at least 10 bp. Any recombinant embryos were scored with additional markers between D15Mit130 and D15Mit111. The following types of markers were used: MIT Simple Sequence Length Polymorphisms (SSLPs) that produce C57 and C3H alleles that differ in size by at least 10 bp; MIT SSLP markers that produce C57 and C3H alleles that differ in size by less than 10 bp; Restriction Fragment Length Polymorphism (RFLP) markers; and Single Nucleotide Polymorphism (SNP) markers. The following polymorphic markers were used: D15Mit252 (22.5Mb), D15Mit280 (24.0Mb), rs3707949 (25.3Mb), rs13482490 (26.7Mb), D15Mit18 (28.4Mb), D15Mit201 (29.4Mb). SSLP MIT markers and their corresponding primer sequences were obtained from www-genome.wi.mit.edu/cgi-bin/mouse/sts-info. Each SSLP marker was amplified by PCR, and products were resolved either by electrophoresis on a 4% Nusieve agarose gel (Amresco) (for D15Mit130 and D15Mit111, D15Mit138), or on a 6% polyacrylamide gel (for D15Mit252, D15Mit280, D15Mit18, D15Mit201) depending on the size difference between C57 and C3H specific alleles. For the latter method, products were detected using Southern Blot analysis with P32 end-labeled primer probes using standard procedures (Figure 12A). SNP marker rs13482490 was identified from the NCBI SNP database (www.ncbi.nlm.nih.gov/projects/SNP). For this SNP marker, primers were designed to span 400-600bp around the polymorphic nucleotide. RFLP marker rs13482490 was PCR amplified and digested with PvuII (Figure 12A). All primer sequences are available in the Appendix A. In total additional 154 E12.5 embryos from gumby heterozygous intercrosses were genotyped with all or some of these polymorphic
markers, and the final critical interval was reduced to 1.7Mb on chromosome 15 between markers rs13482490 (26.7Mb) and D15Mit18 (28.4Mb).

2.5.3 Sequencing the genes within gumby critical interval

Based on the publicly available mouse genomic sequence (www.ensembl.org/Mus_musculus/), there are 10 transcripts within the 1.7Mb gumby critical interval (Figure 10). Q8BQ70, Tiaf2, Q8C9X7, Fam105b, BC052328, Q8CDT1, ENSMUSG000000726630 genes in the critical interval were sequenced as follows: overlapping primers were designed to amplify the entire coding region of each gene, either by sequencing the exons, or by sequencing cDNA, depending on the exon-intron genomic organization of each gene. Genomic DNA was obtained from embryonic yolk sacs as described in Section 2.4. RNA for generation of cDNA was obtained from whole E11.5 gumby embryos, using Trizol reagent (Invitrogen) according to manufacturer’s instructions (1.5μg of RNA used in 20μl Reverse Transcriptase reaction; 2μl used in PCR reaction). For genomic sequencing, primers were derived from intron sequences and were designed to amplify exons and intron-exon junctions. For cDNA sequencing, overlapping primers were designed within exons to amplify the whole coding region, as well as the 5’ and 3’ untranslated regions (UTRs). 100-800bp PCR products were analyzed by direct sequencing. The DNA sequence was compared to the Ensembl sequence database. Primers used for sequencing genes within the critical interval are provided in the Appendix A.

2.5.4 Sequencing Fam105b
Seven exons of *Fam105b* gene were amplified by genomic PCR. The primer sequences are available in the Appendix A. PCR products were analyzed by direct sequencing and a T>A nucleotide substitution was found at nucleotide position 285 (mutation further referred to as T285A). To confirm that this was not a common polymorphism in normal mice I sequenced 10 additional *gumby* and unaffected siblings, as well as pure C57Bl6/J and C3H/HeJ reference strains. The primers used to amplify the third exon where the mutation resides are F:5’-CCTAGGAAGGCAGTGTCTC-3’, R:5’-CCTTATCTTTGCAGTCTGTTTCC -3. Forward primer was used in the sequencing reaction.

**2.5.5 Genotyping the T285A mutation using ARMS-PCR analysis**

To identify the mutation without the need for direct sequencing, I designed a PCR assay that can distinguish single nucleotide difference based on the method called tetra-primer ARMS-PCR described in Ye *et al.*, 2001 [150]. Briefly, this method employs two primer pairs (outer and inner) to amplify, respectively, the two different alleles of a SNP in a single PCR reaction (Figure 12B). PCR conditions used for this assay are as follows: For 50μl reaction: 5μl of 10 x Invitrogen MgCl₂(-) PCR Buffer, 3μl of 50mM MgCl₂, 0.5μl of 25mM dNTPs, 1.5μl of 10pmol each of the outer primers, 5μl of 10pmol A allele primer, 4μl of 10pmol T allele primer, ~100ng of genomic DNA, 0.25μl of *Taq* DNA polymerase (Invitrogen), dH₂O. PCR reaction was incubated for 3 min at 95°C, followed by 35 cycles of 1 min denaturation (95°C), 1 min annealing (55°C) and 1 min extension (72°C), and an additional 7 min extension at 72°C at the end. The product is resolved on 3% agarose gel. Primers were designed using the computer program developed by the authors accessible in [http://cedar.genetics.soton.ac.uk/public_html/primer1.html](http://cedar.genetics.soton.ac.uk/public_html/primer1.html).
The primer sequences are:

**Forward outer** 5’- ATTAAGAGAGCCTGTTTATAGAGG-3’;

**Reverse outer** 5’- TTAAGACCAATATTTACACCTGTC-3’;

**T allele** 5’- GCTTTCTGAATTTCTCTGCA-3’; and

**A allele** 5’- GATATCATGGACTACTGCAAAAAAGTAA-3’.

### 2.6 Cloning of Fam105b

#### 2.6.1 Synthesis of first strand cDNA.

Whole embryo RNA was prepared from +/+ and gumby/gumby E10.5 embryos using Trizol™ reagent (Invitrogen). First strand cDNA was prepared as described in Section 2.3.

#### 2.6.2 Cloning of Fam105b-pBluescript SKII

*At the time when Fam105b was cloned, the starting ATG based on Ensembl was reported as ORF bp 162. Therefore, primers for amplification were designed to encompass this starting ATG, and nothing upstream. Because of this all Fam105b clones used in this study correspond to base pairs 168-1159 (aa 56-352). Currently the starting ATG based on Ensembl is reported to be ORF bp 1.*

Wild-type and T285A forms of Fam105b cDNA were cloned in two steps: First, base pairs 162-469 were PCR amplified using the primer pair: F:5’-CCGTCGAC(Sall)CGATGACCGTGCAGATGAAATAG-3’ and R:5’-ACTGCTTGATCCAGGTATAC-3’ (bp 519), the fragment was digested with Sall and SacI (internal site at bp 469) and subcloned into Sall/SacI digested modified pBlueskript SKII(+) (Stratagene) vector to produce the clone Fam105b-pBluescript SKII (bp169-469). In the second step, base pairs 469-1159(stop) were PCR amplified using the primer pair F:5’-GCTGCAGGACGTTGGAAGCTC(SacI)A-3’ and R:5’-
GCGAATCC(EcoRI)CTCTGGCAGTCACATGTGTGTC-3’, digested with EcoRI and SacI and subcloned into EcoRI/SacI digested Fam105b-pBluescript SKII (bp169-469), producing the final plasmid Fam105b-pBluescript SKII. The integrity of each clone was checked by sequencing. Fam105b-pBluescript SKII plasmid was used as a PCR template to generate the following plasmids used in this study:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector used for cloning</th>
<th>Primers used for cloning</th>
</tr>
</thead>
</table>
| Fam105b - GFP (N-terminal) | pDONR201—pDEST53 (Invitrogen) | F:GGGGACAAGTTTGTACAAAAAAGCAGGC(AttB)TACCGTGCTGCAGATGAAATAG  
R:GGGGACCACTTTGTACAAGAAAGCTGGG(AttB)TTCTTGGCAGTCACATGTGTG |
| GFP – Fam105b (C-terminal) | pDONR201—pDEST47 (Invitrogen) | F:GGGGACAAGTTTGTACAAAAAAGCAGGC(AttB)TACCCCACAGCCAGGTCAA  
R:GGGGACCACTTTGTACAAGAAAGCTGGG(AttB)TCCACACTGATCTCCTCACACA |
| MBP- Fam105b        | pMAL-C2 vector (NEB)     | F:GGAGGAATTC(EcoRI)TACCGTGCTGCAGATGA  
R:TGTCTAGAG(XbaI)TCACACACTGATCTCCTCAC |
| GST- Fam105b        | pGEX-HTa (Pharmacia)     | F:GAGGCCATGG(NotI)CCGTGCTGCAGATGA  
R:CGACTAGT(SpeI)TCACACACTGATCTCCTCAC |
| FLAG- Fam105b       | 3xFLAGpcDNA3.1 (Dr. A.C. Gingras) | F:GCGTTTAAAC(Pmel)TACCGTGCTGCAGATGAAATAG  
R:TGTCTAGAG(XbaI)TTCTTGGCAGTCACATGTGTG |

2.7 Histological Analysis

H&E staining was done by the staff at the Toronto Centre for Phenogenomics, CMHD Pathology Department. Embryos were fixed in 10% formalin and processed for paraffin embedding. 5μm microtome sections were stained with hematoxylin and eosin according to standard procedures.

2.8 Northern Blot analysis

Tissues for RNA extraction were prepared from 6 month old mouse tissues, stage E9.0, E10.5, E14.5 embryos, the yolk sac, and the placenta. Total RNA was purified using Trizol™ reagent according to manufacturer's instructions (Invitrogen). 20μg per lane
total RNA were loaded on a 1% agarose/2.2M formaldehyde gel, separated by electrophoresis and transferred to Hybond N membrane (Amersham Pharmacia Biotech) in 10 x SSC by capillary elution and fixed onto the membrane by UV crosslinking. Hybridization was performed in Church buffer (7% SDS, 10mM EDTA, 1% BSA, 0.25M sodium phosphate buffer, pH 7.2) O/N. Fam105b cDNA fragment (bp 162-1159) was used as a probe. The membrane was washed once in 1xSSC, 0.1% SDS for 10 min at RT, and twice with 0.1xSSC, 0.1% SDS for 15 min at 63ºC and autoradiographed using Denville HyBlot CL film after exposure at -80ºC for 3 days. The membrane was stripped, and hybridized with GAPDH probe for loading control, which was generated by PCR using F:5’-CCAATATGATTCCACCCATG-3’ and R:5’-AGGTCCACCACTGACACGTT-3’ primer pair.

2.9 Apoptosis and Proliferation Assays

Apoptosis assay was performed using ApopTag Plus Fluorescein In Situ Apopotosis Detection Kit (S7111, Millipore), according to manufacturer’s instructions. Phospho-Histone H3 immunofluorescence was performed as described in Section 2.14.1 using rabbit anti-phospho-Histone H3 (Ser10) Mitosis Marker (1:200, 06-579, Upstate cell signaling solutions). Apoptotic and proliferation indexes were calculated by dividing the number of stained nuclei by the number of DAPI positive nuclei.

2.10 Whole-mount in situ hybridization (WISH)

2.10.1 Fam105b in situ probe synthesis

Fam105b probes were synthesized using the riboprobe DIG-labeling kit (Roche) according to the manufacturer’s instructions. Fam105b cDNA region 459-1059bp, corresponding to aa153-352 was PCR amplified using the primers: F:5’-
GCTGCAGGACTTGGAGCTC(SacI)A-3’ and R:5’-GAGAATTC(EcoRI)CTCTTGCCAGTCACATGTGTGTC-3’, digested with SacI/EcoRI and cloned into SacI/EcoRI digested modified pBlueskript SKII vector (Stratagene). This vector was combined with XhoI and T7 or ClaI and T3 to generate Fam105b antisense and sense probes respectively.

Remaining in situ probes used in this study are described elsewhere and include: Hoxb1 [151], Phoxb2 [152], Sox10 [153], Pitx2 [154], PlexinA2 [155].

2.10.2 Whole-mount in situ hybridization protocol

All glassware used for in situ hybridization was baked at 220ºC for 6 h. All solutions were made from di-ethyl-pyrocarbonate (DEPC) treated water, and all washes were carried out at RT unless stated otherwise.

Embryos stored in -20°C in methanol, were re-hydrated through methanol/PBT (0.1% Tween-20 in PBS) washes. Embryos were bleached with 6% hydrogen peroxide in PBT, and after several washes in PBT, partially digested in 10μg/ml proteinase K for 8 (E9.0) to 20 min (E11.5). The reaction was stopped by washing the embryos with 2mg/ml glycine/PBT. After rinsing twice in PBT, the embryos were re-fixed in 0.2% glutaraldehyde/ 4% PFA for 20 min. After several more PBT washes, the embryos were pre-hybridized with hybridization buffer {50% formamide (Invitrogen), 5xSSC pH 4.5, 50μg/ml yeast tRNA (Sigma), 1% SDS, 50μg/ml heparin (Sigma), 0.1% CHAPS (Sigma), 5mM EDTA} for 1 h at 70ºC with gentle rocking. DIG-labeled probe was diluted to 1μg/ml in hybridization buffer, denatured at 85ºC for 10 min and added to the embryos. Embryos were hybridized O/N at 70ºC with agitation.
After hybridization, the embryos were washed twice in wash buffer I (WBI) (50% formamide, 5xSSC pH 4.5, 1% SDS, 0.1% CHAPS) for 30 min at 70°C with gentle rocking. Embryos were washed once with 1:1 WBI:WBII (0.5M NaCl, 10mM Tris-HCl pH 7.5, 0.1 % Tween-20, 0.1% CHAPS) for 10 min at 70°C. After rinsing several times in WBII, embryos were treated with 100μg/ml RNase A for 1 h at 37°C. Embryos were rinsed once in WBII and then washed twice in WBIII (50% formamide, 2xSSC pH 4.5, 0.1% CHAPS) for 30 min at 65°C. The embryos were rinsed three times in TBST (25mM Tris pH8.0, 125mM NaCl 0.1% Tween-20) and then blocked in TBST with 10% heat inactivated sheep serum (Sigma) for 1 h with agitation. Finally, the embryos were incubated O/N at 4°C with anti-DIG-AP antibody (Roche) (1:5000 in TBST, and 1% sheep serum) which was pre-incubated with 1.5g/ml embryo powder, and 1% sheep serum, O/N at 4°C. Next day embryos were rinsed twice in TBST with 2mM levamisole (Sigma), and then washed with this solution 8 times for 1 h, with gentle rocking. The embryos were last washed with NTMT-levamisole (100mM Tris pH 9.5, 100mM NaCl, 50mM MgCl2, 0.1% w/v Tween-20, 2mM levamisole three times for 10 min. For colour development, embryos were incubated in NTMT containing 1:50 dilution of ready-to-use NBT/BCIP solution (Roche, 18.75mg/ml NBT and 9.4mg/ml BCIP). The reaction was stopped with three washes of PBT containing 1mM EDTA. Stained embryos were re-fixed in 4% PFA for 2 h, washed in PBS, imaged using Leica MZFIII microscope equipped with Qimaging 1300C digital camera and analyzed with Adobe Photoshop.

*For in-depth examination of Fam105b expression, E10.5 embryos were embedded in 3% agar, and sectioned sagitally 200μm thick using the vibratome.
*Embryos processed for Phox2b and Hoxb1 in situ hybridization were flat mounted under a square coverslip before visualization.

*E10.5-E11.5 embryos with high background were dehydrated through a series of methanol/PBST washes, and left in methanol for a few days. If the background persisted, embryos were cleared further by one of two methods: (i) Transferred to benzyl benzoate:benzyl alcohol (2:1), or (ii) Re-hydrated through a series of methanol/PBS washes, and kept in 80% glycerol/PBS solution prior to photographic documentation.

2.11 Generation of rabbit anti-Fam105b polyclonal antibody

2.11.1 Generation of GST-Fam105b fusion protein

To generate Glutathione S-transferase (GST)-tagged Fam105b protein (GST-Fam105b), Fam105b cDNA was subcloned in-frame into pGEX-HTa vector (Pharmacia). The ~65kDa GST-Fam105b fusion protein was expressed in E. coli BL121 cells by 0.2mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Sigma) induction at 30ºC for 4 h. Expression was monitored by SDS-polyacrylamide gel electrophoresis followed by coomassie blue staining. Cells were lysed by sonication (6 times with 30 sec bursts) and GST-Fam105b was purified over glutathione-Sepharose™ 4B beads (Pharmacia Biotech) and eluted with 25mM reduced glutathione (Sigma) according to the manufacturer’s instructions. The protein was dialyzed in three changes of PBS using cellulose dialysis membrane (Fisher Scientific) according to manufacturer’s instructions, before being injected into rabbits. Injection was done by the staff at the laboratory of Division of Comparative Medicine, University of Toronto

2.11.2 Generation of MBP-Fam105b fusion protein
To generate the maltose binding protein (MBP)-tagged Fam105b fusion protein (MBP-Fam105b), Fam105b cDNA was subcloned in-frame into pMAL-C2 vector (New England Biolabs). The ~70kDa MBP-Fam105b was expressed in *E. coli* BL121 cells by 1mM IPTG induction at 30ºC for 5 h. Expression was monitored by SDS-polyacrylamide gel electrophoresis followed by coomassie blue staining.

After induction, cells were spun for 10min at 9,000xg and pellet resuspended in ice cold HCB buffer (20 mM HEPES pH 7.4, 200 mM NaCl, 1mM EDTA, 1 mM DTT) at 10ml/gram. Cells were lysed by sonication (6 times with 30s bursts). Sonicate was spun for 30 min at 9,000xg at 4ºC, and the supernatant was added to prewashed amylose resin (New England Biolabs). After 1 h of binding at RT, the beads were spun at for 5 min at 1,000xg, and the pellet was washed 5 x 30 min with 10 volumes of HCB buffer. The last two washes were done in poly-prep chromatography columns (Biorad). MBP-Fam105b fusion protein was eluted using 50mM maltose, 1mM DTT and analyzed by SDS-gel electrophoresis.

### 2.11.3 Antibody purification using Affi-Gel15 column coupled to MBP-Fam105b

For antibody purification ~3mg of MBP-Fam105b was bound to 1ml of Affi-Gel15 (Biorad Cat. #1536052), according to manufacturer’s instructions. After antigen binding, the column was washed with 1M NaCl-PBS and non-specific binding sites were blocked by washing the column with 0.1M ethanolamine, pH 8.0 for 1 h at RT. After several PBS washes, 6ml of crude rabbit antiserum was added to the column and rocked at 4ºC O/N. After several PBS washes, the antibody was eluted with 100mM glycine, pH 2.5 in fractions that were immediately neutralized by the addition of 0.1M Tris pH 8.5. Protein concentration of each fraction was estimated using Coomassie protein assay reagent.
(Thermo Scientific). Fractions with the highest amount of protein were pooled and
dialyzed in three changes of PBS using cellulose dialysis membrane (Fisher Scientific)
according to manufacturer’s instructions Antibody was concentrated by lyophilization,
resuspended in dH₂O and dialyzed O/N in PBS (pH7.4) at 4°C using Slide-A-Lyzer
dialysis cassette (Pierce). The final antibody concentration was estimated to be
0.25mg/ml. NaN₃ was added to 0.05% and antibody aliquots were stored at -80°C. A
working antibody aliquot was kept at 4°C and proven to be stable in these conditions for
at least one year.

2.12 Western blot analysis

2.12.1 Preparation of cell and tissue lysates

Neuro2A cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum
(Gibco). The plasmid encoding the Fam105b-GFP fusion protein was transfected using
Effectene transfection reagent (Qiagen) and cells were lysed after 24 h of transfection.
Neuro2A cells and mouse tissues were lysed in RIPA buffer {1% NP40, 0.5% sodium
deoxycholate, 0.1% SDS, in PBS pH 7.4 with protease inhibitor cocktail tablet (Roche)}. One ml of RIPA was used for 10 cm plate; 3 ml of RIPA was used for 1mg of tissue.

2.12.2 Western blot

SDS-polyacrylamide gel electrophoresis was carried out using 10-15μg of total lysate and
the protein bands were electroblotted onto a polyvinylidene difluoride membrane (PVDF)
(Pall Corporation). Membrane was blocked for 1 h at RT with 5% Bovine Serum
Albumin (BSA) (Sigma) in TBST (25mM Tris pH 8.0, 125mM NaCl 0.1% Tween-20),
then incubated with a primary antibody diluted in block O/N at 4°C with gentle agitation.
Blots were washed 5 x 10 min in TBST and incubated with secondary antibody diluted in
TBST for 1 h at RT. After 5 x 10min TBST washes antibodies were detected using ECL SuperSignal (Pierce) and visualized using Denville HyBlot CL film. To control for loading, membranes were stripped using TBST pH 2.5, and probed with Tubulin antibody. Antibodies used in Western blots were rabbit Fam105b (1:5,000); rabbit GFP (A11122 Molecular Probes, 1:1,000); mouse Tubulin (Clone DM1A Sigma, 1:2000); goat HRP-IgG (anti-rabbit or anti-mouse) (Jackson Immunoresearch, 1:10,000).

2.12.3 Immunoprecipitation

Immunoprecipitation using Fam105b antibody was performed using standard protocols with 50μl of Protein A/G Plus Agarose (Santa Cruz Biotechnology, SC-2003), 400μg of Neuro2A cell lysate and 3μl (0.75μg) of Fam105b antibody. 400μg of Neuro2A lysates transfected with empty pEGFP-C1 (Clontech) was used as control.

2.13 Testing Fam105b antibody specificity by preblocking with GST-Fam105b

To test the specificity of Fam105b antibody the following method was used: Fam105b antibody diluted in the appropriate buffer (5% non-fat instant milk powder/TBST for Western blot, 10% Normal Goat Serum/PBST for immunofluorescence) was incubated with either GST-Fam105b or GST alone, immobilized to glutathione-Sepharose™ 4B beads (Pharmacia Biotech). After 1.5 h of rocking at RT beads were spun down, and the supernatant was used as a primary antibody.

2.14 Immunohistochemistry

2.14.1 Tissue immunofluorescence

For immunofluorescence experiments embryos or tissues were dissected, fixed overnight in 4% PFA, dehydrated in 30% sucrose/PBS, and frozen in Tissue-Tek OCT (Miles laboratories). Tissues were sectioned 16μm thick and kept at -80ºC until further use.
Slides were washed in PBS, and blocked in PBST (PBS-0.3% Triton-X 100) with 10% Normal Goat Serum (NGS) for 2.5 h at RT. Slides were incubated with primary antibodies diluted in PBST-1% NGS O/N at 4°C. Slides were washed in PBS, and incubated with fluorescently labeled secondary antibodies for 1 h at RT. Finally, slides were washed and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories). Sections were analyzed with Olympus BX61 microscope equipped with a Hamamatsu ORCA ER digital camera and images were analyzed with Adobe Photoshop. For double fluorescent experiments both possible combinations of secondary antibodies were used to exclude fluorescent dye effects.

*For placenta only, antigen unmasking technique was required which consisted of boiling sections for 10 min in 10mM Na-citrate prior to blocking.

