Author

Mona Yazdi began her research during her freshman year, eager to take advantage of the opportunity to pursue an interest she had developed early in her life. Intending to move on to medical school after graduation, Mona considers her undergraduate research experience to be a tremendous preparation for her future education. She hopes to be able to make a continuing contribution to the scientific community as she pursues a career as a researcher and physician.

Downregulation of *Nipbl* in the Cardiac Primordium Gives Rise to Heart Defects in *Nipbl* Conditional Mutant Mice

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Abstract

Cornelia de Lange Syndrome (CdLS) is a developmental disorder that affects multiple organ systems and can range in severity from undiagnosed to failure to thrive. Incidence of congenital heart defects in people with CdLS is as high as 20–30%, and these defects can range from minor atrial septal defects (ASD) to more serious forms such as Tetralogy of Fallot, which leads to a mixing of oxygenated and deoxygenated blood in the ventricles due to ventricular septal defects (VSD). A majority of CdLS cases are due to mutation in one copy of the Nipped B-like (*NIPBL*) gene, which encodes a cohesion-associated protein that is conserved among all eukaryotes. In this study we use the FlEx gene-trapping technology to establish conditional alleles of *Nipbl* in mice. Our initial objectives were to establish the *Nipbl FlEx*/+ line and then toggle the *Nipbl* allele *in vivo* from mutant form to wildtype and then back to mutant form using different recombinases. *Nipbl FlEx*/+ mice, with the *Nipbl FlEx* allele in the mutant conformation, displayed many of the phenotypes observed in our original *Nipbl RRS564*/+ line, making it a reliable model for CdLS. Our second objective was to use the *Nipbl FlEx* series of alleles to identify in which tissues heart defects initiate during development. We found that lowering levels of *Nipbl* in the heart leads to large ASDs and VSDs.

Key Terms

- Atrial Septal Defect
- Conditional Mutant
- Cornelia de Lange Syndrome
- Genetic Rescue
- Haploinsufficiency
- Recombinase
- Ventricular Septal Defect

Faculty Mentor

Cornelia de Lange syndrome (CdLS) is a disabling multisystem genetic disease that results from a deficiency in a protein called NIPBL. Individuals with CdLS show a wide range of physical and cognitive problems, but among the most prevalent are congenital heart defects (CHD). By manipulating NIPBL levels in mouse models of CdLS, using the FLEX multi-allele system that has been developed in the Calof lab, we hope to discover the developmental timepoints and tissues in which deficiencies in NIPBL expression are most critical for proper heart development. This may help us discover times and tissues in which heart development is most sensitive to developmental perturbations and lead to the discovery of new causes—and hopefully cures—of congenital heart defects in all babies. Ms. Yazdi's work represents an important step toward this goal.

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Introduction

Cornelia de Lange Syndrome (CdLS) is a multisystem developmental disorder that occurs in approximately 1/10,000 to 1/50,000 births. Since the symptoms vary in severity, it can often be misdiagnosed or undiagnosed in people who are mildly affected (Kawauchi et al. 2009). Congenital heart defects are seen in 20–30% of patients with CdLS, compared to 0.8% of all births, making it a common symptom of the disease. Some of the most common heart abnormalities include atrial septal defects (ASD), in which the wall that separates the upper heart chambers does not form properly, and ventricular septal defects (VSD), in which there are one or more holes in the wall that separates the right and left ventricles of the heart. The cause of many non-syndromic congenital heart defect cases is unknown. Studying heart development and the defects associated with CdLS may reveal what causes heart problems in both non-syndromic and CdLS cases (Cornelia de Lange Syndrome Foundation 2010b). Since CdLS may arise from spontaneous mutations and is often not screened for in genetic tests due to its rarity, researching this disease is vital to understanding how congenital heart defects develop in people with CdLS. Future research may lead to potential treatments or cures that can dramatically benefit the lives of patients with CdLS.

The Nipped B-like (*NIPBL*) gene encodes a cohesion-associated protein that is important in keeping sister chromatids connected during metaphase. *NIPBL* haploinsufficiency is thought to be the reason for many of the developmental defects associated with CdLS (Kawauchi et al. 2009). Haploinsufficiency is when a diploid organism has only one functional copy of a gene. This single functional copy cannot compensate for the loss of the other copy, as is seen with the low levels of *Nipbl* in CdLS. Gene expression profiling in human cell lines, *Drosophila*, and mice have shown that *Nipbl* deficiency leads to a global misregulation of gene transcription (Kawauchi et al. 2009).

To determine why developmental defects occur when *Nipbl* is reduced, our lab generated *Nipbl* +/- mice (RRS564 line). This mouse line allowed us to observe many of the defects associated with reduced *Nipbl* levels, particularly congenital heart defects. However, this model proved to be a challenge, as only approximately 25% of mice survived past four weeks of post-natal life (Kawauchi et al. 2009). Furthermore, these mice expressed reduced levels of *Nipbl* throughout their entire bodies, so it became difficult to identify which tissues or cells with *Nipbl* reductions caused developmental defects.