2.14.2 Cell immunofluorescence

Neuro2A cells were cultured in DMEM supplemented with 10% FBS (Gibco). FLAG-Fam105b plasmid was transfected into cells growing on coverslips using Effectene transfection reagent (Qiagen) and processed for immunofluorescence after 24 h of transfection. Cells were fixed in 3.7% paraformaldehyde for 15 min, permeabilized in PBST (PBS-0.1% Triton X-100) for 20 min and blocked in 10% NGS/PBST for 20 min. Cells were incubated with primary antibodies diluted in 1% NGS/PBST for 1 h at RT. Cells were washed in PBS and incubated with secondary antibody diluted in 1% NGS/PBST for 1 h at RT. After final washes cells were mounted with Vectashield mounting media with DAPI (Vector laboratories). Cells were visualized using Nikon D-eclipse C1 confocal microscope system and analyzed with Adobe Photoshop. Antibodies used for immunofluorescence were: rabbit Fam105b (1:500); mouse monoclonal anti-
human Smooth Muscle Actin (Clone 1A4 DAKO, 1:1,000); monoclonal mouse anti-human Desmin (Clone D33 DAKO, 1:1,000); mouse FLAG M2 (Clone F1804 Sigma, 1:1000); rat anti-mouse CD31 (PECAM-1) (Clone Mec13.3 BD PharMingen, 1:50); biotin conjugated Lectin from Dolichos biflorus (Clone L6533 Sigma 50μg/ml); DyLight 488 Streptavidin (Jackson Immunoresearch 1:400); goat anti-rat FITC (Jackson Immunoresearch, 1:200); donkey anti-mouse AlexaFluor 488 (Molecular Probes, 10μg/ml); goat anti-mouse AlexaFluor 594 (Molecular Probes, 10μg/ml); goat anti-rabbit AlexaFluor 488 (Molecular Probes, 10μg/ml); and goat anti-rabbit AlexaFluor 594 (Molecular Probes, 10μg/ml).

2.14.3 Whole-mount PECAM-1 immunohistochemistry

Embryos were fixed in 4% PFA O/N at 4ºC, dehydrated through a graded methanol series and bleached for 5 h in 5% H₂O₂-methanol at RT. After rehydration embryos were washed three times in PBST (PBS-0.1% Triton X-100) and blocked in (PBST-2% instant milk powder, 0.25% BSA, 0.05% Tween-20, 5% NGS), for 3 h at RT. Embryos were incubated O/N at 4ºC with CD31 (PECAM) antibody (Clone Mec13.3 BD PharMingen; 1:50) diluted in PBS-MT (PBST-2% non-fat instant milk powder). After 6 x 30 min washes in PBS-MT, embryos were incubated with Biotin-SP-conjugated AffiniPure goat anti-Rat (Jackson Immunoresearch; 1:250) for 3 h at RT, washed five times in PBS-MT at RT, and once O/N at 4ºC. Embryos were then incubated with Streptavidin Alkaline Phosphatase (AP) conjugate (Boehringer Mannheim; 1:1000) for 2 h at RT, washed five times in PBST, twice in AP wash buffer (100mM Tris-HCl, 100mM NaCl, 5mM MgCl₂, 0.05% Tween-20, pH 9.5) and stained for AP using ready-to-use NBT/BCIP solution (Roche, 18.75mg/ml NBT and 9.4mg/ml BCIP). The reaction was stopped with three
washes of PBT-1mM EDTA. Stained embryos were re-fixed in 4% PFA for 2 h, washed in PBS, imaged using Leica MZFIII microscope equipped with Qimaging 1300C digital camera and analyzed with Adobe Photoshop.

2.14.4 Slide immunohistochemistry

For immunohistochemistry experiments embryos and tissues were prepared as for immunofluorescence. Frozen sections were washed twice in PBS, bleached for 1 h in 0.3% H2O2-methanol at RT, washed four times in PBS and blocked in Histoblock [PBS-3% BSA, 1% BM Blocking solution (10% Blocking Reagent (Roche) stock made in 100mM Maleic Acid), 20mM MgCl2, 0.3% Tween-20] for 1 h at RT. Sections were incubated with Fam105b antibody (1:1000) in Histoblock O/N at 4ºC, washed twice in PBS and incubated with Biotin-SP-conjugated AffiniPure Goat anti-Rabbit (1:250, Jackson Immunoresearch) for 1 h at RT. Sections were incubated with Vectastain ABC solution (Vector Laboratories) for 45 min at RT. After two washes in PBS slides were developed using 3,3’-diaminobenzidine (DAB) reagent (Sigma D4168). Reaction was stopped by washing the slides in distilled water. Slides were dehydrated from ethanol to xylene, and mounted with Cytoseal-60 (VWR). The sections were analyzed with Olympus BX61 microscope equipped with an Olumpus DP71 camera and images were analyzed with Adobe Photoshop.

2.15 BAC rescue analysis

2.15.1 Generation of BAC transgenic mice

To confirm that T285A mutation is responsible for the gumby phenotype, I performed rescue analysis using Fam105b containing Bacterial Artificial Chromosome (BAC).
bMQ-396D3 BAC (Chr.15: 27,544,413-27,629,541bp) from AB2.2 embryonic stem (ES) cell DNA (129S7/SvEvBrd-\textit{Hprt}^{b-m2}), was generated in Alan Bradley's lab, in the Wellcome Trust Sanger Institute, Hinxton, UK [156]. Circular BAC was purified using NucleoBond AX500 purification kit (Clontech), and injected in Michigan University Transgenic Core into (C57BL/6 X SJL)F2 females to generate transgenic mice for rescue experiment. Several genotyping assays were used to identify mice carrying the BAC transgene: 1) An internal RFLP marker rs31960302, that distinguishes between BAC 129 and endogenous C57BL/6 strains, 2) Two PCR assays with primer pairs complimentary to the genomic sequence and BAC vector sequence at both the 5’ and the 3’ ends of the BAC. The primer sequences are: F:5’-GCCCGTTGACTTTGTTCCC-3’ (genomic) with R:5’-CAGCTGTCCCACACATCAAG-3’ (vector), and F:5’-CCTCCTAAGGTTTTCACAAATGC-3’ (genomic) and R:5’-CTTAATTAAGGATCGATCCGGCG-3’ (vector). Out of 182 mice born, 4 were identified as positive for the presence of the BAC transgene. Three of these founders transmitted the BAC to their progeny, with 10-20% transmission frequency. Breeding of G1 progeny resulted in the expected 50% frequency of transmission. G1 transgenic BAC progeny were mated to heterozygous \textit{gumby}/+ mice to generate BAC carriers that are heterozygous at the \textit{gumby} locus.

\textbf{2.15.2 Estimation of BAC copy number using sequencing}

To estimate BAC copy number for each of the three BAC lines (BAC Line 1-3), I sequenced across SNP rs31960302. In a mouse line carrying a single copy of the transgene, the ratio of 129 specific A allele to C57/C3H specific G allele is 1:2. Each
additional BAC increases the amount of T by 1. Using this method I determined that lines BAC2 and BAC3 carry one BAC transgene, while line BAC1 carries 8-10 copies of the transgene. To confirm this I also sequenced across the *gumby* mutation (T285A). Using similar rationale, in *gumby/+* heterozygotes the ratio of wild-type T allele to mutant A allele is 1:1. Each BAC copy increases the amount of wild-type T allele by 1 (i.e. 2:1 if single copy).

2.15.3 Quantitative PCR

Total RNA for generation of cDNA was obtained from whole E11.5 BAC */gumby* and */gumby* embryos, using Trizol reagent (Invitrogen) according to manufacturer’s instructions. 1.5μg of RNA was used in 20μl Reverse Transcriptase (RT) reaction as described in Section 2.3. Real-time PCR was performed on the Applied Biosystems 7900HT Real-Time PCR system. Samples were prepared using SYBR Green PCR Master Mix (Applied Biosystems) with a total volume of 20μl and were run for a total of 40 cycles with an annealing temperature of 60°C. Gene expression was normalized using mouse GAPDH. Primers pairs used to amplify Fam105b cDNA were: F:5'-GGTTAAGTGCTGCCTGA-3’ and R:5’-CTGACACCTCCCTCATAGC-3’. Primer pairs used to amplify control GAPDH were F:5’-TGTGAACGATTTGGCCTAT-3’ and R:5’-CATAGACCATGTAGTTGAG-3’. Statistical comparisons were performed by Student's t test. p<0.05 was considered significant (n=3).

2.15.4 Western blot analysis of BAC transgenics
15μg of embryo lysates from E11.5 BAC +/gumby and +/-gumby embryos were run on a western blot and probed with α-Fam105b antibody (n=3). α-Tubulin was used as a loading control.

2.15.5 Testing for BAC transgenic rescue

To test for rescue I set to determine whether BAC; gumby/gumby animals are viable beyond E13.5, the stage at which gumby/gumby mutants die. However, because both BAC and the gumby mutation are carried on C57Bl6/J strain, and because wild-type T allele carried on the BAC masks homozygosity at endogenous gumby locus, I needed a way to distinguish between C57gum/C57^+/BAC and C57gum/C57gum;BAC; rescue mice, since both of these are heterozygous at the T285A locus, and C57/C57 at a SNP site closely linked to the gumby locus (rs13482490). To get around this, for each BAC line I performed two generation breeding crosses outlined in Figure 35 to generate mice that are C57gum/C3H^+/BAC (G2). These mice were intercrossed, and their progeny (G3) were scored for rescue at E14.5, past weaning age, and at E10.5 by PECAM-1 immunohistochemistry. Each G3 animal was genotyped at two loci: 1) At rs31960302 for the presence of BAC, and 2) At rs13482490 to infer genotype at the gumby locus.

2.16 Fosmid rescue of the gumby phenotype

Fam105b-containing fosmid G135P62329F7 (EpiFos Fosmid Library) was ordered from the CHORI BACPAC Resource Centre, purified using Plasmid Maxi Kit (QIAGEN), and digested with NotI to snap out the genomic fragment. The 38.6Kb DNA fragment spanning Chr.15:27,589,751–27,628,382bp, was purified using GeneClean Turbo Kit (qbiogene) and injected in the Mount Sinai Transgenic Facility into FVB females to generate transgenic mice for rescue experiment. To identify mice carrying the BAC
transgene I used an internal RFLP marker rs31576805 that distinguishes between Fosmid C57Bl/6J strain, and the endogenous genomic FVB strain. Out of 20 mice born, two males were identified as transgenic carriers and bred to FVB females. Unfortunately, both males did not produce any transgenic progeny and therefore could not be tested for rescue activity.

2.17 Designing a screening strategy to identify novel gumby mutations

To screen the frozen sperm archive of ENU-mutagenized (G1) “mutant mouse library” created by RIKEN Genomic Sciences Center (GSC), I designed and validated primer pairs that can be used for “multiplexing PCR”. To enhance the capacity and throughput of the screen, the primers were designed in a way that multiple primer pairs can be mixed in a single PCR reaction and still give each corresponding clear band, with no background. Primers combination that gave the best results amplified exons 2, 3, 6 and 7 (Figure 38).

2.18 Fam105b knockdown using siRNA technology

The short hairpin RNA (shRNA) expression plasmid (H1 RNA pol III promoter-shRNA-pcDNA3.1(+)) used for siRNA knockdown was obtained from the Laboratory of Dr. Rossant and is described in [157]. Fam105b-specific shRNA transgene, consisting of sense and antisense 23bp region, was generated using the primers: F:5’-gtacc(Asp718)AACTCATACTGTACCAGAGATT(sense)caagagaTCTCTGGTAACATGTATGAGTT(antisense)-3' and R:5’-ctagATTTTCCAAAAACTCATAACTGTACCAGAGATT(antisense)caagagaTCTCTGGTAACAGTATGAGTT(sense)-3'. 10μl of each primer (10μg/ml) was mixed and boiled for 5 min, annealed at RT, and digested with Asp718/XbaI and cloned into Asp718/XbaI digested H1 RNA pol III promoter-shRNA-pcDNA3.1(+) vector. Stable ES
cell lines were generated by electroporation of the ScaI linearized Fma105b shRNA transgene into G4 (129S6 x C57BL/6) ES cells. ES lines were tested for Fam105b mRNA expression using semi-quantitative PCR, with Fam105b specific primers: F:5'-CACGAGGAGGACATGTACC-3' and R:5'-GTTTCCACTGCTTGATCCAG-3' and control Actin primers: F:5'- AGGCCAACGTGAGAAGATG-3' and R:5'- TCCATCACGATGCCAGTGGT-3'. Three ES lines showed 30-50% downregulation of Fam105b and the line with the lowest Fam105b level was used to generate ES-cell-derived embryos by aggregating ES cells with B5/EGFP tetraploid embryos (described in [158]) in the Mount Sinai Hospital Transgenic Facility.
RESULTS

Chapter 3: Identification and characterization of the *gumby* candidate gene

3.0 Overview

The *gumby* mutant was identified in an ENU forward recessive screen on the basis of its cranial deficits: its facial nerve appears to sprout abnormally at E10.5. In addition to that, *gumby* embryos are embryonic lethal in the homozygous state, thus, the gene responsible for the mutation is essential for embryonic development.

Ultimately, to be valuable, new mutations need to be mapped and the gene responsible must be identified. Then the expression and the function of the identified gene are studied and used to unravel the pathways in which the gene might act. The *gumby* mutation was mapped using standard positional cloning techniques that initially localized it to a ~11.5Mb region on mouse chromosome 15. To refine this region further, I used positional cloning together with a candidate gene approach, and identified a point mutation in a novel gene – *Fam105b* - that likely causes the *gumby* mutant phenotype.

This chapter describes the identification of *Fam105b* as the gene likely responsible for the *gumby* mutation, as well as the analysis of the expression and function(s) of *Fam105b* in the mouse. Together these data indicate that *Fam105b* encodes a novel cytoplasmic protein, highly conserved between vertebrate species, which plays a role in the facial nerve axon guidance, angiogenesis, and based on its expression pattern, other developmental processes that will be analyzed in the future. In addition, preliminary evidence suggests that *Fam105b* might act in the highly conserved Wnt pathway, and may contribute to the phenotype of human Cri du Chat Syndrome.

3.1 *Gumby* mutants have hypersprouting facial nerve, and die between E11.5-E12.5
*Gumby* segregates as a simple recessive Mendelian trait, yielding ~25% affected offspring from heterozygous intercrosses. Heterozygotes of both sexes are viable, fertile and overall indistinguishable from unaffected homozygotes.

In E10.5 *gumby* homozygote embryos the facial nerve appears to sprout abnormally [4]. Otherwise, before E11.0 *gumby* mutants are morphologically indistinguishable from their wild-type and heterozygous littermates, with no obvious abnormalities or difference in size (Figure 8A,B). Heartbeats were observed in all freshly dissected embryos. From ~E11.0-E12.0, *gumby* mutants are progressively growth retarded, the first branchial arch (BA1) is slightly reduced (asterisk in Figure 8D,F), their head smaller, and blood vessels often appear grossly malformed (dilated and/or bloody patches). In many the heart is engorged with blood (Figure 8F), suggesting defective blood flow. When isolated at E12.0-E12.5, mutant embryos are either completely resorbed or are severely necrotic, and their yolk sacs are completely devoid of all blood (Figure 8H). No *gumby* homozygous embryos were recovered after E13.0. Thus, the *gumby* mutation is lethal in the homozygous condition between E11.5-E12.5 and therefore affects a gene essential for embryonic development.
Figure 8: Phenotype of gumby embryos at E10.5-E12.5.

Control (+/+) embryos are shown in the left column, gumby homozygous (gumby/gumby) mutants in the right column. At E10.5, mutant embryos (B) are morphologically indistinguishable from unaffected controls (A). At E11.0, mutants (D) appear slightly growth delayed, and have a mildly reduced 1st branchial arch (BA1) (asterisk in D). (E-F) At E11.5 gumby mutants are growth retarded, have reduced BA1 (asterisks in F), and often exhibit hemorrhaging, dilated vessels and hearts filled with blood (F). (G,H) Between E12.0-12.5, mutants are necrotic or resorbed (H), suggesting the time of
lethality between E11.5-E12.5. Stage matched embryos are taken at the same magnification.

3.2 Mapping the *gumby* mutation

3.2.1 Refining *gumby* critical interval using meiotic recombination

Our initial mapping located the *gumby* mutation to a ~11.5Mb region between markers D15MIT130 (20.7Mb) and D15MIT111 (32.2Mb) on mouse chromosome 15. To locate the *gumby* mutation more precisely, I screened 154 additional progeny from *gumby/+* heterozygous intercrosses using up to five additional simple sequence length polymorphisms (SSLPs) and single nucleotide polymorphisms (SNPs) markers. The list of polymorphic markers used for mapping and their primers is available in (Figure 9) and Appendix A. One informative recombinant placed the mutation proximal to D15Mit18 (28.4Mb) and 3 informative recombinants placed the mutation distal to marker rs13482490 (26.7Mb), therefore refining the *gumby* critical interval to a 1.7Mb region on chromosome 15 between 26.7Mb and 28.4Mb.
Figure 9: Fine mapping of the gumby mutation.
Meiotic mapping was performed on a total of 154 progeny from gumby/+ intercrosses. E12.5 progeny were analyzed with polymorphic markers shown here, surrounding the gumby critical interval on mouse chromosome 15. Four recombinants helped refine the critical interval to a 1.7Mb region between markers D15Mit18 (26.7Mb) and SNP rs13482490 (28.4Mb) based on Ensembl assembly version 35.

3.2.2 Analysis of gumby critical interval

3.2.2.1 Gumby critical interval is syntenic to human region deleted in Cri du Chat Syndrome

Genes identified within the gumby candidate interval establish conservation between this region on mouse chromosome 15 and human chromosome 5p15.2. This region is deleted.
in patients with Cri du Chat Syndrome (CdCS) (OMIM 123450). CdCS is a common deletion disorder with an incidence of 1 in 20,000 to 50,000 live births. The core clinical features include a high-pitched cry, characteristic facial features including microcephaly, micrognathia, hypertelorism, epicanthal folds, mild to severe mental retardation, speech, and motor delays, and various behavioral abnormalities including destructive behavior, tantrums, repetitive movements, and aggression. Other common findings include difficulty swallowing and suckling, low birth weight and poor growth, strabismus, excessive salivation, and congenital heart conditions including atrial and ventricular septal defects (ASD, VSD), and patent ductus arteriosus (PDA) [58].

Genotype-phenotype comparisons have identified and refined chromosomal regions involved in specific clinical features. Loss of the band 5p15.2 (CdCS critical region) correlates with all clinical features of the syndrome except for the catlike cry, which maps to band 5p15.3 (1.5Mb in distal 5p15.31 between 7.5Mb and 8Mb). The region associated with facial dysmorphology has been mapped to 2.4Mb in 5p15.2 between 9Mb to 11.4Mb by one study [61], although another study has reported an association of a larger region (11Mb-15Mb) with craniofacial deficits [159]. Several 5p regions have been associated with mental retardation (MR) of varying severity. In general, MR depends approximately on the 5p deletion size and location, although in many cases the retardation is disproportionately severe, given the 5p deletion. One region strongly associated with severe MR lies between 9.3Mb and 18.3Mb [61]. Deletions that do not include these chromosomal regions present varying clinical phenotypes from severe mental retardation and facial dysmorphism to a clinically normal phenotype [58, 61].
Other CdCS features have not been extensively mapped. *Gumby* is located in a region of conserved synteny with proximal 5p15.2 (10.4 to 15.1 Mb), a region strongly associated with severe mental retardation (Figure 10). Because of the discrepancy between the two studies that have mapped the craniofacial symptoms, it is currently unclear whether the *gumby* region lies within the critical interval implicated in facial dysmorphology.

**Figure 10:** *gumby* critical interval is syntenic to a human region deleted in Cri du Chat Syndrome

(A) Phenotypic map of human chromosome 5p. Vertical lines indicate the critical regions of various symptoms of Cri du Chat Syndrome. Adapted from Mainardi 2006 [58]. (B) Band p15.2 (8.8-15.1Mb) where many of the symptoms map, is syntenic to mouse chromosome 15: 26.75-32Mb. Known genes within this region and Fam105b are shown. *Gumby* critical interval is syntenic to the proximal region of p15.2, between 13.5Mb and 15.1Mb. (C) Ten genes that map to *gumby* critical interval based on Ensembl.
3.2.2.2 Evaluation of genes in *gumby* critical interval

The 1.7Mb *gumby* critical interval contains ten (10) annotated RefSeq genes, only three of which have known functions (Figure 9C, Table 1, Table 2). These genes are: *Triple functional domain protein* (*TRIO*), *progressive ankylosis* (*Ank*), and *axonemal dynein heavy chain 5* (*Dnahc5*). Their roles and mutant pathologies are described in detail in Table 1 and Table 2. Based on their reported functions and mutant pathologies none of these genes were considered as likely candidates for the *gumby* mutation. The remaining seven (7) genes in the critical interval have no annotated function.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Position on Chr. 15 (Mb)</th>
<th>Known or predicted function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tas2R119</td>
<td>Taste receptor type 2 member 1</td>
<td>32.1 - 32.1</td>
<td>Functions as a bitter taste receptor; likely controls the detection of the bitter compound 6-n-propyl-2-thiouracil [161, 162].</td>
</tr>
<tr>
<td>Cct5</td>
<td>T complex protein 1 subunit epsilon</td>
<td>31.52 - 31.53</td>
<td>Member of TCP molecular chaperone complex involved in ATP dependant protein folding of many proteins including actin and tubulin. CCT controls polyglutamine aggregation [163, 164].</td>
</tr>
<tr>
<td>Cmbl</td>
<td>Carboxymethylbutenolidase-like (Pseudomonas)</td>
<td>31.49 - 31.51</td>
<td>In Pseudomonas, it is an enzyme that hydrolyses carboxylic ester bonds. Role in humans is unknown.</td>
</tr>
<tr>
<td>March6</td>
<td>Membrane-associated RING-CH 6 (MARCH E3 family)</td>
<td>31.38 - 31.46</td>
<td>MARCH family has structural similarity to viral E3 ligases [165]. <strong>Physiological role is unknown.</strong></td>
</tr>
<tr>
<td>Ropn11</td>
<td>Ropporin 1-like</td>
<td>31.37 - 31.38</td>
<td>Structurally related to ropporin, a sperm specific protein that functions in spermatogenesis [166].</td>
</tr>
<tr>
<td>DAP</td>
<td>Death associated protein</td>
<td></td>
<td>Contains Death-Domain that interacts with classical death receptor Fas that is implicated in many cellular processes including transcription, cell cycle regulation and apoptosis. <strong>Physiological role is unknown.</strong></td>
</tr>
<tr>
<td>Ctnnd2</td>
<td>Catenin delta-2</td>
<td>30.1 - 30.96</td>
<td>Neural specific protein of the armadillo/beta-catenin superfamily. Component of the cadherin-catenin cell adhesion complex. Involved in cell motility. Interacts with presenilin-1 and cadherin associated protein. Regulates spine and synapse morphogenesis and function in hippocampus. Interacts with Presenilin-1 (PS1), the molecule most frequently mutated in familial Alzheimer’s Disease [167-171].</td>
</tr>
<tr>
<td>Dnahc5</td>
<td>Ciliary dynein heavy chain 5</td>
<td>28.1 - 28.4</td>
<td>Ciliary dynein structural component</td>
</tr>
<tr>
<td>ENSMUSG000000726630</td>
<td>Unknown</td>
<td>27.79 - 27.79</td>
<td>Unknown</td>
</tr>
<tr>
<td>Trio</td>
<td>Triple functional domain</td>
<td>27.66 - 27.95</td>
<td>DH-GEF Trio controls myogenesis and axon guidance of dentate gyrus cells in the hippocampus and mitral cells in the olfactory bulb [172]</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Gene Name</td>
<td>Gene Function</td>
<td>Chromosome Range</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Q8CDT1</td>
<td>Unknown</td>
<td></td>
<td>27.79</td>
</tr>
<tr>
<td>BC052328</td>
<td>Unknown</td>
<td></td>
<td>27.58 - 28.61</td>
</tr>
<tr>
<td>Fam105b</td>
<td>Unknown</td>
<td></td>
<td>27.53 - 27.56</td>
</tr>
<tr>
<td>Q8C9X7</td>
<td>Unknown</td>
<td></td>
<td>27.5 - 27.5</td>
</tr>
<tr>
<td>ENSMUSG00000054546</td>
<td>Unknown</td>
<td></td>
<td>27.5</td>
</tr>
<tr>
<td>Ank</td>
<td>Progressive ankylosis protein; transports intracellular pyrophosphate</td>
<td>27.4 - 27.52</td>
<td>Transports intracellular pyrophosphate thus regulating extracellular calcification; osteoblasts differentiation [173-176].</td>
</tr>
<tr>
<td>Q8BQ70</td>
<td>Unknown</td>
<td></td>
<td>26.78</td>
</tr>
</tbody>
</table>

**Table 1:** Overview of the genes that map to a region syntenic to human chromosome 5p band 15.2, where the majority of the CdCS symptoms have been mapped. Genes outlined in red are located in *gumby* critical interval. Outside of this region only known genes are shown.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Mouse mutant phenotype</th>
<th>Linkage to human diseases</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sema5A</td>
<td>Lethal in homozygous state at E11.5 likely due to a decrease in complexity of cranial vessels</td>
<td>Has been proposed to contribute to mental retardation in Cri du Chat syndrome</td>
<td>[58, 125-127, 160]</td>
</tr>
<tr>
<td>Cct5</td>
<td>RNAi-mediated knockdown of CCTξ subunit in HEK293, Neuro2A and HeLa cells enhanced huntingtin–polyglutamine aggregate formation and cellular toxicity.</td>
<td>Missense mutation in CCT5 associated with recessive mutilating sensory neuropathy with spastic paraplegia characterized by progressive distal sensory neuropathy, osteomyelitis, and a mild paraplegia</td>
<td>[163, 164]</td>
</tr>
<tr>
<td>Ctnnd2</td>
<td>Ctnnd2 +/- mice have impaired cognitive function, abnormalities in LTD and LTP, increased hippocampal synaptic density and decreased tetradotoxin induced cell plasticity.</td>
<td>Overexpressed in prostatic tumor cells. A copy number variant (CNV) that disrupts CTNND2 has been implicated in schizophrenia. <strong>Strong correlation between CTNND2 loss and severe mental retardation of Cri du Chat syndrome</strong></td>
<td>[58, 167-171]</td>
</tr>
<tr>
<td>Dnahc5</td>
<td>Dnahc5 +/- mice exhibit primary ciliary dyskinesia (PCD)-like symptoms including ciliary immotility, random left-right axis specification, respiratory infections and hydrocephalus. Die postnataly.</td>
<td>The main gene that causes primary ciliary dyskinesia (PCD), an autosomal recessive disorder characterized by recurrent infections of the upper respiratory airways, randomization of left-right body asymmetry and male infertility (OMIM 244400)</td>
<td>[177, 178]</td>
</tr>
<tr>
<td>trio</td>
<td>Trio^-/- embryos die b/w E15.5-birth of unknown reasons. Have abnormal skeletal myofibers and slightly aberrant neural organization in the hippocampus and olfactory bulb</td>
<td>Possibly involved in glioblastoma tumor cell migration and invasion</td>
<td>[172, 179, 180]</td>
</tr>
<tr>
<td>Fam105b</td>
<td><em>gumby/gumby</em> mice have reduced lower jaw, hypersprouting facial nerve, and angiogenic deficits. Die at ~E13.0</td>
<td><strong>We hypothesize that Fam105 contributes to the craniofacial deficits, mental retardation and heart abnormalities in Cri du Chat syndrome.</strong></td>
<td>This study</td>
</tr>
<tr>
<td>Ank</td>
<td>ank/ank mice have progressive ossific ankylosis in joints leading to complete rigidity and death at around 6 months</td>
<td>Human gain-of-functions mutations of ank lead to cranio-metaphyseal dysplasia (CMD), a disease characterized by the overgrowth of craniofacial and long bones, suggesting that ANK plays a regulatory role in osteoblast differentiation. <strong>CMD has not been observed in Cri du Chat syndrome.</strong></td>
<td>[173-176]</td>
</tr>
</tbody>
</table>

**Table 2:** Mouse mutant phenotype and/or human disorders associated with genes that map to human chromosome 5p band 15.2 where the majority of CdCS symptoms have been mapped. Genes outlined in red are located within the *gumby* critical interval.
3.3 Sequencing the genes in the critical interval

Q8BQ70, Tiaf2, Q8C9X7, Fam105b, BC052328, Q8CDT1, and ENSMUSG000000726630 were directly sequenced using genomic and/or cDNA analyses, depending on the intron-exon structure of each gene. For cDNA analysis, sequencing was done for the whole coding region and ~200bp upstream and downstream regions. For genomic analysis, sequencing was done for all the predicted exons and ~100-150bp of the intron-exon boundary. These sequencing methods should in theory identify mutations that affect both the coding sequence and splicing of the protein. In six of the genes, the sequence of the coding regions was identical to the one reported on Ensembl and NCBI genome browsers. However, in Fam105b, I identified a T>A transversion mutation in the 3rd exon corresponding to T285A substitution of the cDNA sequence (Figure 11C) This mutation is only found in animals carrying the gumby mutation, and not in unaffected homozygous mice, C57BL/6 or the C3H/HeJ reference strains. At the protein level, this T>A transversion results in a nonconservative substitution of tryptophan (W) at position 96 to an arginine (R). I will further refer to the mutation, depending on the context as either c.T285A, when referring to the nucleotide change or p.W96R, when referring to the mutated amino acid.

3.4 Literature analysis of the Fam105b gene

Fam105b sequence contains a 1,056 bp open reading frame (ORF) that is predicted to code for a protein of 352 amino acids, with a molecular weight of 40.3kDa. Analysis of the cDNA sequence by BLAST, PROSITE and PSORT programs revealed no significant matches in the protein database and no known motifs that could predict its function. The ProtParam program predicted a soluble protein with a 5.5 isoelectric point (pI), average hydropathicity of -0.534 and aliphatic index of 78.49. NetPhos predicts 6 Serine, 4 Threonine, and 2 Tyrosine phosphorylation sites.
with scores above 0.9. Sequence comparison of Fam105b orthologues in chordates revealed a high evolutionary conservation, exemplified by 58% mouse-chicken, 61% mouse-Xenopus, and 94% mouse-human, percent identity. The mutated tryptophan and the sequence surrounding it are also highly conserved between all sequenced species (Figure 11D), suggesting that the role of Fam105b is likely conserved, and that the mutation likely has a functional significance.

Figure 11: T to A mutation in Fam105b changes a conserved tryptophan to an arginine

(A) Comparison of the deduced amino acid sequence of mouse and human Fam105b genes. Identical amino acids are shaded in yellow. (B) Fam105b genomic structure. Fam105b is composed of seven axons, and spans 24.77Kb on mouse chromosome 15. (C) Sequencing traces from +/+ and gumby/gumby embryos showing a T to A transversion that changes tryptophan (W) to an arginine (R). (D) Alignment of Fam105b protein sequence from several vertebrate species.
The mutated tryptophan residue is conserved in all *Fam105b* genes sequenced to date. In all panels, the mutation is marked by an asterisk.

To identify the mutation without the need for direct sequencing, I designed a PCR genotyping assay that can distinguish a single nucleotide difference, based on the method called tetra-primer ARMS-PCR described in [150]. This method employs two primer pairs, outer and inner, to amplify, respectively, the two different alleles of a SNP in a single PCR reaction. Schematic presentation of this method and the results of Fam105b-ARMS-PCR analysis are depicted in (Figure 12C,D)
Figure 12: Genotyping \textit{gumby} embryos.

(A) Examples of PCR assays used for mapping and genotyping the \textit{gumby} mutation. To genotype with the marker D15Mit18, PCR products were separated on denaturing polyacrylamide gel, transferred to a nitrocellulose membrane, and hybridized with gamma $^{32}$P-labeled forward primer. To genotype with the marker D15Mit111, PCR products were separated on a 4% NuSieve agarose gel. PCR products were digested with \textit{PvuII} to score the RFLP corresponding to SNP rs13482490. (B) Schematic representation of the tetra-primer ARMS-PCR method used to genotype carriers of the \textit{gumby} mutation (T$>$A substitution). Adapted from Ye \textit{et al}, 2001 [150]. Two allele-specific amplicons are generated using two pairs of primers, one pair (indicated by purple and red arrows) produce an amplicon representing the T allele, and the other pair (indicated by indigo and green arrows) produce an amplicon representing the A allele. Allele specificity is conferred by a mismatch between the 3’-terminal base of an inner primer and the template, and to enhance allele specificity, a second deliberate mismatch at position -2 from the 3’-terminus is also introduced into the inner primers. (C) Results of tetra-primer ARMS-PCR used to genotype the T285A mutation. PCR products corresponding to A and T amplicons were separated on a 3% agarose gel.

3.5 Analysis of \textit{Fam105b} expression and localization

3.5.1 \textit{Fam105b} is expressed in the embryo and adult mouse tissues

Expression of \textit{Fam105} mRNA was first examined by Northern blot analysis of total RNA isolated from embryonic and adult tissues (Figure 13A). \textit{Fam105b} specific cDNA probe detected a single 1.4kb transcript, matching the predicted size of full \textit{Fam105b} cDNA. Expression was examined and detected in E9-E14.5 embryos, as well as in the yolk sac and the placenta. In the adult, \textit{Fam105b} transcript was detected in all tissues tested except for the muscle. To examine \textit{Fam105b} protein distribution, tissue extracts from several adult organs were analyzed on a Western blot using \textit{\alpha}-\textit{Fam105b} polyclonal antibody (Figure 13B). A doublet at ~40kDa, matching the predicted size of \textit{Fam105b}, was detected in all tissues analyzed. An additional band at ~50kDa was repeatedly observed in the heart, and remains to be characterized further in the future. Based on the combined results of Northern and Western blots, \textit{Fam105b} is widely expressed in embryos and extraembryonic tissues. In adult animals, the highest \textit{Fam105b}}
expression is observed in the liver, kidney, ovary and testis; lower expression is observed in the brain, heart and the muscle.

**Figure 13: Northern and Western blot analyses of *Fam105b* expression.**

**A** Northern blot on total RNA from embryos and mouse tissues hybridized with *Fam105b* cDNA probe. A single *Fam105b* transcript of ~1.4Kb was observed in all tissues analyzed, except for the muscle. GAPDH probe was used as an internal control for sample quality. **(B)** Western blot analysis of adult mouse tissue lysates using α-Fam105 antibody. A close doublet at ~40kDa was observed in all tissues analyzed. In the heart, an additional band of ~50kDa was detected. α-Tubulin was used as a loading control.

### 3.5.2 Whole mount *in situ* hybridization analysis of *Fam105b* in mouse embryos.
Northern and Western blot results suggest that *Fam105b* is expressed at embryonic stages relevant to the *gumby* mutation. To test whether *Fam105B* is expressed in regions affected by the *gumby* mutation, I performed whole-mount *in situ* hybridization (WISH) in E9.0-E11.5 embryos using a 600bp probe corresponding to position 459-1059 of *Fam105b* cDNA sequence (Figure 14). *Fam105b* mRNA message was first detected at E9.0, at which stage the expression was confined to the developing vasculature, including the dorsal aorta, outflow tract (OFT) and the intersomitic vessels (ISVs) (Figure 14A,D). Between E10.0 to E11.5, in addition to expression in the vasculature, signal was also detected in the developing heart (Figure 14B,C,F), distal branchial arches (Figure 14C,F), and in the telencephalon (Figure 14B,C). In addition there was expression in a stream of cells that resembled migrating neural crest cells at the level of rhombomere (r) 4 and r6/r8 (asterisks in Figure 14B,C), although additional markers are required to confirm this. Detailed analysis of 200μm vibrotome sections prepared from stained embryos revealed staining in all the major blood vessels including the dorsal aorta, ISVs (Figure 15A,E), aortic arches (arrows in Figure 15E), and cranial vessels (Figure 15B), as well as staining of the heart myocardium (Figure 15E) and the inner layer of the telencephalon (Figure 15F). *In situ* hybridization was also performed using a 897bp cDNA probe (corresponding to bp 162-1059), and a 297bp probe (corresponding to bp 162-459). These probes produced an expression pattern similar to that described above, but the background level was higher (data not shown), suggesting that our probes are sensitive, specific and have little cross reactivity. *In situ* hybridization with control sense probe produced minimal background staining (Figure 16).
Figure 14: Analysis of Fam105b expression by whole-mount in situ hybridization

Whole-mount in situ hybridization (WISH) was performed on E9.5 (A,D,E), E10 (B), and E11.5 (C,F) embryos to analyze the expression pattern of Fam105b. (A,D,E) At E9.5 Fam105b was found in the developing vasculature, including the dorsal aorta, intersomitic vessels, and the outflow tract. Rectangles in (A) depict fields magnified in (D) and (E). In addition to vascular expression, between E10.0-E11.5, signal was also detected in the heart (B,C,F), in the first and second branchial arches (C), and in the stream of cells that resemble migrating neural crest cells (asterisks in B,C). (F) Representative image of a cleared and bisected E11.5 embryo showing staining in the distal mesenchyme of the first branchial arch (arrow in F), outflow tract, and the heart. 1,2; branchial arches 1 and 2; a, atrium; CV, cardinal vein; DA, dorsal aorta; ISV, intersomitic vessels; OFT, outflow tract; t, telencephalon; v, ventricle.
Figure 15: Vibrotome sections of embryos processed for whole-mount in situ hybridization

Several embryos processed for WISH were sectioned sagitally 200µm thick to show Fam105b expression in more detail. Representative sections from E10.5 embryos are shown at para-sagital (A-C) and sagital (D-F) levels. (B,C) and (E,F) are magnified from (A) and (D) respectively (boxed area in D is shown in F). Note the expression in the cranial vessels (B), intersomitic vessels (C) the aortic arches (arrows in E) and in the telencephalon (F).

Figure 16: Whole-mount in situ hybridization using the sense probe

Whole-mount in situ hybridization using sense Fam105b probe was done on E9.5 (A), E10.5 (B) and E11.5 (C) mouse embryos. In all stages, background staining was minimal.

These findings show that Fam105b expression is consistent with the temporal and tissue specific deficits of gumby embryos. Specifically, expression in the branchial arches is consistent with the
characteristic reduced 1st branchial arch of *gumby* mutant >E11.0, while expression in the heart and vasculature, correlates with vascular hemorrhaging and engorged hearts often observed in these mutants.

### 3.5.3 Generation and evaluation of Fam105b specific antibody

To analyze the expression and localization of Fam105b protein, I developed a polyclonal anti-mouse Fam105b antibody in rabbits (see Materials and Methods Section 2.11). To test the antibody, Neuroblastoma 2A (Neuro2A) cells were transfected with plasmid encoding Fam105b-GFP fusion protein, and cell lysates were analyzed by Western blotting with α-Fam105b and α-GFP antibodies for comparison. α-Fam105b antibody recognizes a ~70kDa Fam105b-GFP band, also recognized by the α-GFP antibody, and a smaller doublet at ~40kDa, matching the predicted size of Fam105b endogenous protein (Figure 17B). To test for antibody specificity, Fam105b antibody was preabsorbed with either GST-Fam105b or GST alone, prior to hybridization. The signals corresponding to endogenous and transfected proteins were fully eliminated by preabsorbing the antibody with GST-Fam105b fusion protein, but not GST alone, suggesting that the generated antibody is specific to Fam105b (Figure 17B).

To assess the immunoprecipitation ability of the α-Fam105 antibody, I performed a pull-down experiment using cell lysates from Neuro2A cells transfected with Fam105b-GFP fusion construct. Immunoprecipititates were run on a Western blot, and probed with α-GFP antibody (Figure 16C). A single band corresponding to Fam105b-GFP was detected in the input and pull-down lanes, but not in lanes from control experiments: (i) omitting the antibody, (ii) using untransfected cells (Figure 17Ci) or (iii) transfecting empty-GFP vector instead of Fam105b-GFP (Figure 17Cii), suggesting that the antibody can specifically pull-down Fam105b protein in
cultured Neuro2A cells. Other work has demonstrated that the antibody can also pull-down the endogenous Fam105b protein in embryonic lysates (data not shown), and therefore can be utilized in the future to identify and/or confirm potential interactions.

**Figure 17: Western blot analysis of Fam105b antibody**
(A) Fam105b antibody was raised in rabbits against 65kDa GST-Fam105b fusion protein shown here in coomassie stained SDS-polyacrylamide gel. (B) To test the antibody, Neuro2A cells were transiently transfected with Fam105b-GFP fusion constructs (+), and cell lysates were run on the Western blot along with untransfected controls (-). Lysates were blotted for comparison with anti-Fam105b (α-Fam105b) and anti-GFP (α-GFP) antibodies to evaluate the ability of Fam105b antibody to recognize Fam105b-GFP fusion protein. Fam105b antibody recognizes the 70kDa Fam105b-GFP fusion protein, as well a doublet at ~40kDa, matching the predicted size of endogenous Fam105 protein. To test antibody specificity, Fam105b antibody was preabsorbed prior to hybridization with either GST-Fam105b or GST alone. Preabsorbing with Fam105b-GST, but not GST alone, completely eliminated the signal corresponding to endogenous and transfected proteins. α-Tubulin was used as a loading control. (C) Fam105b-GFP proteins were immunoprecipitated with α-Fam105b from Fam105b-GFP transfected (+) Neuro2A cells and blotted with α-GFP. Control immunoprecipitation containing an equal amount of cell lysate and Protein A/G beads but omitting the antibody (Ci), and immunoprecipitation of cells transfected with empty GFP vector (Cii), were also performed.
3.5.4 The *gumby* mutation does not affect expression levels of Fam105b protein

To test whether the mutation affects Fam105b protein levels, embryo lysates derived from E10.5 *gumby* and unaffected littermates were run on a Western blot and probed with α-Fam105b antibody (Figure 18). The size and amount of Fam105b specific bands was comparable between mutant and control embryos. This indicates that the mutation likely has no effect on the overall expression of Fam105b protein.

![Western blot analysis](image)

**Figure 18: Analysis of Fam105b protein levels in *gumby* mutants.** Western blot analysis of embryo lysates from E10.5 *gumby/gumby* homozygotes and +/+ controls using α-Fam105 antibody. The size and amount of Fam105b in the mutant was comparable to unaffected controls. Tubulin was used as a loading control.

3.5.5 *Fam105b* is expressed in vascular endothelial

Whole mount *in situ* hybridization results demonstrated that *Fam105b* is widely expressed in the developing vessels. The walls of vessels are composed of two distinct cell types: vascular endothelial cells (ECs) which form a cell sheath that lines the luminal surface of the vessel, and perivascular cells, including vascular smooth muscle cells (vSMCs) and pericytes, which encircle the outside of the vascular endothelium, providing maintenance and modulatory functions to the vessels [67]. To identify the vascular cell type expressing *Fam105b*, I performed double-immunofluorescence experiments in E11.5 embryonic sections, comparing the distribution of Fam105b with endothelial specific marker platelet endothelial cell adhesion
molecule-1 (PECAM-1) and perivascular markers Smooth Muscle Actin (SMA) and Desmin. Fam105b did not localize with either SMA or Desmin (data not shown). On the other hand, as shown in Figure 19, there was a high degree of co-localization between Fam105b and PECAM-1 (Figure 19), suggesting that Fam105b is expressed in vascular endothelial cells (ECs). Interestingly, the extent of co-localization of Fam105b with PECAM-1 varied between different regions of the embryo. Notably, co-localization was observed in the dorsal aorta, and intersomitic vessels (Figure 19A) as well as in neural tissues such as the neural tube (Figure 19B) and the hindbrain (Figure 19C,D), but not in non-neuronal tissues such as the heart (Figure 19E), or the intestine (Figure 19F). Sections in Figure 19 are taken from E11.5, but, similar results were observed at E12.5 and E15.5. Pre-absorbing the α-Fam105b antibody prior to hybridization with GST-Fam105b (Figure 19G), but not GST alone (Figure 19H) completely eliminated Fam105b signal suggesting that the antibody is specific and does not cross react.
Figure 19: Fam105b co-localizes with endothelial marker PECAM-1 predominantly in blood vessels supplying the neural tissues.

Immunofluorescence was performed to analyze the expression of Fam105b in the vasculature of E11.5 embryo. Embryonic sections were co-stained for Fam105b (red) and PECAM-1 (green). (A) Fam105b is expressed in endothelial cells of dorsal aorta and intersomitic vessels. (B-D) Fam105b is expressed in the vessels that innervate the neural tube (B) and the hindbrain (C,D). Arrows in (B) point to co-localization of Fam105b and PECAM-1 signals. Boxes in (C) are magnified in (D) to show high degree of signal overlap between Fam105b and PECAM-1. (E) Fam105b is not expressed in the vessels in the heart (note the lack of co-localization with PECAM-1 in ‘E merge’). (F) Fam105b is expressed in intestinal epithelial cells, but not in the surrounding vessels (note the lack of co-localization with PECAM-1 in ‘F merge’). (G,H) Controls: Fam105b antibody was preabsorbed with GST-Fam105b (G) or GST alone (H) to assess signal specificity. Representative sections from the hindbrain are shown. Pre-absorbing with GST-Fam105b, but not GST alone, eliminated Fam105b signal (lack of red in H), suggesting that the antibody signal is specific to Fam105b. DA, dorsal aorta; DRG, dorsal root ganglia; ISV, intersomitic vessels.

Another interesting observation was that in some sections, α-Fam105b only labeled a subset of ECs, as only a subset of cells stained for PECAM-1 costained for Fam105b (Figure 20). The nature of these Fam105b-positive ECs remains unknown, but upon closer examination, I observed that cells co-expressing PECAM-1 and Fam105b were often located at the presumptive
leading tips of vessels (arrows in Figure 20B,C) or at the ‘vascular buds’ (arrowheads in Figure 20B,C). Consistent with this, Fam105b expression was highest in the 'roots' of ISVs (i.e. cells that appear to initiate the formation of ISV) in embryos processed for whole-mount in situ hybridization with Fam105b specific probe (asterisks in Figure 15C). Together these data suggest that: (i) Fam105b is predominantly expressed in vascular cells that supply neural tissues, and that (ii) Fam105b is enriched in a subset of endothelial cells – possibly the ‘tip cells’ which are present at the leading edge of vascular sprouts and define the direction in which the new sprout grows. In the future, this hypothesis can be tested in the zebrafish and mouse retinas where this can be assessed more readily.
Figure 20: **Fam105b is expressed in a subset of endothelial cells.**

Several representative images are shown to illustrate the expression of *Fam105b* in a subset of endothelial cells. (A) Low magnification image of sagittal sections of the caudal neural tube of E11.5 embryo. Boxed areas are magnified in (B). (C) A high magnification image taken from a sagittal section of hindbrain of E12.5 embryo. *Fam105b* expression is increased at the presumptive tips of vessels (arrows in B,C) and at the vascular buds (arrowheads in B,C). The drawing of the mouse embryo on the right indicates the regions shown in each of the panels.