To aid us in identifying the cell types in which these defects originate, we created a conditional mouse model for CdLS using FlEx (Flip Excision) gene-trapping technology (Schnutgen et al. 2005). The process uses gene-trapping vectors with specific recombinases (enzymes that control gene expression). The FlEx DNA sequence, called the FlEx cassette, is flanked by recombination sites that allow the sequence to be flipped. When the FlEx cassette is flipped, it is no longer recognized so it gets spliced out, effectively resulting in a normal allele (Schnutgen et al. 2005; Schostak 2011). This FlEx technology allows the *Nipbl* gene to toggle through generations from mutant to wildtype with specific recombinases, then back to the mutant form when the gene is exposed to different recombinases (Figure 1).

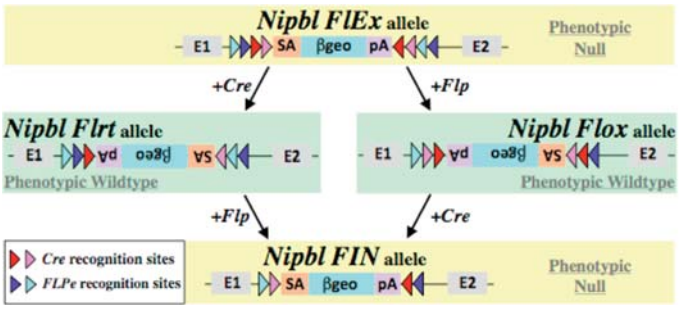

Figure 1

Schematic of conditional *Nipbl* "FlEx" alleles showing how the *Nipbl* gene can be manipulated from mutant (*FlEx*) to wildtype (*Flrt* or *Flox*) and back to mutant (*FIN*) by using *Cre* and *Flp* recombinases. Adapted from unpublished data (R.Santos, A.D. Lander, A.L. Calof et al. 2012).

Using *Actin-FlpE* or *Nanog-Cre* mice, which express the DNA recombinase in all the cells of the body, we inverted the mutant *Nipbl FlEx*/+ allele to yield the wildtype form of the *Nipbl* allele (*Nipbl Flox*/+ or *Nipbl Flrt*/+, respectively). The mice carrying these alleles were indistinguishable in body size from their wildtype littermates, showing that the FlEx system was working as predicted. *Nanog-Cre* was used to re-invert the *Nipbl Flox* allele to the mutant *Nipbl FIN* allele, displaying *Nipbl* haploinsufficient phenotypes. Here we report the success of toggling these alleles and how it indicates that FlEx technology works *in vivo* in the mammalian mouse model. This genetic technology provides us with a new tool to manipulate *Nipbl* levels under specific conditions.

Using this technology, we aimed to determine if reduced *Nipbl* levels, specifically in cardiac tissue, cause heart defects. This would allow us to determine if the low levels of *Nipbl* in heart specific tissues or other tissues (*e.g.* endodermal and

neural crest tissue) were responsible for the defects seen in *Nipbl* heterozygous mice. The goal of this research was to determine which tissue or cell lineage is responsible for the congenital heart defects seen in CdLS.

Materials and Methods

Mice

All experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine, and were consistent with Federal guidelines.

Embryonic Stem (ES) cells were administered into blastocysts from C57B6/J mice (Jackson Laboratory) to generate chimeric mice at the UC Irvine transgenic mouse facility. Chimeric males, identified by coat mosaicism, were mated with CD1 (Charles River) female mice to generate germline transmitted offspring with a grey-*chinchilla*-coat color, and mice carrying the *Nipbl FlEx* gene-trap allele were identified by Polymerase Chain Reaction (PCR). These *Nipbl FlEx/+*, offspring were maintained under normal laboratory conditions and an extensive line was established by continuous crosses with CD1 females.

To generate mice in which *Nipbl* levels were reduced in cardiac specific tissue, phenotypically wildtype *Nipbl Flox/+* animals were crossed with heart specific Nkx2.5Cre to yield conditional mutant offspring (Moses et al. 2001).

Genotyping

Mice and ES cells were confirmed to be *Nipbl* heterozygous using PCR. All PCRs were performed using a C1000 Thermal Cycler (BioRad). DNA was extracted from tail samples from weaned mice or upper limb tissue from embryos and genotyped for the complimentary strands of the following: *LacZ* Forward (5'-TGATGAAAGCTGGCTAG-3'), *LacZ* Reverse2 (5'-ACCACCGCACGATAGAGATT-3'), MHPRT Forward (5'-AAGCCTAAGATGAGCGCAAG-3'), and MHPRT Reverse (5'-AAGCGACAATCTACCAGAGG-3'); MHPRT was used as PCR internal control. A sample of each animal's DNA was denatured for one minute at 94ºC and 30 repeats of the following cycle: 94ºC for 30 seconds, 58ºC for 30 seconds, and 72ºC for 30 seconds, followed by a 5-minute incubation period at 72ºC. Positive results for *LacZ*, showing a heterozygous *Nipbl* allele, were identified by two bands: one at 550 base pairs and the other at 234 base pairs (MHPRT internal control band). DNA negative for *LacZ* was identified by only one control band at 234 base pairs.