3.5.6 **Fam105b is expressed in the kidney tubules and intestinal epithelia**

Fam105b immunofluorescence in sections of mouse embryos at E10.5-E16.5 revealed expression in several other tissues, specifically the kidney, the intestine and the liver (Figure 21). Expression in these tissues was observed starting at ~E11.5, and in all embryonic stages examined afterwards (E12.5, E15.5), as well as in kidney and intestine tissues derived from adult mice (adult liver was not examined) (Figure 21E-H). Once again, the specificity of the signal was confirmed by pre-absorbing the antibody with GST-Fam105b (Figure 21I-L) and GST (Figure 21M-P) peptides.
Figure 21: *Fam105b* is expressed in the kidney, intestine and liver in embryos and adult mice.

Immunofluorescence with α-Fam105b antibody (green in A-D, I-P, red in E-H) was used to examine its expression in sections of E16.5 embryos and adult intestine and kidney. **(A-D)** At E16.5, *Fam105b* is expressed in the kidney (B), intestine (C) and in the liver (D). Low magnification (A) and high magnification (B-D) images are shown. **(E-H)** *Fam105b* is expressed in adult intestinal epithelia (E,F), and in kidney tubules (G,H).  **(I-P)** Controls: Signal is eliminated when α-Fam105b antibody is pre-absorbed with GST-Fam105b (I-L) but not GST alone (M-P) confirming signal specificity. int, intestine; kid; kidney; liv, liver.
Intestinal cells are arranged in a folded sheet known as an epithelium. The bases of the folds, the crypts, contain stem cells and their transiently proliferating daughters. As the cells differentiate, they migrate upward along the lengths of the folds - the villi - to form various cell types that absorb nutrients and perform the epithelium's other functions [181]. A closer examination of the intestine at E16.5 demonstrated that Fam105b expression is highest in the villi, with much lower expression in the crypts (Figure 22A, B). This suggests that Fam105b is predominantly expressed in differentiated intestinal epithelial cells, and not in proliferative stem cells at the crypts. As mentioned previously, Fam105b is not expressed in vascular endothelial cells of the intestine (note the lack of signal overlap between Fam105b and PECAM-1 in Figure 22A, B).

The basic structural and functional unit of the mammalian kidney, the nephron, is composed of the renal corpuscle (consisting of glomerulus and Bowman’s capsule) and the renal tubules. The glomerulus consists of numerous capillaries containing fenestrated endothelial cells that allow the passage of material from the blood vessels into the renal corpuscle. Endothelial cells sit on glomerular basement membrane (GBM), which is lined by podocytes that form a crucial component of the filtration barrier. The glomerulus is enclosed in the Bowmans’ capsule, which collects fluids from the blood in the glomerulus (called “glomerular filtrate”) and passes it along the renal tubules. Renal tubules are specialized for reabsorption and secretion and process the glomerular filtrate to form urine by ultrafiltration [182]. In the kidney of both embryos and adult mice, Fam105b is expressed throughout the kidney tubules (Figure 22C,D) but not in the endothelial cells that surround the kidney tubules, or in the capillaries of the glomerulus (note the lack of overlap between PECAM-1 and Fam105b in (Figure 22C,D).
Figure 22: *Fam105b* is expressed in the kidney tubules and in the intestinal villi. Immunofluorescence with α-Fam105b antibody (*red*) was used to examine in more detail its expression in the intestine and kidney. Representative images from E16.5 intestine (A,B) and adult kidney (C,D) are shown. α-PECAM-1 antibody (*green*) was used to visualize vessels, and DAPI (*blue*) labeling was used to visualize tissue morphology. (A,B) In the intestine, Fam105b labeling is detected primarily in the epithelial cells at the villi, but not in the crypts or in the blood vessels marked by PECAM-1 (A,B merge). Boxes in (A) are magnified in (B). (C,D) In the adult kidney, *Fam105b* is highly expressed in kidney tubules (t). No expression in detected in
the glomerulus (g) or in vascular endothelial cells (note the lack of green and red overlap in C,D merge).

3.5.7 Fam105b is expressed in placental endothelilal and uNK cells

In humans and rodents, the placenta is composed of three major layers: the outer maternal layer, which consists of decidual cells, maternal vasculature and uterine natural killer cells; and two fetal layers - a middle spongiotrophoblast layer and an inner labyrinthine layer, composed of highly branched villi designed for efficient nutrient exchange [183]. Examination of mouse placenta at E11.0 and E13.5 for Fam105b expression revealed staining of fetal endothelial cells within the labyrinthine layer (Figure 23H-M) and uterine natural killer (uNK) cells within the maternal decidual layer (Figure 23A-G). This observation was confirmed by co-staining with Dolichos biflorus (DBA) lectin which has been widely used to identify uNK cells in the mouse placenta, and which at higher concentration, also labels endothelial cells within the labyrinthine layer [184]. uNK are specialized lymphocytes that become transiently activated during placenta development during the first half to two-thirds of the pregnancy. They have been shown to positively regulate decidual angiogenesis, possibly through secretion of pro-angiogenic factors such as VEGF-A, and their misregulation has been associated with reproductive failure and recurrent miscarriages [185, 186]. High degree of overlap was observed between Fam105b and DBA lectin within the placenta at both E11.0 (Figure 23A-G) and E13.5 (data not shown), although as predicted, Fam105b was found solely within the cytoplasm, whereas DBA lectin was found both within the cytoplasm and in the membrane (Figure 23G). At E11.0 Fam105b is expressed in the endothelial cells within the labyrinthine layer, which at this stage of placenta development appear somewhat dispersed, but which can be identified by their co-expression of DBA lectin (Figure 23H-J). At E13.5, Fam105b is highly expressed in the endothelial cells
within the labyrinthine layer which at this stage have formed the characteristic highly branched villi within the labyrinthine layer (Figure 23K-M).
Figure 23: Fam105b expression in the placenta.
Immunofluorescence with α-Fam105b antibody (red) was used to examine its expression in the mouse placenta at E11.0 and E13.5. At E11.0 signal was most pronounced in the maternal layer (m in A, C-G) and in the endothelial cells of the fetal labyrinthine layer (H-J). Boxes in (A) and (B) indicate the fields enlarged in (C) and (D) respectively. (E-G) High magnification images showing high degree of overlap between Fam105b and DBA lectin (green), a known marker of uterine natural killer (uNK) cells, but which also labels endothelial cells within the labyrinthine layer (I). (K-M) At E13.5, Fam105b clearly labels labyrinthine layer endothelial cells which have formed the characteristic branched villi shown here at low (K) and high (L-M) magnification. The drawing of the placenta on the right indicates the regions shown in panels A-M. m, maternal; f, fetal. Except for (K-M), which are taken from E13.5, the rest of the images are taken from E11.0. Controls: α-Fam105b antibody was preabsorbed with GST-Fam105b (N-O) or GST alone (P-Q) to assess signal specificity.

3.5.8 Fam105b is expressed in several neural tissues

Fam105b expression was also analyzed by biotin-streptavidin based immunohistochemistry, as this method is more sensitive, and can sometimes reveal regions of low expression. Using this method I detected Fam105b in several neuronal regions (Figure 24, Figure 25). At E11.5, signal was detected in dorsal root ganglia (DRG) (Figure 24A), and sympathetic chain ganglia (arrowhead in Figure 24B). In the brain of postnatal day (P) 21 mouse, Fam105b signal was
detected in cerebellar purkinje and granule cells (Figure 25C), hippocampal cells (Figure 25E), uncharacterized cells within the hindbrain (Figure 25G), and cells within the cortex (Figure 25I). Even though these data are preliminary, they suggest that *Fam105b* is expressed in a subset of neuronal cell types, and future experiments should clarify their precise identity. Preblocking the primary antibody with Fam105b-GST produces minimal background staining (Figure 24C,D, Figure 24B, D, F,H,J,L).

**Figure 24: Fam105b is expressed in dorsal root ganglia and sympathetic ganglia at E11.5.** Immunohistochemistry using α-Fam105b antibody was performed on sagital (A,C) and transverse (B,D) sections of E11.5 mouse embryo. At E11.5, Fam105b labeled the dorsal root ganglia (DRG) (A), and sympathetic nerve ganglia (arrow in B). Preblocking the primary antibody with Fam105b-GST produces minimal background staining (C-D). BA, 1st branchial arch; DA, dorsal aorta; T, trachea.
**Figure 25: Fam105b is expressed in the adult brain.**

Immunohistochemistry using α-Fam105b antibody was performed on postnatal 21 (P21) sagital mouse brain. (A-J) represents sagital view, (K,L) represents coronal view. Boxes in (A,B) indicate fields enlarged as labeled. Boxes in (I,J) indicate fields enlarged in (G,H) respectively. *Fam105b* is expressed in cerebellar purkinje and granular cells (C), in a subset of cells within the hindbrain (E), in a subset of cortical cells (G,I), and in the hippocampus (K). Preblocking the primary antibody with Fam105b-GST produces minimal background staining (B,D,F,H,J,L). GC, granule cells; PC, purkinje cells.

### 3.5.9 Gumby mutation does not change the cellular localization of Fam105b

In all tissues tested, and Neuro2A cells stained with α-Fam105b antibody, Fam105b appears to be localized to the cell cytoplasm (Figure 26A-C). This is consistent with the lack of a known nuclear localization signal or transmembrane domain in Fam105b protein sequence. To test the effect of W96R mutation on Fam105b cellular distribution, Neuro2A cells were transfected with FLAG-tagged wild-type and W96R Fam105b constructs and processed by immunofluorescence using α-FLAG antibody (Figure 26D,E). The distribution of mutant protein (Figure 26D) in the cytoplasm was similar to wild-type control (Figure 26E), suggesting that the mutation does not alter protein localization. Similar results were obtained using COS7 and NIH3T3 cells (data not shown).
Figure 26: Fam105b is localized to the cell cytoplasm.
The cellular distribution of Fam105b was examined by immunofluorescence using α-Fam105b antibody (green in A, red in B,C). Fam105b is localized in the cytoplasm in Neuro2A cells (A), adult intestine (B) and vascular endothelial cells (C). (D,E) Neuro2A cells were transiently transfected with FLAG-tagged wild-type and W96R Fam105b constructs and the cells were immunostained with α-FLAG antibody (green). Both wild-type (D) and W96R (E) forms of Fam105b show similar distribution in the cytoplasm.

3.6 Analysis of the gumby phenotype

3.6.1 Analysis of Hoxb1, Phox2b, and Sox10 expression in gumby embryos

Gumby homozygous mutant embryos have a hypsprouting facial nerve (cranial nerve VII) at E10.5. To define the cellular events responsible for this phenotype, I performed whole-mount *in situ* analyses with probes for a number of genes known to play a critical role in the specification and development of the facial nerve. Specifically, I analyzed the expression of *Hoxb1*, *Phox2b*, and *Sox10* in gumby mutants at E10.5. The branchiomotor components of the facial nerve (FBM) are born in rhombomere 4 (r4). *Hoxb1*, is expressed selectively in r4, and is a key regulator for the specification and identity of r4 and FBM neurons [26, 27, 187]. *Paired-like homeobox 2b* (*Phox2b*) is expressed in the progenitors of all branchiomotor (BM) and visceromotor (VM) neurons and is required for BM and VM neuron generation [21, 28, 152]. In *Phox2b*-mutant mice, all BM and VM neurons are absent, and, in *Phox2a* into *Phox2b* knock-in mouse line the FBM neurons differentiate but fail to migrate correctly, suggesting a more specific role of *Phoxb2* in FBM caudal migration [188]. As shown in (Figure 27A-D), mutant hindbrain is properly segmented into rhombomeres and the expression of *Hoxb1* and *Phox2b* in the hindbrain is comparable between mutant and control littermates.
The transcription factor *Sox10* is necessary for development of neural crest (NC) derivatives. NC cells migrate from the developing spinal cord to various regions in the embryo, where they give rise to many different types of cells including cranial sensory neurons. *Sox10* is initially expressed in cranial and trunk NC cells and NC derivatives and is later confined to the peripheral nervous system and glial cells [189]. At E10.5 *Sox10* specific signal is found in all sensory cranial ganglia and nerves, and in the trunk dorsal root ganglia (DRG). Overall, as was observed with the anti-neurofilament antibody, all cranial nerves project normally, however, the facial nerve appears to sprout abnormally in *gumby* embryos compared to littermate controls (Figure 27C,D,G,H). This result confirms the observation that the facial nerve hypersprouts in *gumby* mutants, however, since *Sox10* is not otherwise misexpressed, this indicates that the facial nerve deficits are not due to a major defect in NC development.
Figure 27: Expression analysis of Hoxb1, Phoxb2, and Sox10 in gumby embryos.

E10.5 gumby embryos were examined for expression of Hoxb1, Phox2b, and Sox10 using whole-mount in situ hybridization. +/- embryos are shown in the top row, gumby/gumby embryos are in the bottom row. Flat mounted hindbrains hybridized with probes specific to Hoxb1 (A,E) and Phox2b (B,F). Embryos hybridized with probe specific to Sox10 (C,D,G-H) with high magnification images showing expression in the facial cranial nerve (VII) (D,H). Gumby embryos have normal expression of Hoxb1, Phox2b, and Sox10. Note that compared to the facial cranial nerve of +/- embryos (D), the facial cranial nerve of gumby embryos appears 'hypersprouty' (H). DRG, dorsal root ganglia; OV, otic vesicle; r4, rhombomere 4. Cranial ganglia (trigeminal, v; facial/vestibuloacoustic,vii/viii; glossopharyngeal, ix., and vagus, x) are marked.

It remains unclear how Fam105b deficiency translates into the facial migratory deficits observed in gumby mutants. However, these findings and the observation that gumby mutants only show a subtle defect in facial nerve migration, argue against a major defect in hindbrain patterning or cranial nerve development. Instead, it is more likely that deficits in signals responsible for the
targeted guidance of the facial nerve through its environment (i.e. loss/gain of important
guidance cues, and/or ability to respond to them) account for the facial nerve phenotype of
gumby mutants.

3.6.2 gumby mutants likely die because of vascular deficits

3.6.2.1 Embryonic and extraembryonic morphology of gumby embryos at E10.5-E11.0

Lethality at mid-gestation often results from abnormalities in extraembryonic tissues, and/or
defects in cardiovascular development and function [190]. Freshly dissected yolk sacs from
mutant E10.5 embryos were overall comparable to wild type controls, with clearly visible
vitelline vessels, and well-organized vascular networks (Figure 28), although we could not rule
out subtle vascular abnormalities, which might be uncovered by more sensitive assays such as
PECAM-1 immunohistochemistry.
Figure 28: Yolk sac appears overtly normal in gumby mutants.

Bright-field images of freshly dissected yolk sacs from E10.5 control (A,C) and mutant (B,D) embryos showed pervasive vascularization by highly branched vessels in both cases. (C) and (D) are high magnifications of boxed areas in (A) and (B) respectively.

Serial histological examination showed a seemingly normal development of the placenta. The thicknesses and morphology of the labyrinthine, spongiotrophoblast, giant trophoblast and maternal decidua layers were similar in normal and mutant embryos, with proper innervations of fetal (arrowheads in Figure 29C,D) and maternal vessels (asterisks in Figure 29C,D). However, this assay might not be sensitive enough to exclude more subtle placenta defects, and in light of Fam105b expression in the placenta, it could be beneficial in the future to undertake a more thorough investigation of placental development of gumby mutants.
Figure 29: Placenta appears normal in *gumby* mutants at E11.0.

Hematoxylin-eosin-stained sections of E11.0 placentas from normal (A,C) and mutant (B,D) embryos showed a normal thickness and organization of the maternal layer (m) and fetal (f) labyrinthine layer (ll), spongiotrophoblast (sp) and giant trophoblast layer (gi). (C) and (D) are high magnifications of boxed areas in (A) and (B) respectively. Asterisks indicate maternal enucleated blood sinus; arrowheads indicated fetal nucleated blood vessel.

Examination of serial cross sections through mutant embryos did not reveal any gross abnormalities at E10.5 with normal morphology of the heart chambers, the outflow tract
cushions, and the large vessels including the aorta, aortic arches and anterior cardinal vein (Figure 30). Histological examination of paraffin sections at E11.5 revealed that at this stage gumby embryo is necrotic, and the ventricles in the head are collapsed (Figure 31).
Figure 30: H&E stained *gumby* embryos at E10.5 appear similar to normal littermates.

Hematoxylin-eosin staining of transverse histological sections of +/+ (left column) and *gumby/gumby* (right column) embryos at E10.5. Representative sections are shown in the posterior (top) to anterior (bottom) order. Overall, mutant embryos appear indistinguishable from littermate controls, with normal structures of the branchial arches (A,B) neural tube (C,D), major blood vessels (E,F) and the heart (G-J). ACV, anterior cardinal vein; AS, aortic sac; BA, 1st branchial arch; BP, branchial pouch; CVC, common ventricular chamber; DA, dorsal aorta; LV, left ventricle; PHV, primary head vein; NE, neuroepithelium; RA, right atrium; RV, right ventricle; OFT, outflow track; VS, ventricular septum.

![Image](image_url)

Figure 31: H&E stained *gumby* embryos at E11.5.

A hematoxylin-eosin stained sagittal sections of E11.5 +/+ (A) and *gumby/gumby* (B) embryos. *gumby* embryo appears partially necrotic and its ventricles are collapsed (asterisks in A, B).

3.6.2.2 *gumby* embryos have normal levels of apoptosis or proliferation

At E11.0-E11.5 *gumby* embryos have a slightly hypoplastic first branchial arch (BA1) and smaller heads. To test whether this is caused by decreased cell proliferation, and/or increased cell death, terminal-deoxynucleotide-transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining and PhosphoHistoneH3 immunohistochemistry were performed to detect cell apoptosis and proliferation respectively. Overall, at E10.5, apoptosis and proliferation levels were
comparable between *gumby* embryos and controls (Figure 32). To quantify this observation, I performed cell counts in the 1<sup>st</sup> branchial arch (BA1) and in the head (Figure 32E, H). The apoptotic index was calculated by dividing the number of positively stained nuclei by the total number of nuclei in BA1 and in the head. Because there was a significant difference in the number of apoptotic cells in sagital and para-sagital regions of BA1, I distinguished between these regions when performing cell counts (Figure 32E). Proliferation index was calculated as for apoptosis in the BA1, and in a 50μm region of the telencephalon (Figure 32H). No significant difference was detected between *gumby* and littermate controls, suggesting that at this stage there is no change in apoptosis or proliferation.
Figure 32: *gumby* embryos have normal levels of proliferation and apoptosis.

*gumby* embryos and their littermate controls were examined for apoptosis (A-E) and proliferation (F-H) using TUNEL and PhosphoHistoneH3 assays respectively. (A-D) Representative section of E10.5 *gumby* embryo showing apoptosis at the 1st branchial arch (BA1) (B) and in the head (D). No obvious difference was observed when compared to the BA1 (A) and the head (C) of littermate controls. (F,G) A representative section of E10.5 *gumby* embryo showing proliferation in the BA1 and in the head (G). No difference was observed when compared to littermate controls (F). (E) Apoptotic index of BA1 at sagittal and para-sagittal...
levels, and in the head. (H) Proliferative index of BA1 and a region in the telencephalon. For each assay, mean was calculated from 3 different sections from 3 embryos of each genotype, and *gumby* vs. normal comparison failed to detect significant differences. BA1 and telencephalon regions used for calculations are outlined by white punctate lines and boxes respectively.

3.6.2.3 *gumby* embryos exhibit vascular defects in the head and in the trunk

Mutations affecting blood vessel formation often result in embryonic lethality at mid-gestation. This, in combination with finding strong *Fam105b* expression in vascular endothelial cells, compelled me to examine the developing vasculature in *gumby* embryos. Vascular development was visualized in E10.0 and E11.0 *gumby* embryos and littermate controls by whole-mount immunohistochemistry with endothelial specific marker platelet endothelial cell adhesion molecule-1 (PECAM-1) antibody (Figure 33, Figure 37). The major structures of the vascular system were similar in control and mutant embryos, endothelial cells were present in comparable numbers, and were assembled into tubes, suggesting that vasculogenesis and initial phases of angiogenesis are not affected by the mutation. However, closer examination revealed improper organization of branching vascular networks in the head and in the trunk. Notably, in the medial region of the head of control embryos, several large diameter cranial vessels branch to form a hierarchical vascular network (Figure 33A, Figure 37D). In contrast, in mutant embryos, large vessels in the head appeared dilated, and branching was reduced (Figure 33D, Figure 37E). In the trunk region of control embryos, intersomitic vessels (ISVs) have a highly fasciculated appearance, and between ISVs there is an intricate 'capillary' network that extends and surrounds the neuroectoderm called perineural vascular plexus (PNVP) (Figure 33C, Figure 37G). PNVP forms around the neural tube in response to VEGF and PNVP-derived vessels go on to form the BBB that is critical for proper CNS function in the adult [191]. In mutant embryos, the ISVs appeared more defasciculated (arrowheads in Figure 33E), and extensions between the somites...
(Figure 37H) and the perineural vascular plexus (Figure 33F) were less elaborate. In both the cranial and the trunk regions, many of the primary vessels showed 'blebs' where secondary branches should form (arrows in Figure 33D, arrowhead in Figure 37H). These vascular abnormalities were observed at both E10.0 and E11.0, making it unlikely that the phenotype results from a developmental delay. These findings suggest that Fam105b plays a role in angiogenesis.

These early vascular malformations likely lead to vessel dilation and hemorrhaging observed at later stages of gumby development. Because many mutations affecting blood vessel formation result in the mid-gestation lethality, we hypothesize that these vascular abnormalities largely contribute to the growth retardation and eventual death of gumby embryos, although alternative possibilities cannot be completely excluded.
Figure 33: Branching of vessels in the head and the trunk is impaired in gumby embryos.

PECAM-1 immunohistochemistry was used to analyze the developing vasculature in the normal (A-C) and gumby (D-F) embryos at E11.0. Compared to the complex cranial vasculature of normal embryos (A), the cranial vessels of mutant embryos (D) have thick primary branches and show many ‘blebs’ (indicated with arrows in D), where secondary branches should form. Lateral (B,E) and dorsal (C,F) views of the trunk are shown for each embryo. Compared to the fasciculated intersomitic vessels (ISVs) of normal embryos (B), in mutants (E) the ISVs appear less fasciculated. In addition, perineural vascular plexus, (PNVP) is less intricate in mutants (F) compared to controls (C). PNVP, perineural vascular plexus; VA, vertebral artery.

3.7 Fam105b interacts with Dishevelled 2

In 2005 an interaction between human Fam105B and Dishevelled 2 (Dvl2), was identified in a genome-wide yeast-two-hybrid screen [8]. The mammalian Dvl2 gene is an ortholog of the Drosophila segment polarity gene Dishevelled, a member of the highly conserved Wingless/Wnt signaling pathway. Dvl2-deficient mice present with 50% lethality at early postnatal period.
Mutants are presented with severe cardiovascular outflow tract (OFT) defects including double outlet right ventricle (DORV), transposition of the great arteries (TGA) and persistent truncus arteriosus (PTA) [192]. Between E11.5 and E12.0, the developing OFT (also known as conotruncus) first rotates, followed by the development and fusion of the conotruncal cushions. A type of neural crest (NC) cells called the cardiac neural crest (CNC) cells originate in the dorsal neural tube, migrate into the truncal cushions and participate in the formation of the connective tissue that separates the OFT into mature ascending aorta and proximal pulmonary artery. Expression of CNC cell markers Pitx2 and PlexinA2 was attenuated in Dvl2−/− mutants at E10.5, suggesting that the OFT defects likely result from cardiac neural crest abnormalities. If Fam105b interacts with Dvl2, then similar OFT defects might be present in gumby embryos. Unfortunately gumby embryos die before OFT septa are formed and therefore could not be assessed for OFT defects. However, because it appears that the OFT defects present in the Dvl2−/− mutants are due to CNC abnormalities, I examined the expression of Pitx2 and Plexin2A in gumby embryos at E10.5 (Figure 34). Pitx2 was detected in the migrating CNCs at the level of the sixth branchial arch and in the outflow tract, and its expression was comparable between mutant (Figure 34C,D) and control embryos (Figure 34A,B). In control embryos, PlexinA2 signal was observed in migrating CNCs at the level of sixth branchial arch (Figure 34E,F) and as two prongs of CNC cells extending into the OFT, rostral to the right ventricle (Figure 34G). However in all gumby embryos tested, this expression was greatly reduced compared to littermate controls (Figure 34H-J).

It is unclear why only Plexin2A, but not Pitx2 expression was reduced in gumby embryos. Expression of Fam105b in the OFT and the downregulation of Plexin2A in gumby embryos
suggests that *Fam105b* may play a role in OFT formation, possibly by regulating CNC migration into the OFT. However, the precise role of *Fam105b* in OFT development is not clear from these experiments and needs further investigation.
Expression of Pitx2 and PlexinA2 was compared between gumby and control embryos at E10.5 by whole-mount *in situ* hybridization. (A-D) Pitx2 signal was detected in the migrating cardiac neural crest cells (CNC) (arrowheads in B,D) and in the outflow tract (arrows in B,D) in control (A,B) and gumby (C,D) embryos. Boxes in (A) and (C) represent enlarged fields in (B) and (D) respectively. (E-J) In control embryos (E-G), PlexinA2 signal was detected in the dorsal root ganglia (DRG) (E), in the cardiac neural crest cells at level of sixth branchial arch (CNCs) (arrowheads in F), and as two ‘prongs’ within the OFT (arrows in G). In gumby embryos (H-J), PlexinA2 expression was dramatically reduced in the DRGs (H) and in migrating CNCs (arrowhead in I, arrows in J) compared to littermate controls. Boxes in (E) and (H) represent enlarged fields in (F) and (I) respectively. Hearts in (G) and (J) are dissected from (E) and (H) respectively. (n=3) for each genotype
Chapter 4: Functional confirmation of the *gumby* gene and other genetic analyses of *Fam105b*.

4.0 Overview

Association of expression patterns with mutant phenotypes and detecting mutations in the gene provide circumstantial evidence, but they are not sufficient to prove that a gene is responsible for the genetic defect. To confirm that the correct gene has been identified, a variety of genetic experiments can be employed, each with its own strengths and weaknesses.

The use of transgenic technology to rescue a mutant phenotype provides a powerful means to prove that a candidate gene is indeed responsible for a particular mutant phenotype, particularly in cases where a partial or complete loss-of-function scenario is suspected. Recently, many transgenic resources have become available, and great progress has been made in generating transgenic mice by insertion of large DNA segments such as Fosmids, BACS, and even YACS, thus making such studies truly feasible. The caveats for transgenic rescue include: (i) if the allele of interest is neomorphic/gain-of-function, rescue might not occur or be incomplete and, (ii) the transgene must provide the gene with sufficient regulatory sequences to guarantee correct expression of the wild-type gene compliment. Because *Fam105b* regulatory regions have not been characterized, this is based on an educated guess.

The causative nature of the candidate gene can be further strengthened if similar phenotypic manifestations are observed in multiple mutant alleles of the gene of interest. These alleles may be generated via various reverse genetic approaches, such as ENU mutagenesis and RNAi-based gene silencing. The advantages of these experiments are: (i) non-complementation analyses with these additional mutations will add further support to the W96R-*Fam105b* being causative of the
**gumby** phenotype, and (ii) because these series of alleles might differ in certain parameters, they can be useful for further genetic and biochemical analyses. The caveats include: (i) if the W96R-Fam105b mutation affected other functions of *Fam105b* than the new *Fam105b* allele or if the mutation is neomorphic/gain-of-function, the results might be difficult to interpret and, (ii) these experiments are time consuming, and generally require multiple mouse lines to confirm that phenotypic effects observed in RNAi or ENU experiments are indeed due to the specific silencing or mutations of the targeted gene.

Thus, to validate that *Fam105b* indeed is the *gumby* gene, and to generate additional *Fam105b* alleles that can be utilized in future experiments, I performed transgenic rescue experiments, generated a new allelic series by reverse genetics, and attempted knockdown of *Fam105b* in ES cells using RNAi technology. These experiments are described in detail in this chapter.

### 4.1 Rescue analysis of *gumby* mutation

#### 4.1.1 Attempting Fosmid rescue of *gumby* mutation

I first attempted to rescue the *gumby* mutation by generating transgenic mice carrying a Fosmid containing *Fam105b*. Like bacterial artificial chromosomes (BACs), Fosmids are based on low-copy F episomes propagated in recombination deficient *E.coli*. Smaller than BACs, Fosmids carry on average ~40Kb of genomic DNA. They are therefore easier to manipulate, allow linearization before being injected into oocytes, and should in theory result in a higher proportion of transgenic carriers and intact transgenic insertions. From 20 animals screened, two transgenic male carriers were identified and crossed to FVB/N females for line maintenance. Unfortunately, both males did not transmit the transgene to their progeny, likely because of mosaic transgene insertion, therefore precluding further analysis.
4.1.2 BAC rescue of *gumby* mutation

To compliment the Fosmid analysis, I also performed rescue experiment with a bacterial artificial chromosome (BAC) bMQ-396D3 (Figure 35). This BAC was chosen for rescue experiments because: (i) It has large enough flanking regions to likely include all the necessary regulatory sequences for proper gene expression; (ii) *Fam105b* is the only intact functional gene present within this BAC (it also carries 7 out of 8 axons of *BC052328*) which allows us to make unambiguous statements about gene rescue; and (iii) It allows the identification of BAC carriers using SNPs between the BAC, which was generated using genomic DNA from 129Sv/J mice, and the C57 strain, on which the *gumby* mutation was generated. This BAC was injected into (C57BL/6 X SJL)F2 mice, and four transgenic carriers were identified.

![Figure 35: Schematic representation of BAC bMQ-396D3](image)

*Figure 35: Schematic representation of BAC bMQ-396D3*

The 85,128bp BAC starts 6,229bp downstream of *Fam105b* exon 7, and ends 54,207bp upstream of exon 1. In addition to full *Fam105* sequence, the 3' end of the BAC also contains the first 7 exons (from a total of 8) of the BC052328 gene, and no genes 5' to *Fam105b*. ‘SNP’ represents indicates the location of rs31960310, used to identify transgenic carriers.

Three out of four founders (BAC Lines 1-3) had germ-line transmission with 10-20% transmission frequency suggesting mosaicism of the germline. G1 progeny were used for rescue breeding according to the scheme outlined in Figure 36. Before I could assess rescue I had to overcome several constraints in my ability to identify mice that are *gumby/gumby*;BAC. Normally, *gumby* mice are identified by the ARMS-PCR assay described above. This assay distinguishes between the wild-type 'T' allele and the mutant 'A' allele at ORF bp 285. However,
because the wild-type ‘T’ allele carried on the BAC masks the ‘A/A’ homozygosity at the endogenous *gumby* locus, I could not distinguish between *gumby/gumby*:BAC and *gumby*/+:BAC using this strategy. Instead I had to use a closely linked marker rs13482490, which is polymorphic between the C57 and C3H strains used to generate and to outcross the *gumby* mutation respectively. However, because BAC transgenics were also generated on a C57 background, I needed to find a way to distinguish it from the C57^gum^ carrier strain. To get around this, for each BAC line I performed a two generation cross, and generated animals that were C57^gum^/C3H^+^;BAC which were then intercrossed and their progeny were scored for rescue.
Figure 36: BAC rescue breeding strategy.

(A) Representation of the breeding strategy used to test whether BAC transgenic mice can rescue the gumby phenotype. For BAC Lines 1-3, two generation crosses were required to generate gumby heterozygous mice carrying the BAC transgene, with wild-type ‘T’ allele carried on the C3H strain, and mutant ‘A’ allele carried on the C57 strain (G2:C57\textsuperscript{gum}/C3H\textsuperscript{T};BAC). Presence of BAC was determined by SNP rs31960302 (black) that distinguishes between BAC 129Sv/J (129) strain and endogenous C57/C3H strains. The genotype at the Fam105b-bp285 locus was inferred from a closely linked SNP rs13482490 (green/blue). When scoring progeny from G2...
intercross at >E13.5, a mouse that is viable and has a genotype C57/C57;BAC indicates rescue of the *gumby* phenotype. Numbers beside mouse icons indicate the expected ratio of each genotype in the G3 progeny. (B) PCR assays used to genotype for rescue. To determine the presence of BAC transgene, PCR products were digested with *Apol* to score the RFLP corresponding to SNP rs31960302. To infer the genotype at the Fam105b-bp285 locus, PCR products were digested with *Pvu*II to score the RFLP corresponding to SNP rs13482490.

4.1.2.1 BAC rescue of *gumby* lethality and vascular deficits

BAC Line 1 was able to rescue two hallmarks of *gumby* embryos - its embryonic lethality and vascular deficits (Figure 37). *gumby/gumby*;BAC rescue animals were recovered at expected numbers (ratio: ~3/16 in a C57<sup>gum</sup>/C3H<sup>+</sup>;BAC intercross). At E14, *gumby/gumby* embryos are nonviable and partially resorbed (Figure 37B). In contrast, *gumby/gumby*;BAC embryos are viable and appear overall indistinguishable from *gumby/+* littermate controls (Figure 37A, C). While no *gumby/gumby* adult mice were ever recovered, *gumby/gumby*;BAC animals of both sexes were born at expected numbers, were fertile and had no gross morphological defects (Figure 37J,K). At E10.5 *gumby* embryos have defects in vascular patterning in the head and trunk regions. Specifically, as evident from staining of endothelial cells with PECAM-1 antibody, in *gumby* homozygous embryos there are fewer vessels in the head (cranial vessels) and they appear dilated compared to controls (Figure 37D,E). In the trunk of control embryos intersomitic vessels (ISVs) branch off and form a 'secondary' network between the somites (Figure 37G). In *gumby* mutants the 'secondary' ISV network appeared less intricate, with fewer secondary branches arising from ISVs (Figure 37H). In contrast, in *gumby/gumby*;BAC embryos the pattern of cranial vessels and capillary network formed between ISVs appeared similar to *gumby/+* and +/+ controls (Figure 37F,I). Together, these results indicate that introducing wild-type *Fam105b* gene into *gumby/gumby* background fully rescued the embryonic lethality and
corrected the vascular abnormalities of these mice and, therefore, confirms that the T285A mutation causes the *gumby* phenotype.

**Figure 37: BAC rescues lethality and vascular abnormalities of *gumby* embryos.**
BAC rescue was scored at E14.0, at adult stage and by PECAM-1 analysis at E10.5. (A-C) At E14.0 *gumby/gumby* embryos are nonviable and partially resorbed (B), whereas *gumby/gumby* embryos carrying the BAC transgene (*gumby/gumby;BAC*) (C) appear normal and overall indistinguishable from *gumby/+* controls (A). (D-I) PECAM-1 stained *gumby/+*, *gumby/gumby* and *gumby/gumby;BAC* embryos at E10.5. Compared to the complex cranial vasculature of normal embryos (D), the cranial vessels of *gumby* mutants (E) have fewer branches, and the vessels appear more dilated (arrow in E). In the trunk, a web of vessels branches off the primary intersomitic vessels (ISVs) in normal embryos (G), but in mutants (H), rather than migrating away from primary vessels at branch points, endothelial cells appear to aggregate at them (arrowheads in H). In contrast, in *gumby/gumby;BAC* embryos the vascular pattern is similar to *gumby/+* controls (F,I). While no *gumby/gumby* embryos are recovered as adults,
gumby/gumby; BAC adult mice (K) were obtained at expected ratios and appeared overall comparable to gumby/+ littermates (J).

### 4.1.2.2 Analysis of Fam105b expression in BAC carriers

BAC insertion number for each founder line was estimated by sequence analysis of the polymorphic SNP marker rs31960302 that distinguishes between endogenous C57 and BAC 129SvJ strains. In a mouse line carrying a single copy of the transgene, the ratio of the 129 specific ‘A’ allele to the C57 specific ‘G’ allele is 1:2. Each additional BAC insertion increases the amount of ‘A’ by 1. Using this method I determined that BAC Lines 2 & 3 carry a single transgenic insertion, and BAC Line 1 carries 8-10 insertions (Figure 38A). This was also confirmed by sequencing across the Fam105b-T285A locus (data not shown).

Transgene expression was evaluated by qPCR using Fam105b specific primers and by a Western blot analysis using α-Fam105b antibody. In BAC Line 1, there was approximately an eight fold increase in Fam105 mRNA expression over non-transgenic control (Figure 38B), consistent with the estimated number of transgene insertions. mRNA levels in BAC Line 2 were not increased. Currently no data are available for BAC Line 3. Surprisingly, Western blot analysis showed no increase in Fam105b protein levels in all three BAC lines (Figure 38C). This suggests the possibility that Fam105b protein levels are tightly regulated posttranscriptionally, by a mechanism that is not yet understood.

For BAC Line 2, after screening 23 adults and 20 embryos, I did not identify any rescue animals, which most likely results from a non-functional insertion of the BAC transgene, consistent with the qPCR results. As of yet, I could not get enough mice from BAC Line 3 to assess its rescue capability.
Figure 38: Analysis of *Fam105b* expression in BAC transgenic mice.
(A) BAC transgene copy number was estimated in three BAC lines (L1-L3) by sequencing across SNP rs31960302 (indicated by an asterisk), which is polymorphic between BAC 129SvJ and genomic C57 and C3H mouse strains. A 2:1 ratio of genomic ‘G’ allele to transgenic ‘A’ allele indicates the presence of a single copy of the transgene (BAC L2 and L3). A 1:4-5 ratio in BAC L1 suggests the presence of 8-10 copies of the transgene. (B) *Fam105b* mRNA levels were measured by qRT-PCR in BAC L1 and L2 embryos at E11.5 (n=3 for each value). Results are shown as abundance relative to internal GAPDH control. Levels of non-transgenic carriers are set to 100%. In BAC Line 1, levels were significantly increased compared to non-transgenic controls (**, p<0.01). (C) E11.5 BAC transgenic embryos BAC were examined for *Fam105b* protein levels on a Western blot. For each BAC Line, BAC carriers (BAC) were compared to non-transgenic littermates (-). Tubulin was used as a loading control. No difference in protein levels was observed in any of the three lines.

4.2 Generation of *Fam105b* allelic series
The analyses of *gumby* mutation and *Fam105b* expression during early prenatal stages have uncovered an important role of *Fam105b* in early embryonic development, and more specifically a role in facial nerve migration and angiogenesis. Our analyses also showed expression of
Fam105b in a wide range of mouse tissues in late perinatal and postnatal periods. However, because gumby mutants die early in gestation, the requirement for Fam105b in later development remains largely unknown.

While our experiments testing the ability of BAC bMQ-396D3 to rescue the gumby mutant phenotype were in progress, we decided to develop an allelic series of Fam105b to test whether other Fam105b mutations would cause a “gumby-like” phenotype and to perform non-complementation tests with these and the W96R-Fam105b allele. This analysis would provide an alternative, independent validation of having identified Fam105b as the true gumby gene. In addition, the allelic series can potentially prove invaluable for examining the full spectrum of biologic roles of Fam105b in embryos and adults and for performing structure-function analyses.

To generate the allelic series, we have established a collaboration with Dr. Yoichi Gondo at the RIKEN Genomic Sciences Center (GSC) to identify additional point mutations in Fam105b by using a gene-based approach. The RIKEN GSC mutagenesis center has prepared a library of genomic DNA and cryopreserved sperm from over 7000 G1 male mice (http://gsk.riken.go.jp/Mouse/main.htm) [193]. Mutations are identified by PCR amplified products of genomic DNA by using the Temperature Gradient Capillary Electrophoresis (TGCE) method (Figure 39A). To enhance the throughput of the screen, I designed and optimized multiplex PCR primers that were mixed in a single PCR/TGCE reaction. Primer sequences and their amplicons are shown in Figure 39B. In total, five new mutations in Fam105b were identified. At present, three of these mouse lines are being tested by Teresa Maclean in our laboratory and so far two mutants phenocopy the angiogenic defects observed in gumby homozygotes.
Figure 39: Generation of *Fam105b* allelic series.

(A) Outline of ENU-based gene driven mutagenesis. C57BL/6 males (G0) were treated with ENU and mated to produce G1 offspring and their sperm were cryopreserved. To successfully screen the heterozygous mutations in target genes, G1 genomic DNA was screened by Temperature Gradient Capillary Electrophoresis (TGCE). Once a mutation was identified, G1 mouse lines carrying the mutation were revived by *in vitro* fertilization or embryo transfer, and bred to generate mice homozygous for the mutation (blue mouse) (diagram obtained from [www.brc.riken.go.jp/lab/mutants/genedriven.htm](http://www.brc.riken.go.jp/lab/mutants/genedriven.htm)). (B) Primers sequences used to screen the RIKEN ENU library for mutations in exons 2, 3, 6, and 7 of *Fam105b*. The four primer pairs were used together in a single PCR reaction. (C) Five mutants were identified, their positions marked by asterisks. Original *gumby* mutation (W96R) is shown in blue.

4.3 Attempting to knock down *Fam105b* using RNA interference technology

To complement our allelic series studies, I have attempted to knock-down *Fam105b* using embryonic stem (ES)-cell-mediated transgenic RNA intereference (RNAi) [157, 158]. This technique has gained popularity over the past few years for several reasons. It is generally faster and more efficient than the conventional knock-down methods because it doesn’t require the construction of complex vectors, and doesn’t rely on homologous recombination. Because tetraploid aggregation results in the production of embryos nearly 100% derived from genetically modified ES cells, it eliminates the need for multi-generation breeding, usually required with conventional knock-down strategies. Although this method has several limitations, if successful, it would have a two fold advantage. First, if RNAi embryos are phenotypically similar to *gumby* mutants it would add additional confirmation of having identified *Fam105b* as the true *gumby* gene. Second, it would allow us to see to what extent the CdCS symptoms can be reproduced in embryos with *Fam105b* levels reduced to ~50%, therefore unraveling the effect of *Fam105b* deletion in the context of CdCS. Three *Fam105b* 'knockdown' ES cell clones with 30-50% wild-type mRNA amounts were identified, and one was used to generate entirely ES-cell-derived
embryos [158]. Unfortunately, during the time of these experiments, the G4 ES cells used to generate tetraploid embryos proved unable to contribute to viable embryos not just in our hands but in all other laboratories. This was presumptively caused by problems with the culturing conditions used by the transgenic facility. Thus, regrettably these experiments were unproductive.
5.0 DISCUSSION

Here I described the molecular and genetic analysis of the mouse mutant *gumby* which was identified in our laboratory in a recessive ENU forward mutagenesis screen for mouse mutants with cranial deficits. Analysis of *gumby* has uncovered a role for the novel, vertebrate specific gene *Fam105b*, in facial nerve axon guidance, blood vessel development and potential roles in Wnt signaling and human Cri du Chat Syndrome.

5.1 The vital role of ENU mutagenesis screen in identifying the *gumby* mutation

Before its application in the mouse, mutagenesis screens using chemical agents such as ENU or Ethylmethanesulphonate (EMS) mutagenesis have been successfully employed with invertebrate model systems such as *C. elegans* and *D. melangaster*, and have tremendously contributed to our understanding of human biology and disease. For example, mutagenesis screens have greatly advanced our understanding of embryonic patterning, organogenesis, and specific cellular functions such as nerve and muscle function. However, because of obvious differences between invertebrates and mammals, not all human traits can be effectively modeled in an invertebrate system.

Although not without its drawbacks, the mouse has become the mammalian species of choice in studying the molecular basis of disease because it is closely related to humans (the two are ~85% identical), and because it is relatively amenable to genetic manipulation.

In 1980s Russel and colleagues [194] conducted extensive ENU chemical mutagenesis studies in mice and many groups have demonstrated that these approaches, accompanied by comprehensive methods to detect and score phenotypes, are feasible, practical and immensely invaluable [4, 7]. Because ENU produces primarily point mutations, it can provide alleles which may affect gene
function in many different ways ranging from complete loss-of-function to partial loss-of-function or gain-of-function. Studying genes represented by these various alleles may reveal a more complete range of gene activity than knockouts. Furthermore, ENU-induced nucleotide substitutions that result in missense mutations that change amino acids, often define functional domains required for protein interactions or biochemical activity.

The first large-scale ENU mouse mutagenesis programmes were launched in 1997, at the Helmholtz Zentrum Munchen German Research Center for Environment and Health (GSF) and at the Medical Research Council (MRC) in the UK. This was quickly followed by similar programmes in RIKEN in Japan and at the Australian National University (ANU). Early in the mutagenesis projects scientists have focused predominantly on dominant screens, and approximately 2-4% of the G1 progeny exhibited some dominant phenotypes during basic screening in the first 18 months affecting most systems/organs of the mouse. The success of dominant screens encouraged many research centers to carry out recessive phenotypic screens as well. Although recessive screens are more laborious, requiring three generations of breeding, there are several advantages to recessive screens. First, ENU induces roughly 1,000-fold more recessive mutations than dominant ones, and second, many human genetic diseases are recessive [195].

Since the sequencing of the mouse genome and the emergence of an arsenal of reagents used in mapping have accelerated genetic mapping of mutations, it has become feasible for smaller research groups to carry out more specialized, phenotype-driven screens such as ours. For example, screens were performed identifying mice with deficits in embryonic morphology at
mid-gestation [7], sensory systems [196], neurological and behavioural abnormalities [197, 198], hematopoiesis [199, 200], immune system [201] and others.

Several genes with diverse functions have been identified in ENU mutagenesis screens, and several of these have led to the identification of specific human disease genes and have yielded a broader understanding of human development and human diseases. For instance, the identification of the \textit{Clock} gene has led to our understanding of the circadian rhythm [202].

To complement forward genetic screens, once the technologies for generating transgenic and gene-targeted or knockout mice have become available, reverse genetics (gene-driven) mutagenesis screens were also initiated. Although, these reverse genetic screens have greatly advanced our knowledge of human development and disease pathogenesis, they are limited by the difficulty of predicting which genes or alleles will affect a particular behaviour. Our knowledge of gene function is often limited and biased by particular scientific assumption. One of the principal advantages of a phenotype based forward genetic screen is the power to precisely select a phenotype(s) of interest and discover gene function in an unbiased manner. This is exemplified by two large-scale screens that incorporated tests for auditory and vestibular functions, and identified mutant mice with deafness resulting from patterning deficits in the developing inner ear. In both cases, the mutant gene was identified as the \textit{Notch} ligand \textit{Jagged1}, for the first time implicating this previously characterized gene in the signaling process that patterns the inner ear neuroepithelium [196, 203, 204]. This demonstrates that the use of such screens can be significantly useful not only in assessing the function of previously uncharacterized genes, but also to reassess the function of genes that might be considered well characterized [204]. In our small-scale mutagenesis screen, after screening only 40 G1 males, we
have identified seven novel mutations that affect cranial nerve development. Further studies have led to the identification of a novel vertebrate gene *Fam105b* that is critical for embryonic development, and has roles in facial nerve development and embryonic angiogenesis. It is likely that in the absence of our screen and subsequent positional cloning of the *gumby* mutation, these *Fam105b* functions would have remained unknown because genetically it would have been very difficult to identify *Fam105b* in any other organism but the mouse, or via other approaches (such as gene-targeting). First, because it is a chordate specific gene, it would not have been identified in any of the non-chordate screens or possibly in the zebrafish screen due to its duplicated genome and partial redundancy of *Fam105b* paralogues. Second, even if it was identified by other approaches, because it has no similarity to any known proteins, and has no previously identified domains, it is unlikely to have been selected as a candidate for further analysis. Although due to its role in vascular development, it could have been identified in a screen for mutants with angiogenic deficits, such screens have not been reported so far in the mouse (a screen for mutations in angiogenesis has been performed in the zebrafish [205].) Finally, it was not identified in any expression based screens, such as microarray screens performed to analyze changes in gene expression in response to VEGF stimulation in myometrial microvascular endothelial cells (MMECs) [206], or human umbilical vein endothelial cells (HUVEC) cells [207], likely because *Fam105b* is not present at high levels in all endothelial cells, and because its regulation appears to be primarily posttranscriptional. Therefore, our results further support the value of such screens in assigning important functions to novel genes and provide further impetus for the use of ENU mutagenesis screens as tools for dissecting the genetic basis of human development.
5.2 The facial nerve abnormalities of \textit{gumby} mutant embryos

In \textit{gumby} mutants the cranial facial nerve has a mildly ‘sprouty’ appearance, although it follows its normal trajectories. Although axonal defasciculation and abnormal sprouting of cranial nerves have been reported in other mouse mutants such as \textit{Neuropilins} and \textit{Sema3A} mouse mutants [50, 52, 117, 118], no cranial abnormalities identical to \textit{gumby} embryos have been identified to date. This suggests that future analyses of \textit{gumby} facial deficits may lead to the identification of novel signaling cascades and/or molecular factors with previously uncharacterized roles in axon guidance. Overall, although I have not been able to characterize the facial nerve deficits in great detail, normal expression of \textit{Hoxb1}, \textit{Phox2b}, \textit{Sox10} and other markers (not shown) suggests that the hindbrain patterning and the initial generation of branchiomotor/visceromotor (BM/VM) neurons is normal in \textit{gumby} mutants. Instead, consistent with the lack of deficits in other cranial nerves or hindbrain segmentation, it is likely that the facial nerve deficits represents either: (i) a cell-autonomous defect specific to facial cranial nerve or, (ii) a defect in local environment thought which the nerve travels (i.e. misspecification of guidance cues). Consistent with the latter possibility, \textit{Fam105b} is expressed in the second branchial arch, but its expression was not detected in the facial cranial nerve at the time of axon migration. However, while in my current experiments I have not been able to find \textit{Fam105b} expression in the facial nerve, this does not exclude the possibility that \textit{Fam105b} might be expressed transiently or in later embryonic stages. Its expression in the facial nerve will be examined more carefully in the future. Alternatively, it is possible that the facial nerve guidance defects are secondary to defects in vascular development. The peripheral nervous system and its vasculature develop coordinately in part through common cues, and often run alongside each other in the body [62, 110]. Expression of
*Fam105b* in the aortic arches presents a possibility where vascular signals help guide the facial nerve to its respective targets, and disruption of this signal in the mutant causes the facial nerve to sprout.

### 5.3 Possible roles of *Fam105b* in angiogenesis

One of the most exciting findings were the expression of *Fam105b* in endothelial cells and the vascular deficits of *gumby* embryos, which together suggest that *Fam105b* plays an important role in embryonic angiogenesis. Given the interaction of Fam105b with Dishevelled-2, and the emerging role of the Wnt pathway as a major regulator of angiogenesis, it is possible that the function of *Fam105b* in vascular development is mediated through its role in Wnt signaling.

Several intriguing observations were made regarding *Fam105b* expression in the vasculature. First, it appears that *Fam105b* is predominantly expressed in endothelial cells within the neural tissues (such as the neural tube and the brain) but not in the endothelial cells of non-neural tissues such as the heart, the intestine or the kidney. There is considerable evidence that although endothelial cells (ECs) display many common features, they also exhibit remarkable heterogeneity, and the endothelium is uniquely adapted to communicate regionally with specific underlying organ tissues [66]. The heterogeneity is most evident at the morphological level, and EC vessel phenotype is classified as continuous, fenestrated or discontinuous. There is also functional heterogeneity as ECs in different organs are required to perform different organ-appropriate functions. CNS ECs interact with astrocytic feet to produce the BBB, a process which has been recently shown to require the canonical Wnt pathway [68]. Of course many questions regarding EC diversity still remain, such as: what is the underlying cause of EC heterogeneity, and how does EC heterogeneity arise? Are there subpopulations of ECs in the
early embryo that could be molecularly defined? [66]. Although, our work is preliminary, the expression of *Fam105b* in neural ECs further supports the heterogeneity of ECs in the embryo, and its analysis in the future may shed new light on novel molecular mechanisms that control this largely uncharacterized phenomena.

Another intriguing observation was the apparent enrichment of *Fam105b* at the presumptive leading edge of vascular sprouts and at vascular buds. The existence of specialized vascular cells at the tips of angiogenic sprouts, the endothelial tip cells (ETC) has been described by several groups [72, 73, 85, 86], primarily through work in the adult mouse retina where the vascular architecture is well characterized and the angiogenic processes are more accessible for monitoring and manipulation [208]. ETCs display morphological and functional features distinct from endothelial stalk cell: ETCs lack a lumen, are not perfused by blood, extend many protruding filopodia, and unlike stalk cells, they migrate but do not proliferate in response to VEGF-A [73]. Several genes are preferentially expressed in tip cells, including *VEGFR2*, *platelet-derived growth factor B (PDGF-B)*, *Dll4*, and *Unc5b*, suggesting that tip cells have a gene expression profile that is distinct from the stalk cells [72, 73, 88, 145]. The pattern of *Fam105b* expression and the observations that *gumby* have reduced vascular complexity, collectively suggest a model where *Fam105b* promotes angiogenesis primarily in neural tissues, although the exact mechanism has not been delineated. In *gumby* embryos, vascular sprouting of cranial vessels is less elaborate and the vessels appear dilated compared to controls. This is consistent with the observation that defects in angiogenic sprouting frequently lead to an increase in vessel diameter, presumably because additional ECs are incorporated in the wall of existing tubes and are no longer directed into new sprouts [209]. Similarly, in the trunk of *gumby*
embryos, there are fewer vessels in the perineural vascular plexus, further supporting the idea that vascular sprouting is reduced. In theory, Fam105b can act by several mechanisms – to induce or regulate tip cell behaviour, to induce proliferation, to promote maturation and stabilization of formed blood vessels, to inhibit regression, or a combination of these. To test these hypotheses, a systematic and comprehensive analysis of Fam105b expression with other vascular markers will be performed in the future. In the mouse embryo, the vascular patterns are complicated and because the embryonic vasculature expands in a three-dimensional manner, the distinction between immature, sprouting and mature vessels is often difficult, especially in embryonic sections. Thus, some future analyses addressing the role of Fam105b in angiogenesis at the cellular level may be performed in other experimental systems, namely the mouse retina and the zebrafish.

There are many advantages of using the zebrafish to study vascular development: its external development and optical transparency makes it ideal for high-resolution imaging studies of vascular development, especially using transgenic strains expressing EGFP in endothelial cells (ex. Fli:GFP); zebrafish embryos receive enough oxygen via passive diffusion to develop normally for several days in the absence of blood circulation, thus allowing studies on the vascular system even upon perturbation of angiogenic processes; and, the structure and developmental of zebrafish vascular system is known in great detail [210].

The advantage of the mouse retina is that unlike mouse embryos, it provides a two-dimensional model of angiogenesis, and the blood vessels develop mainly after birth in a highly reproducible spatial and temporal pattern. In addition, retina vasculature displays all of the morphological hallmarks of angiogenesis (sprouting, branching, fusion, remodeling, and maturation), and
vasculature is accessible both for observation and for experimental procedures using drugs and other agents, making it an excellent platform in which to examine the expression of signaling molecules in relation to each stage of the angiogenic process [211, 212]. Together with the mouse, the study of Fam105b in these experimental systems will likely provide new insights into the precise role of Fam105b in vascular endothelial cells.

5.5 Fam105b and the Wnt pathway

The Wnt signaling pathway induces various cellular responses from cell proliferation to cell fate determination and differentiation, and has been shown to regulate many crucial aspects of embryonic development and adult homeostasis. Its ability to affect such a wide range of biologic activities is in part dependent on the cooperation of Wnt pathway with other factors and signal transduction pathways [213]. In addition, it appears that some molecules act in a temporal and spatial restricted pattern to modulate specific aspects of Wnt signaling. The interaction between Fam105b and Dvl2 suggests that Fam105b might be involved in the Wnt pathway in vertebrates. It is unlikely that Fam105b represents a core element of the Wnt pathway. More likely, Fam105b plays a vertebrate specific role in regulating Wnt-dependant events in a time- and tissue-dependent restricted manner. Alternatively, Fam105b might be a component of the basic biology of the cell that is used by Wnt signaling in the vertebrates, but whose role is not dedicated solely to the Wnt pathway. At this instance we can not exclude the possibility that the Fam105b functions identified in our studies are independent of Wnt signaling. However, based on the current knowledge of the Wnt pathway, and our analysis of Fam105b expression and gumby phenotype, we speculate that Fam105b may be involved in Wnt signaling in several developmental contexts discussed below.
5.5.1 The role of Wnt signaling in the migration of the facial nerve

Based on the *gumby* mutant phenotype, we postulate that *Fam105b* may regulate facial nerve axon guidance and possibly cell migration. Facial branchiomotor (FBM) neurons are born and differentiate within rhombomere 4 (r4). FBM neurons extend axons via the facial nerve between E10.5 and E13.5, concomitantly, commencing at E10.5, neuronal somata migrate caudally from r4 to r6, and finally migrate radially within r6 to form the facial motor nucleus at ~E13.5 [22]. Several lines of evidence point to a role of non-canonical Wnt-PCP pathway in the rostracaudal migration of FMB somata (discussed above).

Because the caudal migration of the FBM somata occurs primarily between E11.5 and E13.5, the early lethality of *gumby* mutants makes it difficult to determine whether *Fam105b* is required for this process. It has been suggested that axon guidance and neuronal migration require similar guidance molecules to transduce the guidance signal [214]. However, the existence of many mutants that only show defects in either axon pathfinding or somata migration [14, 30], and given that axon outgrowth precedes the migration of FBM neurons, it is possible that these processes are not necessarily coupled and may involve different factors. One piece of evidence suggesting that the two might be linked comes from the analysis of *Tbx20* mutant mice [31]. *T-box transcription factor (Tbx) 20* is expressed in the migrating BM/VM neurons, and is required for the rostracaudal migration of FBM neurons, the lateral migration of the trigeminal nerve, and the trans-medial migration of vestibuloacoustic neurons. Its requirement in the FBM migration is associated with its role in positively regulating the PCP signaling pathway which, as mentioned earlier, is involved in FBM migration. Interestingly, in addition to migratory defects of FBM somata present in *Tbx20* mutants, the peripheral motor axon projections of the facial nerve also
display subtle axon guidance errors. Specifically, while the overall pathfinding of the facial neurons appears normal, the distal ends of facial motor axons extend beyond their normal stopping point and are occasionally misrouted and loop back [31]. Defects in both axon guidance and somata migration are also seen upon the loss of Nrp1. Loss of Nrp1 compromised rostracaudal migration of FBM somata, and resulted in the formation of malpositioned FBM nuclei. In addition, the FBM axons (and other cranial nerves) were severely defasciculated [52].

Interestingly, by analyzing $VEGF_{164}$ and $Sema3A$ mouse mutants, it was shown that Nrp1 controls somata migration through its affinity to the $VEGF_{164}$ ligand, whereas FBM axon guidance is controlled through its affinity to the $Sema3A$ ligand [36]. These observations suggest that the migration of somata and axon guidance might be partly linked and warrants further analysis to test whether Fam105b might also regulate the rostracaudal migration of FBM somata possibly via its role in the PCP pathway.

5.5.2 The role of Wnt signaling in angiogenesis

The precise role of Wnt signaling in vascular development is not yet fully understood, however, several molecules of the canonical and non-canonical Wnt pathways have been implicated in the complex series of events that lead to angiogenesis (reviewed in [215, 216]). Although a systematic survey of expression or function of Wnt signaling components in vascular cells has not been conducted, reports from multiple investigators have shown that many Wnt ligands and their Frizzled receptors are present in endothelial and vascular smooth muscle cells, including $Wnt7a$, $7b$, $1$, $3a$, $5a$ and Frizzled receptors 4, 6, and 8 [68, 79, 100, 215, 216], and several Wnt components regulate endothelial cell proliferation and survival in vitro and in vivo. A recent study demonstrated that Wnt/\(\beta\)-catenin signaling is required for the formation of CNS blood
vessels, but not for the formation of blood vessels in non-neural tissues [68]. Although the mechanism is not yet clear, preliminary evidence suggest that Wnt/β-catenin regulates EC expression of the BBB-specific transporter Glut-1, thus suggesting that Wnt/β-catenin might be involved in integrating the formation of neural specific endothelium with BBB development. Although gumby mutants do not appear phenotypically similar to these β-catenin mutants, the expression of Fam105b in endothelial cells within the neural tissue and the specific vascular deficit of gumby mutants further support the idea that Fam105b might be involved in Wnt-related signaling in vascular remodeling, although precisely how Fam105b modulates this process is not yet clear.

The role of PCP in vascular development is less well understood. It is known that endothelial cells express Wnt5a, a member of non-canonical Wnt ligands. Wnt5a has been shown to promote EC proliferation and survival in cell culture, and suppression of Wnt5a by either a neutralizing antibody or RNAi, inhibited EC proliferation and migration [217, 218]. In addition, zebrafish Wnt5 mutant pipetail (ppl), and zebrafish microinjected with Wnt5 antisense morpholino (MO) display disrupted vascular development [219].

Dvl2 has been shown to promote EC proliferation in cell culture [219], but interestingly no vascular deficits have been reported for Dvl2 or other Dvl mouse mutants. This might be due to a functional redundancy between three mouse Dvl3s, demonstrated by the more pronounced cardiac phenotypes of mice deficient in both Dvl2 and Dvl3 [220], and more pronounced skeletal abnormalities present in mice deficient in both Dvl2 and Dvl1 [192].

5.5.3 The role of Wnt signaling in cardiogenesis
**Fam105b** expression in the heart and the outflow tract (OFT) and the attenuation of cardiac neural crest (CNC) marker *PlexinA2* in *gumby* embryos suggest that *Fam105b* may be involved in CNC-dependant events of heart development. Defects in CNC development or migration lead to a spectrum of distinct cardiac phenotypes in mice and humans, including patent ductus arteriosus (PDA) that is a common defect in CdCS. In addition to PDA, CdCS patients often present with anterior septal defects (ASD) and ventricular septal defects (VSD) that have multiple possible etiologies including defects in CNC formation. At present, none of the known genes within the CdCS critical interval have predicted roles in heart development, thus, it is attractive to speculate that a novel gene(s) such as *Fam105b* is responsible for the cardiac manifestations of CdCS patients. This hypothesis is further reinforced by the observation that Wnt signaling plays many critical roles in cardiogenesis, some of which are discussed below.

The heart is formed from two distinct mesoderm populations or "heart fields" that arise from a common origin and express both distinct and overlapping molecular markers. Cells from the first heart field (FHF) contribute to the left ventricle (LV) and atria (A). Cells from the second heart field (SHF) contribute primarily to the cardiac outflow tract (OFT), right ventricle (RV) and atria [221]. As the heart matures, the atrioventricular valves arise from the cardiac cushions (CC), the ventricular septum (VS) arises from myocardium from the left and right ventricles, and the atrial septum (AS) arises by the growth of two septa: the septum primum which grows from the ventral and posterior walls of the atrium and the septum secundum, which forms a ridge on the dorsal and posterior walls of the atria [222-226]. The OFT is developed from SHF cells and the CNC cells. CNCs originate in the dorsal neural tube, migrate and surround the branchial arch arteries, and a subset continues to migrate into the OFT where they produce the connective tissue that
separates the OFT into mature ascending aorta (connected to the LV) and pulmonary artery (connected to the RV) [222-226]. Recent findings in chick, mouse and zebrafish embryos provide evidence that both canonical and non-canonical Wnt signaling pathways play critical roles in various events of cardiogenesis. Several components of the pathway are expressed within the developing heart, and mutations in genes encoding several pathway members in mice and zebrafish have been shown to disrupt cardiac development [83, 84]. The Wnt/β-catenin pathway plays temporally distinct roles during various stages of cardiogenesis including repressing early cardiac specification at pre-cardiac mesoderm, and later inducing the proliferation of SHF derivatives including the OFT, and RV [227, 228]. Subsequently, loss of β-catenin in the heart results in lethality attributed to defects in SHF derivatives: OFT and RV [224, 228-231]. Non-canonical Wnt signaling also plays a critical role in cardiac development. Wnt11 is required for the induction of cardiac tissues through JNK and PKC signaling, and when it is blocked in *Xenopus* embryos, the expression of early cardiac genes is attenuated [223, 232]. In addition to β-catenin and Wnt11 other members of both canonical and non-canonical Wnt signaling cascades have been implicated in OFT development. These include *Vangl2* [84], *Wnt5* [233], *Dvl2* [192], and *Dvl3* [220]. The OFT defects result from defective Wnt signaling in either the heart myocardium, and/or signaling in CNCs.

In the canonical pathway, Wnt/β-catenin pathway directly induces the expression of *Pitx2* in CNCs [234]. *Pitx2* is expressed in the CNCs as these cells migrate from the dorsal neural tube through the third to sixth branchial arches, and *Pitx2*-expressing cells exhibit defective proliferation in both *Wnt1-Cre/ β-catenin* flox [CNC-depletion of β-catenin] and *Pitx2*+/− mice [234, 235]. This leads to a marked diminution of CNC cells arriving in the outflow tract and as a
consequence leads to lack of proper septation in these mutants. OFT deficits of $Dvl2^{-/-}$ are similar to $Pitx2^{-/-}$ and $Wnt1$-Cre/$\beta$-catenin flox mice suggesting that the Wnt/Dvl/$\beta$-catenin→Pitx2 pathway may account for the defects of $Dvl2^{-/-}$ mice; however, this remains to be confirmed, because these defects have also been observed in mice deficient for non-canonical Wnts – $Wnt5a$ and $Wnt11$. $Wnt5a^{-/-}$ mice die shortly after birth from a range of abnormalities, and display prominent PTA, VSD and other less common OFT abnormalities including TGA, and DORV [233]. In these mutants, the expression of $PlexinA2$, which is expressed at the time when CNC cell-derived mesenchyme condenses to form the OFT septum, was significantly reduced, suggesting that a lack of proper contribution of CNC cells to the forming septum may account for the cardiac phenotypes observed in these mutants [233]. The exact mechanism by which $Wnt5a$ could affect CNC migration remains unknown. However, because it is expressed in the OFT mesodermal cells adjacent to CNC at the time of their migration, it is conceivable that it serves to provide local positional information for the incoming CNC that will populate and condense to form the OFT septum. $Wnt11$ deficient mutants exhibit several OFT abnormalities, including DORV, TGA, PTA, VSD and aortic arch artery (AAA) defects [136]. Interestingly, $Wnt11$ is a direct target of canonical $\beta$-catenin→Pitx2 signaling cascade in developing heart, suggesting that the canonical and non-canonical Wnt pathways might integrate to achieve correct OFT morphogenesis [136].

Mutations in $Dvl2$ cause OFT defects that resemble those caused by the ablation of chick CNCs such as the DORV and PTA, and as mentioned previously, in these mutants there is a loss of CNCs markers $Pitx2$ and $PlexinA2$ [192]. These deficiencies are likely attributable to defects in canonical and non-canonical pathways, since similar phenotype have been described in mice
deficient in both canonical and non-canonical components. The attenuation of *Plexin2A* in the OFT of *gumby* mutants suggests that *Fam105b* might function in the septation of the OFT, possibly through a mechanism that involves *Dvl2* and other Wnt components. Early embryonic lethality of *gumby* mutants has precluded the analysis of *Fam105b* deficiency on OFT septation, as this process does not commence till around E11.5. It is unclear why in *gumby* embryos the expression of *Plexin2A* was diminished, but not the expression of *Pitx2*. Given that *Dvls* function at the crossroads of canonical and non-canonical Wnt signaling, it is possible that the phenotype observed in *Dvl2*-knockout mice sample both of these pathways. Therefore it is conceivable that the attenuation of *Plexin2A* and *Pitx2* in *Dvl2* mutants represent these two different mechanisms of *Dvl2* signaling, only one of which is *Fam105b* dependant. Alternatively, *PlexinA2* and *Pitx2* might label different populations of CNCs, only some of which require *Fam105b*. The outflow track septum has three identifiable components: the aorticopulmonary septum, which is most caudal and forms in the aortic sac; a more rostral truncal septum, which forms the partition between the aortic and pulmonary semilunar valves; and the conal septum, which helps divide the proximal aorta from the pulmonary artery and contributes to the ventricular septum. Neural crest derived cells from the branchial arches constitute the aorticopulmonary septum. Two prongs of cardiac neural cells extend into the truncal cushions where these columns organize the cells already present to form the truncal septum. A less well-organized group of cardiac neural crest extends into the conal cushions and produces the septum that divides the conus to form the left ventricular aortic vestibule and the right ventricular pulmonary infundibulum [225]. It is possible then, that the interaction between *Fam105b* and *Dvl2* is required for one of these CNC populations (*Plexin2A* positive), while *Dvl2* alone (with other molecules) is required for the
migration of other subpopulations (Pitx2 positive). Finally, the loss of Pitx2 expression in Dvl2 mutants was not absolute [192], thus, it is possibly that a subtle decrease in Pitx2 expression is present in gumby mutants as well. In the future using more reliable CNC cell markers such as Pax3 and Connexin43 (Cx43) [226, 236] or using Wnt1-Cre/ROSA26 transgenic system that has been used successfully to mark the cardiac neural crest cell population [226] will hopefully provide clearer answers to these questions.

5.5.4 Wnt signaling in intestinal epithelium, kidney tubules, and liver

In the embryo and in adult mice, Fam105b is expressed in the intestinal villi epithelial cells, in kidney tubules, and in the liver, all of which depend on Wnt signaling for their proper development. Canonical Wnt signaling is required for the development and homeostasis of both fetal and adult intestinal epithelial cells [181, 237-239], and Wnt signaling underlies many cases of colorectal cancer [239]. In the intestine, genes activated by Wnt are expressed in the proliferative compartment at the crypts, while genes downregulated by Wnt are expressed in the differentiated cells at the tops of the crypts and in the villi. The emerging model is that Wnt signals, presumably emanating from mesenchymal cells beneath the crypt, maintain crypt cells in a proliferative state. Cells exiting this environment are no longer exposed to Wnts, and differentiate rather than proliferate [239].

In kidney development, several studies have provided evidence for an essential role for canonical Wnt signaling in the induction of kidney tubules, and studies have indicated that Wnt4 is both essential and sufficient to trigger kidney tubulogenesis [240].

Although the direct role of Wnt signaling in liver development has not been investigated, several clues in the literature suggest that Wnt/β-catenin may regulate hepatocyte development and liver
growth. In addition, Wnt/β-catenin pathway has been implicated in at least 30% of hepatocellular cancers and about 70% of hepatoblastomas [241]. It was beyond the scope of this study to examine the precise role of Fam105 in these tissues. However, because of the availability of cellular and tissue-based experimental models (such as liver ex vivo culture system, and the use of isolated kidney tubules), in the future, the analysis of these tissues may offer important advantages to study the functional significance of Fam105b in Wnt signaling. In addition, a conditional knockout mouse that will be degenerated in our laboratory in the future should enable us to switch Fam105b on and off exclusively in these tissues, thus examining the requirement of Fam105b in more defined settings.

It has been known for years that Wnt signaling controls proliferation and differentiation of various cell types during embryonic development. However, it is becoming increasingly clear that Wnts do not simply promote these processes but rather regulate and fine tune them in a cell-type and temporally-specific manner. This is likely mediated in part by nonoverlapping expression profiles of Wnt members themselves, as well as by association with other molecules that help distinguish and specify the cellular mechanisms that function in each Wnt signaling pathway and in each tissue. The identification and characterization of these molecules is therefore essential to fully understand Wnt signaling in normal embryonic development and in disease context. Currently it remains impossible to predict the cellular role that Fam105b might play in the Wnt signaling pathway. However, our findings combined with existent knowledge regarding various roles of Wnt signaling help direct future detailed studies to dissect the role of Fam105b in Wnt signaling pathways in specific temporal and cell-specific contexts.
5.6 Fam105b levels are likely controlled posttranscriptionally

One intriguing observation that was made during our analysis of Fam105b expression in BAC-Line 1 embryos was the distinct regulation of mRNA and protein levels. While mRNA level was proportional to the number of transgenic insertions, the protein level was unchanged even in the presence of an 8-fold mRNA increase. These findings indicate that Fam105b protein levels are tightly regulated, and although the underlying mechanism is currently unknown, it suggests that a threshold level of Fam105b protein is critical for its proper function in the embryo. Interestingly, a recent study that has analyzed the expression of Nrarp in the presomitic mesoderm has demonstrated that despite oscillating mRNA levels, Nrarp protein did not oscillate. In the mouse retina Nrarp differentially regulates Notch and Wnt activity in endothelial stalk cells to promote cell proliferation and inhibit cell regression, and Nrarp deficiency leads to vascular abnormalities resulting from defects in stalk cell formation and extensive vessel regression [86]. Therefore, this could represent a novel regulatory mechanism of Wnt signaling, whereby tightly regulated proteins, such as Nrarp and Fam105b, maintain a proper balance of Wnt signaling in a cell. Having such an additional level of control would be especially important in cardiovascular development, a process that is very sensitive to genetic, environmental and dosage perturbations.

5.7 Using the gumby mouse mutant to study human diseases

Ultimately we would like to use the gumby mouse model to understand human relevant physiological processes that occur during normal development and in disease states. Scientists have come to use the mouse for many diverse purposes: to understand development, to examine specific cellular and molecular mechanisms, and to model human disease. Mouse mutants have
been appreciated for decades, and the development of advanced technologies of mouse genetic manipulations, and recent sequencing of the mouse genome have made the studies of mammalian biologic processes in the mouse truly feasible. Mice have been instrumental in probing the immune, endocrine, nervous, cardiovascular, skeletal and other complex physiological systems that mammals share, especially because mice naturally develop diseases that affect these systems. Many mouse disease models faithfully recapitulate the phenotype of the human disease and in many cases, as for example with motor-neuron smooth muscular atrophy (SMA) the study of mouse models not only helped elucidate disease pathogenesis but also provided insights into therapeutic avenues [242].

Mice models have also provided great insights into the pathogenesis of several known human deletion disorders. Mice harbouring targeted deletions or missing individual genes have contributed to the identification of critical genes that contribute to specific subphenotypes manifested in these diseases. For instance various mouse models have led to the identification of $Tbx1$ as the chief haploinsufficient gene in del22q11 deletion disorder [57], $elastin$ as the gene responsible for the supravalvular aortic stenosis (SVAS) phenotype of Williams-Beuren syndrome [243], and $retinoic acid induced 1 (RAI1)$ as the major gene responsible for the Smith-Magenis Syndrome [244].

Of course, not all human traits and diseases are equally amenable to recreating in mice, and many mouse models have failed to replicate the phenotypes that are seen in human. Often this is because the disease-causing DNA aberrations act by mechanisms that differ between mouse models and human disease. For instance, if human disease is caused by gain-of-function mutation, then a loss-of-function mutation in the mouse would not recapitulate the phenotype. In
addition, specific physiological, biochemical and anatomical differences between the two species render some comparisons extremely challenging or impossible. Often, mouse models reproduce only certain aspects of human disease phenotype, or, they might be more severely affected than human cases, have no clinical phenotype at all, or show deficits that are irrelevant to the human disease. Examples of traits that have been challenging to model using mouse models include human behaviour, and cognitive disorders such as mental retardation. The genetic analysis of these traits have been hindered by a number of factors including polygenic inheritance, locus heterogeneity, gene-environment interactions, the challenge of correlating human behaviour with mouse behaviour, and limitation of behavioural and neurological assays. For instance, it is difficult to identify and to fully assess higher cognitive functions in mice that can be used as a surrogate for human intelligence [242]. In the absence of mouse intelligence quotient (IQ) test, researchers have relied on a few tests that assess certain aspects of cognition and social behaviour such as the Morris water maze, and the resident-intruder test. Many mutant mice have shown impairment in these standard tests, but it is not yet clear how these deficits are related to the mental retardations that is seen in the human diseases [242]. Fortunately, much progress has been made in developing and validating neurological assays such as diverse behavioural and motor tests, and mouse functional magnetic resonance imaging (fMRI) assays, which in the future will likely advance our ability to model neurological disease in the mouse [242].

Another phenotype that has been challenging to model in mice is the craniofacial abnormalities which accompany many human disorders. Interestingly, a characteristic craniofacial phenotype is present in many deletion syndromes, including Williams-Beuren (OMIM 194050), DiGeorge (OMIM 188400), Wolf-Hirschhorn (OMIM 194190), and Cri du Chat Syndrome (OMIM
However, little progress has been made in identifying the genes responsible for the craniofacial phenotypes of these diseases. This is likely due to several reasons. First, obvious anatomical differences between mouse and human faces pose serious limitations in identifying human-like facial dysmorphologies in the mouse. Second, whereas even your average person can readily detect even subtle deviation in the human face, our ability to assess mouse faces with similar sensitivity is limited. Third, even when craniofacial deficits exist in mouse models, they often have low penetrance and/or are background dependant. Finally, the disease phenotypes that cause the highest morbidity are of a higher relevance to disease-oriented research, and therefore in general attract more attention and funding than the craniofacial deficits. Some of these challenges are illustrated with examples of Smith-Magenis syndrome (SMS) and Wolf-Hirschhorn (WHS). SMS presents with a characteristic craniofacial phenotype, including midface hypoplasia, a broad nasal bridge, prognathia, a down-turned mouth and bulky philtral pillars [244, 245]. WHS is characterized by a 'Greek warrior helmet' facial appearance which includes prominent glabella, and hypertelorism. Several mouse models of SMS and WHS manifest several craniofacial abnormalities including short concave, and/or curved snouts and hypertelorism, and domed skull, short ears, and hypertelorism, respectively. However, in both cases these mouse features are very subtle and can only be detected using sensitive technologies such as surface three-dimensional craniofacial scanning and skeletal analyses. In addition, the penetrance of these features is low and highly dependant on deletion size and genetic background [244, 245].

Another feature shared by many deletion syndromes is the occurrence of congenital heart diseases (CHD), although CHDs also occur frequently in non-syndromic forms and constitute a
leading cause of infant morbidity and mortality. Mouse models have been instrumental in both understanding the regulatory mechanisms involved in the patterning and formation of the normal heart, and to identifying some of the mechanisms that lead to heart abnormalities. Some of the genes causing the main forms of congenital heart disease have been identified in mice, and mutations that were first discovered in human heart diseases, such as $TBX5$, have been extensively studied in mice to understand their pathogenesis in humans. Fortunately, in many cases mice carrying mutations in genes that are associated with congenital heart disease closely mimic the human conditions. For example $Thx5$ heterozygous mice accurately recapitulate the heart defects found in Holt–Oram syndrome (HOS) that results from $TBX5$ deficiency. However, for other genes, there are differences in the precise heart defects observed in mice and humans, and in the sensitivity of the phenotype to gene dosage. For example, $Thx20$ deficiency in mice results in heart defects only if the dosage falls below 50%, whereas in humans, $TBX20$ mutations that are predicted to cause a 50% loss of function result in a range of structural defects. These phenotypic differences between human and mice are probably due in part to different sensitivities to gene dosage. These might be partly due to differences in species-specific physiology - such as the shorter gestation time or faster heart rate of mice - or it might be influenced by other genetic factors [222]. Another difference between human and mouse models of congenital heart disease is that humans with defects such as PTA or DORV survive to term, whereas mice with these defects rarely do. Thus, mouse models are of considerable value for research on congenital heart diseases, but intrinsic differences between mouse and human physiology need to be carefully taken into account [222].
In the future we would like to use our mouse models to understand the role of Fam105b in human development and disease, such as CdCS. Although the gumby mouse model has been instrumental for understanding certain functions of Fam105b, there are certain limitations associated with this model that prevent us from fully understanding the molecular requirements of Fam105b in the context of normal development or in the context of CdCS. First of all, its early embryonic lethality precludes the analysis of its functions in adult mice and its complete role in Wnt signaling. Second, because gumby may be a hypomorphic allele of Fam105b, it may not faithfully reproduce the ~50% dosage of Fam105b present in CdCS patients. In addition, as mentioned earlier, it is common to see different sensitivities to gene dosage in mice and humans that often result in different phenotypes in the two species. Thus, several Fam105b mouse models might be required to closely recapitulate the CdCS phenotypes that result from Fam105b insufficiency. For example, Tbx1 has been identified as the main gene haploinsufficient in del22q11DS deletion syndrome. However, the phenotype and its variability are extreme in the human syndrome but are very limited, or absent, in the standard knockout Tbx1 models. Interestingly, by combining two different hypomorphic alleles and producing a mouse with ~20% mRNA dosage of wild-type levels, Zhang and colleagues were able to closely reproduce the phenotype of human patients, in terms of severity and variability [57].

To overcome the limitation of the gumby mouse model, and to advance our analysis of Fam105b, our laboratory, in collaboration with Dr. Yoichi Gondo has identified five additional point mutations in Fam105b. Three of these mice are currently being examined by a student in our laboratory, Teresa MacLean. Teresa is also generating a conditional knockout mouse that would enable us to switch Fam105b on and off exclusively in the tissues and at the time of interest, thus
examining the requirement of Fam105b in later stages of development and in a more defined settings. We believe that these various Fam105b mouse models will provide useful insights into the role of Fam105b in normal development and may help uncover the molecular mechanisms that underlie CdCS abnormalities.

5.7.1 The potential role of Fam105b in Cri du Chat Syndrome

Unlike several other deletion disorders where the genetic causes have been well elucidated, CdCS remains poorly understood. No mouse deletions have been or reported, and most of the genes that map within CdCS critical region are still unknown. In order to elucidate the molecular mechanisms of CdCS pathology, a combination of clinical, molecular and genetic methods is required to advance our understanding and treatment of CdCS. Given the knowledge and tools that are now available, we can expect further analyses in the mouse to help elucidate the molecular mechanisms underlying this disorder in the future.

Patients with deletions of unusual size and/or interstitial deletions have enabled refinement of the critical interval for various CdCS clinical manifestations. Since no CdCS patients without the deletion have been reported, it suggests that haploinsufficiency for a single gene may not explain the full spectrum of phenotypes associated with this disorder. This is further supported by the observation that different chromosomal regions have been assigned for different symptoms.

Although the critical region has been greatly refined in the past decade, the molecular mechanisms responsible for CdCS pathology have not been well characterized. To date, only three genes have postulated roles in CdCS pathology. Telomerase reverse transcriptase (TERT), a rate-limiting component for telomerase activity, has been mapped to the 5p15.3 band, a region implicated in the high-pitched cry. Zhang and colleagues have found that induction of TERT
mRNA in proliferating lymphocytes derived from 5 of 7 CdCS patients was lower than that in unaffected control individuals and that patient lymphocytes exhibited shorter telomeres than age-matched unaffected individuals, suggesting a role of TERT in the CdCS phenotype [246]. Two other genes, Semaphorin 5A (Sema5A), and δ-catenin, which have been mapped to the 5p15.2 band, have been suggested to contribute to some CdCS pathologies, such as mental retardation. This is based on several observations: both are large genes that cover a large portion of the CdCS critical interval; both are expressed early in embryonic development and play a role in guiding axons or migrating neuronal precursors in mice, rats and zebrafish [125, 126, 160, 168, 169, 247]. Hemizygosity of δ-catenin is strongly associated with severe mental retardation of CdCS [169], and δ-catenin homozygous knockout mice showed an increased hippocampal synaptic density and impaired cognitive function, further supporting a critical role of this gene in brain function [167, 168].

Fam105b haploinsufficiency alone is unlikely to cause all the deficits specific to the CdCS profile. However based on our analysis of Fam105b and gumby mutant, we speculate that it could contribute to the craniofacial, cardiac and cognitive aspects of CdCS phenotype, although further analyses are required to substantiate this hypothesis. Fam105b is located within the region implicated in mental retardation, but just outside of the critical interval implicated in craniofacial pathologies. However, the genetic analysis that led to the identification of this critical region produced somewhat inconsistent results between different groups involved in these studies. For example, Church and colleagues have placed the critical region involved in craniofacial abnormalities between 11.0Mb and 15.0Mb on chromosome 5 [159, 248], while Zhang and colleagues have placed this critical interval between 9.0Mb and 11.4Mb [61]. In the
latter case, the results were further complicated by one patient, who had a deletion that included this proposed critical region, but did not have the phenotype. In addition, even if *Fam105b* is outside the critical interval *per se*, since it is possible that this region contains regulatory elements that affect the level and/or timing of *Fam105b* expression, we can not yet absolutely exclude *Fam105b* as a potential candidate for the craniofacial abnormalities. In particular, because *Fam105b* is expressed in several craniofacial components during embryonic development, including branchial arches, cranial vessels and possibly neural crest cells, we hypothesize that *Fam105b* might contribute to the spectrum of craniofacial pathologies of CdCS patients. Interestingly, several clues in the literature suggest that the Wnt pathway may be involved in craniofacial development. First, both canonical and non-canonical Wnt pathways have been shown to regulate the development and migration of neural crest cells - including cranial neural crest cells, which give rise to most of the craniofacial skeleton [249-251]. Second, using two Wnt reporter mice - TOPgal and BATgal, it was shown that the canonical Wnt signaling is active in many craniofacial tissues during mouse development from E8.0 to E15.5 These include the branchial arches, cranial neural crest cells, forebrain, hindbrain, midbrain, otic vesicle, maxillary and mandibular promences [252, 253]. Finally, the most direct evidence that Wnt signaling is a crucial regulator of facial morphogenesis came from the study that showed that disruption of Wnt signaling had a profound affect on the facial development. Specifically, in *Lef1/Tcf4* compound homozygous mice or in mice injected with Wnt antagonist Dkk1, there were dramatic midfacial malformations consisting of hypertelorism and a foreshortened midface [253]. Together this suggest that the Wnt pathway plays a role during craniofacial development, and it is attractive to speculate that *Fam105b* may be contributing to the craniofacial deficits of
CdCS via its role in the Wnt pathway. Future experiments utilizing sensitive technologies such as surface three-dimensional craniofacial scan and skeletal analyses on *gumby* heterozygous mice and additional *Fam105b* mutants might reveal craniofacial anomalies similar to those presented in CdCS patients.

Many prominent CdCS features including neurobehavioral anomalies and cardiac defects have not been well mapped. CdCS patients often exhibit difficulty swallowing and suckling, strabismus, and saliva control problems, which are likely caused by as-yet-undefined deficits in cranial nerve development, and perhaps that of the facial nerve specifically. The visceral component of the facial nerve innervates parasympathetic ganglia to control lacrimal gland activity and salivation. Thus, the facial nerve deficits observed in *gumby* mutants could be responsible for the increased salivation manifested in CdCS patients.

Our preliminary analyses suggest that *Fam105b* is expressed in the developing telencephalon and in several brain regions of adult mice. In addition, the facial axon guidance defects observed in *gumby* embryos suggest that *Fam105b* might have a broader role in axon guidance. Thus it would be important in the future to test whether *Fam105b* might contribute to mental retardation of CdCS patients.

Expression of *Fam105b* in the heart and the outflow tract, and the finding that *Plexin2A* expression was attenuated in *gumby* mutants suggest that *Fam105b* might be involved in some aspects of cardiogenesis, and thus might contribute to the congenital heart defects (CHDs) of CdCS, which largely contribute to the morbidity and mortality of these patients. These CHDs PDA, secundum ASD and VSD, tatralogy of Fallot, and DORV [254]. These defects can result from defective signaling in either the heart myocardium, and/or signaling in CNCs [222]. Wnt
signaling has been implicated in multiple aspects of cardiogenesis, and mutations in genes encoding several pathway members have been shown cause similar phenotypes in mice and zebrafish. This raises the possibility that deletion of Fam105b in CdCS might impair Wnt signaling and as a result cause abnormal heart development leading to CHDs. Therefore, future investigation of Fam105b and the Wnt pathway in CdCS may have important implications for understanding the origin of cardiac deficits in these patients.

Finally, although CdCS is a rare disorder, many of its symptoms are seen in other diseases, such as mental retardation, behavioural and cardiac abnormalities. Therefore, the genes which contribute to CdCS may also play a role in these disorders, thus, understanding the genetic basis of CdCS might lead to the development of more effective treatments of CdCS and perhaps more common disorders.

5.8 Future directions and concluding remarks

Finally, I would like to summarize the major findings and contributions of my PhD thesis and outline future directions. The present research examined the expression and function of a novel, conserved vertebrate gene Fam105b and its hypomorphic mutant gumby. Based on my findings, I propose that Fam105b plays a role in angiogenesis and in the axon guidance of the cranial facial nerve. In addition, Fam105b might function in the highly conserved Wnt pathway, by interacting with Dishevelled-2, and may contribute to the pathology of the human Cri du Chat Syndrome. Thus understanding its functions might in the future allow the development of more effective treatments of Cri du Chat Syndrome and possibly other more common disorders.

In the future, we will be further analyzing the role of Fam105b in angiogenesis, facial nerve development, and cardiogenesis. Although our primary focus will be on the mouse, we will
expand our analyses to include in vitro models, as well as the zebrafish and the mouse retina, the benefits of which have been discussed previously. This work will be complimented by various biochemical studies aimed at dissecting the cellular functions of Fam105b using such experiments as the mass spectrometry and crystallization of the protein to dissect its structure. We are also interested in characterizing the biological relevance of Fam105b-Dvl2 interaction in Wnt signaling, and will be performing in vitro experiments using well established cell culture assays and in vivo experiments using TOPgal (canonical Wnt) reporter mice. Finally, we are in the process of characterizing the phenotype of Fam105b allelic series using diverse assays to examine the effect of Fam105b in angiogenesis, facial nerve formation, and heart development.
### Appendix A - Primers sequences

#### (i) Primers used for mapping the *gumby* mutation

<table>
<thead>
<tr>
<th>Marker</th>
<th>5'→3' Primer sequence</th>
<th>C57 specific allele or amplicon (bp)</th>
<th>C3H specific allele or amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D15Mit130</td>
<td>F:CATATTTTGCAATTTTTAGTAATAGGC</td>
<td>185</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>R:CAACACAGAAATAAAAAGTGAGAGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D15Mit252</td>
<td>F:CTTCAACATTTATCATTTGTCACA</td>
<td>124</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>R:CTTCTGTATTCACAGGGTGCTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D15Mit280</td>
<td>F:TCTCTTTCCCTCTCTATCTCGC</td>
<td>150</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>R:TCTCTTTCCCTCTCTATCTCGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs13482490</td>
<td>F:GGGATGATTTGCTGAAGACTGATGG</td>
<td>A allele</td>
<td>G allele; <em>Pvu</em>II RFLP</td>
</tr>
<tr>
<td></td>
<td>R:TGACTGTTCCTGTCCTCCCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D15Mit18</td>
<td>F:GGGCTAATTGATAATGATTAGTGCG</td>
<td>134</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>R:CCCCATCCAGGTTTCTAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D15Mit201</td>
<td>F:TTTTGGAGTCTTTGTCTTCCTCC</td>
<td>101</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>R:TTGAGTGGTATAATGTTTTGATTTACACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D15Mit111</td>
<td>F:GTTCAGAAGGCAATGTCTGG</td>
<td>167</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>R:GCTCAGTGCTAATCTCTGACTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D15Mit138</td>
<td>F:TTCAATCCCTTTTGCTAAATG</td>
<td>149</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>R:CAAGACCCCTAGATTAGTCTACCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(ii) Primers used for sequencing the genes that map to the *gumby* critical interval

<table>
<thead>
<tr>
<th>Gene</th>
<th>Region sequenced</th>
<th>5'→3' Primer sequence</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ENSMUSG000000726630</strong></td>
<td>Genomic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 1, Exon 2</td>
<td>F:GAGGCGGGCTCGTCCGGCCGC R:GTGGATCGCAGAGCCCGAT</td>
<td>357</td>
</tr>
<tr>
<td><strong>Q8CDT1 (ENSMUSG00000052811)</strong></td>
<td>Genomic</td>
<td>F:ACGTCGTCAGCAGTCTCTTTG</td>
<td>985</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TAAGTTCTCGTGTATGAAACCTTG</td>
<td></td>
</tr>
<tr>
<td><strong>BC052328</strong></td>
<td>Genomic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 1</td>
<td>F:GCGCTCCCACAGTGAGCTT</td>
<td>499</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:ACCCGACCAGCTGTCCCACGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 8 (first half)</td>
<td>F:CTCACTGGTGCCAGGTGA</td>
<td>1080</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GAATGGTAATACACATCTGAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 8 (last half)</td>
<td>F:CAAAGAAGCATTGTAAGAGGAG</td>
<td>1105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:CATCAGAAAGCCTTGATGTTTAC</td>
<td></td>
</tr>
<tr>
<td><strong>cDNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exons 2-5</td>
<td>F:GCATGGAAGGCGCAGAGGC</td>
<td>399</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:CGAAACTGTACTGGTGATCACCAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exons 6-7</td>
<td>F:CTTCAAGCAAGCCCTTCTCTTC</td>
<td>541</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TCAGAAAGTCAGTGATTAAAC</td>
<td></td>
</tr>
<tr>
<td><strong>Fam105b</strong></td>
<td>Genomic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 1</td>
<td>F:AGGTAAGTACTACCGGGCATC</td>
<td>711</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TCTTTCCAAAATTCTACGGCTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 2</td>
<td>F:GAGGAGCCATTCAGGAGGT</td>
<td>377</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:AGACTGGATGTATGAGGAGGAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 3</td>
<td>F:CCTAGGAAGGCAGTGCTCTC</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:CCCTCTCTCTTGAGCTGGTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 4</td>
<td>F:GGAGACATTGGTGCAAGCTGT</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TCAAGCAACATGAGCAGAAAGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 5</td>
<td>F:GGACTCTGTAGATTTGGAACC</td>
<td>598</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:AGTTCCCTGTCATGCACCTAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 6</td>
<td>F:TGGCATGTGGCTTACGCTTCCC</td>
<td>505</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TCAGGAAATGCTGGACAGAAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 7</td>
<td>F:GGAGACTGTGTTGCAGCTAAG</td>
<td>815</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:CAAAAGCAGATAGGAGCTATC</td>
<td></td>
</tr>
<tr>
<td><strong>Q8C9X7</strong></td>
<td>Genomic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 1</td>
<td>F:GAACCCAGACCATTAATTCCAAAC</td>
<td>702</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GTGGAGAGGCAACGATCCAA</td>
<td></td>
</tr>
<tr>
<td><strong>Tiaf1</strong></td>
<td>Genomic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 1</td>
<td>F:CTTCCCTTTCTTCTTTTGTG</td>
<td>657</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TCTCCTTAATGAGAATCCTG</td>
<td></td>
</tr>
<tr>
<td><strong>Q8BQ79</strong></td>
<td>Genomic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 1</td>
<td>F:GGCGCTTGTGGGAACACTGA</td>
<td>626</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:AAAGCCCCAGCTCTTTCTAT</td>
<td></td>
</tr>
</tbody>
</table>
### (iii) Primers used in transgenic rescue experiments

#### BAC rescue

<table>
<thead>
<tr>
<th>Marker</th>
<th>5'→3' Primer sequence</th>
<th>C57&amp; C3H specific allele (genomic)</th>
<th>129SvJ specific allele (BAC)</th>
</tr>
</thead>
</table>
| rs31960302 | F:TTAGGGCTTGCTAGAGCT  
R:CCGTTCTTTGTCTTTCTCTCC | G allele | A allele: ApoI RFLP |

#### Fosmid rescue

<table>
<thead>
<tr>
<th>Marker</th>
<th>5'→3' Primer sequence</th>
<th>C57 specific allele (Fosmid)</th>
<th>FVB Specific allele (genomic)</th>
</tr>
</thead>
</table>
| rs31576805 | F:CCCTTAAGTTGTCTCTGTCTG  
R:TGCAATCTTTCTGATCCCCT | A allele | G allele; HinfI RFLP |
REFERENCES


Appendix B

Generation of a transgenic mouse line expressing GFP-Cre protein from a

*Hoxb4* neural enhancer

Elena Rivkin 1,2 and Sabine P. Cordes 1,2*

1 - Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, 600 University Avenue, Toronto, Ontario, Canada M5G 1X5

2.- Department of Molecular Genetics, University of Toronto, 1 King’s Circle, Toronto, ON, Canada

This work has been published in *Genesis*, Volume 46 (2): 119-124
B.1 ABSTRACT

Here we describe a transgenic mouse line, in which expression of green fluorescent protein fused to Cre recombinase (GFP-Cre) is directed by the Early Neuronal Enhancer (ENE) of *Hoxb4*. In E9.0-13.5 transgenic embryos, Cre activity coincided with endogenous *Hoxb4* throughout the neural tube up to the r6/r7 boundary in the hindbrain, the dorsal root ganglia, and the Xth cranial ganglia. Unexpectedly, Cre activity was also consistently detected in the trigeminal (Vth) cranial nerve, which is devoid of endogenous *Hoxb4* expression. Strong GFP dependent fluorescence appeared slightly later in E9.5-E11.5 embryos, and reflected the later expression pattern expected for Hoxb4-ENE directed expression in the neural tube up to the r7/r8 not r6/r7 boundary. Thus, with the exception of the trigeminal nerve, this reporter faithfully reproduces endogenous embryonic neural *Hoxb4* expression, and provides an excellent reagent for *in vivo* gene manipulations in neuronal *Hoxb4* positive cells as well as the developing trigeminal nerve. This transgenic mouse line should prove especially useful for determining the fate map of neuronal populations arising in rhombomeres 7 and 8 on its own and in combination with the small set of other existing rhombomere-specific Cre recombinase expressing lines.
B.2 INTRODUCTION, RESULTS & DISCUSSION

Many of the dynamic events that establish the stereotypic neuronal architecture and connections of the vertebrate hindbrain and spinal cord occur in a tightly defined interval: from E9.0-E13.5 in the mouse. Initially, integration of anterior and posterior signals establishes the basic rostral-caudal coordinates and directly or indirectly governs expression of early axial patterning genes, such as the highly conserved Hox segmentation genes. In the hindbrain, the initiation of Hox gene expression coincides with the morphological appearance of the eight rhombomeres, which are lineage-restricted units that subdivide the hindbrain along its anterior-posterior axis [1, 2]. Classic lineage tracing experiments that relied largely on lipophilic dye injections into individual cells have shown that each cranial motor nerve arises from distinct rhombomere(s) [3]. However, because rhombomeres are only transiently morphologically apparent and because the hindbrain undergoes dramatic transformations during its development, determining the precise origins and, at times, targets of other hindbrain-born neurons, such as the more widely dispersed and less accessible interneurons of the reticular formation, has been challenging. Needless to say, our understanding of the molecular hierarchies that govern the development of these other neuronal populations is cursory. Recently, recombinase dependent gene activation and inactivation techniques have emerged as powerful tools for resolving such more complex fate mapping problems and dissecting the genetic hierarchies that govern region-specific neural development [4-6].

The Cre/loxP system has been used successfully for in vivo gene manipulations in the mouse [7]. This method relies on expression of Cre recombinase, which mediates irreversible recombination
of loxP site-flanked DNA sequences, most commonly resulting in deletion of such sequences. Any cell tagged in this fashion and its progeny carry the resulting DNA rearrangement permanently, allowing determination of lineage relations between embryonic and adult tissues or analyses of region-specific gene requirements. Modification of Cre by fusing green fluorescent protein (GFP) to its amino terminus allows immediate fluorescent scoring of living cells and transgenic animals, in which loxP-flanked DNA sequences are destined to be removed from the genome [8]. Although GFP-Cre fusion proteins have been tested in embryonic stem cells, they have not been examined extensively in transgenic animals.

At present some neural-specific Cre recombinase mouse lines exist, but additional ones are needed to help us understand neuronal development better. The regulatory regions required for precise early neural expression of several Hox genes have been defined and offer unique tools for region-specific Cre expression. In the mouse, 39 Hox genes are organized in four distinct clusters (HoxA, B, C and D). Analyses of mouse mutants have shown that the combinatorial expression of genes from the HoxA and B clusters controls rostralcaudal identity along the neural tube between E8.0-E10.5 and appears to be most relevant to early neural development, [1, 2, 9]. By contrast, deletion of the entire HoxC gene cluster had only mild effects on overall axial patterning [10]. More recent observations have suggested that, in the spinal cord, the HoxC cluster genes may direct the regional identity of specific motor neuron pools and, thereby, their connectivity with target muscles between E10.5-13 [9]. However, the roles of HoxC genes in determining motor neuron connectivity remain to be confirmed in HoxC gene cluster deletion mutants. And whether HoxC cluster genes play a similar role in the vertebrate hindbrain also remains to be determined. In the vertebrate hindbrain, rostrally expressed Hox genes display
rhombomere-specific expression with anterior limits coincident with specific rhombomere boundaries [1, 2]. So, for example, Hoxb4 expression extends up to the rhombomere 6 and 7 (r6/r7) boundary [11]. Rhombomere-specific regulatory elements for some Hox genes have been defined and present ideal reagents to direct expression of Cre recombinase in specific rhombomeres or subsets of rhombomeres. For example, a regulatory element from Hoxa1 can direct transgene expression in rhombomere 2 [12], while enhancers from Hoxb1 and Hoxb3 can govern transgene expression in r4 and r5, respectively [13, 14]. Similarly, rhombomere-specific enhancers from the earlier acting segmentation genes, Egr2/Krox20 and MafB/Kreisler, that regulate expression of some Hox genes directly, can drive transgene expression in r3+5 and r5+r6, respectively [15, 16]. The regulatory elements from Hoxb2 and Egr2/Krox20 have been used to generate rhombomere-specific Cre-expressing mouse strains, but so far no Cre-expressing lines that can be used to dissect the fates of neurons from the more posterior hindbrain have been generated.

Previous analysis of Hoxb4 regulation identified enhancers capable of directing neural reporter gene expression equivalent to that of endogenous Hoxb4 [11]. Hoxb4 expression can first be detected in E8.5 mouse embryos and peaks between E12.5-E14.5. Hoxb4 is expressed along the spinal cord with an anterior limit of expression coincident with the r6/r7 boundary in the hindbrain, in dorsal root ganglia, the Xth cranial ganglia (vagal nerve), and also in the stomach, lung, kidney, longitudinal muscle layer of the gut, and developing limb buds [17]. Early neural Hoxb4 expression is governed by two enhancers, which are located downstream of the Hoxb4 gene: the Early Neuronal Enhancer (ENE) initiates Hoxb4 expression and that of LacZ reporter genes in the neural tube of 9-10 somite stage embryos (E8.25-8.5) and the Hoxb4 Late Neuronal
Enhancer (LNE) maintains \textit{Hoxb4} expression starting at E9.0 [11]. The ENE has been localized to a 300bp \textit{StuI-SacI} that is sufficient for early neural expression. Interestingly, while endogenous \textit{Hoxb4} and LNE directed expression always extends up to the r6/r7 boundary in the hindbrain, ENE directed expression extends up to the r6/r7 boundary at E8.25-8.5 but recedes to the r7/8 boundary by E9.0-9.5 and is extinguished by E10.5. In the present study, we describe the generation and analysis of transgenic mice, which express GFP-Cre recombinase fusion protein under the control of a ENE element of \textit{Hoxb4} and can act as a lineage tracer for Hoxb4 positive neurons.

To label \textit{Hoxb4} expressing neural cells we used the ENE enhancer combined with the hsp68 minimal promoter to direct expression of the GFP-Cre fusion protein in transgenic mice (Figure 1a). Using standard DNA microinjection into oocytes, we generated one transgenic founder animal, from which the Hoxb4-ENE-GFP-Cre transgenic line was derived. We reasoned that the GFP-Cre fusion protein would allow both robust tracking of enhancer activity in live animals, and precise Cre recombinase activity, which could be harnessed to fate map \textit{Hoxb4} positive neurons and for precisely timed and regionally restricted gene manipulations.

To test GFP-Cre recombinase activity, we crossed Hoxb4-ENE-GFP-Cre mice to the Z/AP reporter strain [18]. In Z/AP mice, \textit{placental alkaline phosphatase (PLAP)} expression can be activated and LacZ expression inactivated in a Cre recombinase dependent manner. In E9.0 - E14.5 embryos doubly transgenic for the Z/AP reporter and Hoxb4-ENE-GFP-Cre, Cre recombinase activity, as assayed by PLAP staining, was compared with endogenous \textit{Hoxb4} expression, as detected by whole-mount RNA \textit{in situ} hybridization. In E9.0-14.5 doubly
transgenic embryos, \textit{PLAP} expression was activated in the developing spinal cord and the hindbrain with a sharp anterior boundary at r6/r7 (Figure 1b,c) coincident with that of endogenous \textit{Hoxb4} (Figure 1d). PLAP staining was localized to the regions in which motor and interneurons reside and also labeled motor neuron projections and contralateral interneuronal projections in E12.5 embryos (arrows and asterisks, respectively, in Figure 1e). Motor neuron projections were also labeled in whole mount embryos beginning at E10.5 (Figure 1b,c,e). In general, PLAP staining in E9.5-E14.5 transgenic embryos corresponded to previously described \textit{Hoxb4} expression [17]. PLAP staining was also present in neuronal projections extending from the posterior neural tube up towards the anterior hindbrain (Figure 1f,g). Specifically, we observed PLAP in the spinal cord, the dorsal root ganglia, motor neurons, and the Xth cranial nerve. Cre recombinase activity was not detected in any non-neuronal derivatives.
Figure 1. Schematic representation of the Hoxb4-ENE-GFP-Cre transgene construct and detection of Cre recombinase activity. (a) The 880-bp Hoxb4 Early Neuronal Enhancer (ENE) and hsp68 minimal promoter were cloned upstream of the GFP-Cre fusion protein and a splice and polyadenylation sequence from SV40 to generate the Hoxb4-ENE-GFP-Cre expression construct. BamHI, B; ScaI, S. Panels (b-i) show detection of Cre recombinase activity. Alkaline phosphatase staining of embryos transgenic for Z/AP and Hoxb4-ENE-GFP-Cre at E10.5 (b) and E11.5 (c) revealed PLAP staining throughout the posterior neural tube with an anterior limit at r6/r7, indicated by an arrow. Within the neural tube, PLAP coincided with endogenous Hoxb4 expression, as detected by RNA in situ hybridization of whole mounts at E11.5 (d). Beginning at E10.5 and especially prominent after E11.5, PLAP staining was present in motor neurons (b, c and e), and in the Vth cranial ganglia (b, c, h and i) and Xth cranial nerves (c). In transverse sections of E12.5 embryos (e), PLAP staining labeled motor and interneurons and their projections. Projections of motor neurons are marked with arrowheads and contralateral interneuronal projections with asterisks. Sagital sections of an E12.5 embryo (f,g) show a distinct staining boundary within the hindbrain, and individual neuronal projections (arrowheads) especially evident at higher magnification (g). The ectopic PLAP staining of the trigeminal (V) nerve was consistently detected starting at E10 (b), can be seen clearly in E11.5 embryos (c) and at higher magnification in (h). In (c, h and i), black arrowheads mark the trigeminal nerve. The transverse section shown in (i) was taken at the level indicated by the arrowhead in (c). Optic cup, oc; Otic vesicle, ov, Rhombomere; r.

We further defined the overlap of Cre activity with endogenous Hoxb4 expression by comparing PLAP staining with Hoxb4 expression detected with an α-Hoxb4 antibody in serial sections of Hoxb4-EN-GFP-Cre; Z/AP transgenic embryos. We found complete overlap of Hoxb4 and PLAP expression in the spinal cord and in the hindbrain with an anterior expression limit at the r6/r7 boundary (Figure 2). This anterior r6/r7 limit of PLAP staining and Hoxb4 expression coincided in all stages examined (Figure 2a,b). In E14.5 sagital sections, both PLAP and Hoxb4 protein were detected in the dorsal root ganglia (Figure 2c,d). On the subcellular level, there appeared to be partial co-distribution of PLAP and Hoxb4, which can be explained on the basis
of protein differences: Hoxb4 is a transcription factor found primarily in the nucleus, while PLAP, as a GPI-linked protein, labels neurons in their entirety.

**Figure 2. Colocalization of Hoxb4 protein and Cre activity in the neural tube Hoxb4-ENEGFP-Cre; Z/AP transgenic embryos.** Hoxb4 protein was detected with a Hoxb4 monoclonal antibody and compared to PLAP expression. In serial sagittal sections of E10.5 embryos, Hoxb4 (a) and PLAP (b) colocalized within the hindbrain, with an anterior r6/r7 boundary. In E14.5 embryos, Hoxb4 (c) and PLAP (d) were also co-expressed in the dorsal root ganglia (DRG). These sections are located just above the hindlimb bud. PLAP also labeled motor neuron projections (d, arrowheads) that could not be detected by antibody staining alone. Rhombomeres 4-7, r4-r7; DRG, dorsal root ganglia.

In addition to predicted regions of *Hoxb4* expression, PLAP expression was also observed in the developing trigeminal nerve of E10.5-11.5 embryos (Figure 1c,h,i). This discrepancy could be caused by insertion site-dependent ectopic expression of the transgene. Alternatively, *Hoxb4* may be expressed too weakly or transiently in the trigeminal nerve to be detected by RNA in situ.
experiments or immunohistochemistry. Thus, except for the Vth cranial ganglia, the Hoxb4-ENE-GFP-Cre reporter line recapitulates endogenous neuronal Hoxb4 activity and can serve as a reagent for conducting in-depth lineage analysis, gene activation and inactivation experiments.

To visualize GFP activity, E8.5-E12.5 transgenic embryos were examined for GFP-Cre expression by conventional fluorescent microscopy. Fluorescence was first detectable at E9.0, peaked at E10.5, and extinguished by ~E12.0. Surprisingly, we could not detect GFP-Cre expression prior to ~E9.0, as we had expected based on earlier promoter analysis [11]. Given that Cre recombinase activity coincided precisely with endogenous Hoxb4 expression and extended up to the r6/7 boundary, this discrepancy can be reconciled by the fact that, in contrast to previously used methods, which involved enzyme amplification steps, GFP fluorescence is a direct, linear readout of promoter activity, and sufficient levels of detectable protein must be present. Perhaps with higher power fluorescence microscopy, GFP expression might be detected earlier. GFP fluorescence was localized exclusively to the neural tube and the hindbrain with a sharp anterior limit between rhombomeres 7 and 8 (r7/r8) at E9.5. The expression at the anterior r7/r8 boundary was maintained through E11.5 (Figure 3), while the posterior boundary expanded caudally from E9.5 to E11.5 (Figure 3a, b marked by asterisks). In transverse sections of E10.5 embryos, the highest level of GFP expression was seen in the central neural tube (Figure 3d). GFP fluorescence was undetectable in all mesodermally derived tissues, including the somites, which express endogenous Hoxb4 [17]. Furthermore, no GFP fluorescence could be detected in sections of adult brains nor could the GFP-Cre protein be detected by immunohistochemistry with an anti-GFP antibody (data not shown). Thus, overall GFP expression correlated well with
previous descriptions of later ENE-directed expression patterns, while Cre recombinase activity recorded the early and later ENE directed expression profiles *in toto*.

![Figure 3. Detection of GFP-Cre by fluorescence.](image)

**Figure 3. Detection of GFP-Cre by fluorescence.** Brightfield and GFP overlay of whole mount embryos (a-c) and a transverse section at the brachial level (d) illustrate GFP localization in the Hoxb4-ENE-GFP-Cre transgenic animals. Lateral (a,b) and dorsal (c) views are shown. In E10.5 (a) and E11.5 (b) animals, GFP-Cre fluorescence showed a sharp anterior r7/r8 boundary in the hindbrain and more diffuse posterior expression that continued to extend more caudally in E11.5 embryos (a, b) and is marked by an asterisk. A transverse section of an E10.5 embryo (d) showed that GFP was confined to the neuronal tissues. Floor plate, fp; Otic vesicle, ov; Somite, s.

Our ENE-Hoxb4-GFP-Cre reporter line now joins a small group of validated mouse lines that express Cre recombinase specifically in a subset of rhombomeres: Cre recombinase is expressed in rhombomere 1 of Wnt1-Cre mice [19], rhombomere 2 of Hoxa2-Cre mice [4, 12], rhombomeres 3 and 5 of Egr2-Cre mice [15], and in rhombomeres 7 and 8 of our ENE-Hoxb4-
GFP-Cre mice. Of these lines, the ENE-Hoxb4-GFP-Cre line is the only one that allows tracking of Cre expression and Hoxb4 expressing cells by GFP expression. Because GFP expression is absent after E12.5 and could not be detected in adult brains of ENE-Hoxb4-GFP-Cre mice, GFP fluorescence from the GFP-Cre protein may well not interfere with the tracking of other GFP-based reporters in later development. Reagents exist to complete this series by generating lines that express Cre in rhombomeres 4 and 6. Thus, together with existing lines, ENE-Hoxb4-GFP-Cre mice should allow fairly complete and precise tracking and fate mapping of derivatives from the posterior hindbrain, as has been done for some regions derived from the anterior hindbrain already [4, 6].

In conclusion, these results demonstrate the exquisite neural specificity of GFP-Cre expression and activity in our ENE-Hoxb4-GFP-Cre reporter mice, and that, with one minor, but potentially useful, exception - the trigeminal nerve, GFP-Cre recombinase expression mirrors the neural expression of endogenous Hoxb4. Taken together, the GFP-Cre protein should allow fate-mapping of and gene manipulations within the transiently Hoxb4 expressing neural tube. This study also indicates that visual detection of GFP fluorescence may be a slightly less sensitive method for detecting GFP-Cre recombinase protein than direct measures of Cre activity itself. And this should be borne in mind in other analyses of transgenic animals expressing GFP or GFP fusion proteins.
B.4 Materials and Methods

B.4.1 Generation of ENE-Hoxb4-GFP-Cre transgenic mice

The 600bp minimal hsp68 promoter was obtained by PCR from the hsp68-LacZ-KSII plasmid [20] by using the following primers: Hsp68F 5’-CTGTCGACAACCGTGCCGAATTCTTAGATCTGTAGTTCTGAACCTTAGCCATGAG-3’ and Hsp68R 5’-CTGTCGACAACCGTGCCAAGCTTTTCGGATCCAGATTTGGTTCTGAGTAGCTGTC-3’, and inserted into the SalI site of the pBluescript KSII+ plasmid (Stratagene). The GFP-Cre fusion gene with SV40-IVS-polyA was digested from GFP-Cre-pCMV vector (provided by Dr. David Threadgill) with AflI and BglII and cloned into the HindIII/BamHI sites of hsp68-pBluescript. An 880bp XbaI/NcoI fragment containing the Hoxb4 early neuronal enhancer (ENE) (provided by Dr. Alex Gould) was cloned into the BglII site of hsp68-GFP-Cre-polyA. The ~3.2kb HoxB4-ENE-hsp68-GFP-Cre insert was purified as a MluI fragment and used to generate transgenic mice by standard pronuclear injection. All mice were maintained in accordance with CACC regulations. One founder animal was identified by Southern blot analysis of genomic DNA isolated from tail biopsies using a GFP-Cre probe and by PCR analysis using GFP-Cre specific primers: GFP-CreF 5’-ACTCTGGTCAGAGATACCTG-3’ and GFP-CreR 5’-TTGAAGAAGATGTTGCGCT-3’.

B.4.2 Scoring GFP expression
GFP expression was directly observed under the Leica DM IRB inverted microscope with a Hamamatsu digital Camera.

**B.4.3 Alkaline phosphatase staining**

Alkaline phosphatase (AP) staining on whole embryos and 16µm cryosections was performed as previously described [18]. At times, E10.5 and E11.5 embryos were cleared in Benzyl Benzoate: Benzyl Alcohol (2:1) before imaging. All pictures were taken using a Leica DM IRB inverted microscope with a Hamamatsu digital Camera.

**B.4.4 RNA in situ hybridization**

The Hoxb4 probe was prepared as described in [21] and RNA in situ hybridization on whole mounts and sections was performed as described in [22] and [23], respectively.

**B.4.5 Immunohistochemistry**

Immunohistochemistry was performed as described by [11] with a monoclonal antibody directed against murine Hoxb4 (I12, Developmental Studies Hybridoma Bank) and with a rabbit anti-GFP antibody (1:2000 dilution; Molecular Probes) and a Cy3-donkey anti-Rabbit secondary antibody (1:250; Jackson Immunoresearch Laboratories).
B.5 Acknowledgments

We would like to thank David Threadgill for the GFP-Cre fusion construct, Alex Gould for the ENE Hoxb4 enhancer containing construct, Corinne Lobe and Andras Nagy for the Z/AP reporter mice, and Sandra Tondat and the SLRI transgenic facility for generation of transgenic mice. This research was funded by CIHR grant #14312 to S.P.C.
B.6 REFERENCES