Primers used for FlpE PCR include: FlpE Forward (5'-CACTGATATTGTAAGTAGTTTGC-3'), FlpE Reverse (5'-CTAGTGCGAAGTAGTGATCAGG-3'), IL-2 Forward (5'-CTAGGC CACAGAATTGAAAGATCT-3'), IL-2 Reverse (5'-GTAGGTGGAAATTCTAGCATCATCC-3'); IL-2 was used as PCR internal control. These primers were used for identifying *Actin-FlpE* under cycling conditions of 35 cycles of 94ºC for 30 seconds, 58ºC for 60 seconds, 72ºC for 60 seconds, and 72ºC for 5 minutes. Expression of two bands, one at 324 base pairs for the IL-2 control and one at 725 base pairs for the ActFlpE transgene, confirmed the presence of the *Actin-FlpE* allele.

To identify specific gene trap conformation and distinguish among *Nipbl FlEx*, *Flox*, *Flrt* and *FIN* alleles, PCR was conducted using the following primers: BO45 (5'-CTCCGCCTCCTCTTCCTCCATC-3'), BO48 (5'-CCTCCCCCGTGCCTTCCTTGAC-3'), and BO50 (5'-TTTGAG GGGACGACGACAGTCT-3') under cycling conditions of 30 cycles of 94ºC for 60 seconds, 72ºC for 60 seconds, and 72ºC for 7 minutes. A band at 652 base pairs indicates *FlEx* conformation, 735 base pairs indicates *Flrt* conformation, 782 base pairs indicates *Flox* conformation, and 518 base pairs indicates *FIN* conformation.

For PCRs requiring the identification of the Nkx2.5Cre transgene, the following primers were used: Cre forward (5'-GCACTGATTTCGACCAGGTT-3'), Cre reverse (5'-GCTAACCAGCGTTTTCGTTC-3'). DNA was replicated under cycling conditions of 30 cycles of 94ºC for 30 seconds, 62ºC for 30 seconds, and 72ºC for 30 seconds. Positive results for *Cre* genotypes were identified by one band at 200 base pairs.

All PCR amplicons were run on a 2% agarose gel at 100 volts for 30 minutes.

Weighing

Nipbl FlEx/+ offspring from chimeras of F_0 progeny were weighed daily beginning post-natal day 1 to day 21 (weaning age). Following weaning, litters were weighed weekly for six months, then once a month.

Histological Analysis for Heart Structure

Female mice were plug-checked every morning, and midday of plug discovery was considered day 0.5 of development. Anatomical and histological analysis was performed on manually dissected hearts from embryos taken at embryonic day 17.5 (E17.5). Hearts were fixed in 4% paraformaldehyde for one week and then placed in phosphate buffered saline (PBS) before being embedded in 1% agarose. Embedded

hearts were sent to the UC Irvine Pathology Department to be sectioned and Hematoxylin and Eosin stained. Heart sections were analyzed for ASDs and VSDs by determining the number of sections that displayed a defect and measuring the size of the defect using a Zeiss microscope and Axiovision 4.8 software.

Results

Reduced Survival Rate of Germline Transmitted Nipbl FlEx/+ Animals

ES cells were obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM) and identified as a cell line containing the FlEx gene-trap cassette. Similar to the original *Nipbl RRS564/+* line, the FlEx gene-trap cassette (FlpRosaβgeo) was inserted into intron 1 of the *Nipbl* gene, which is 14.5kb downstream of exon 1 (Yaramanoglu 2011). The original conformation is mutant (*FlEx*) and, when the vector is exposed to Cre or Flp recombinase, the gene-trap cassette is inverted and locked into place, making the allele phenotypically wildtype (*Flrt* or *Flox*, respectively). Exposing the gene to the second recombinase re-inverts the allele back to its mutant conformation (Figure 1). These recombinases are recombination enzymes used to manipulate the structure of the mouse genome and to control gene expression.

To establish a *Nipbl FlEx/+* mouse line, male chimeras were crossed with CD1 females to generate *Nipbl FlEx/+* mice. Successful germline transmission was identified by chinchilla (grey) coat color. To determine if the chinchilla coat colored offspring carried the *Nipbl FlEx* allele, *LacZ* and FlEx specific PCRs were performed to determine their genotype. Mice positive for the *LacZ* (550 bp) band and the FlEx (635 bp) band indicated the presence of the *Nipbl FlEx* allele. A successful *Nipbl FlEx/+* line was established; however, of the 221 total chinchilla coat colored offspring, only 11 were *Nipbl FlEx/+*, yielding a rate of 4.97% germline transmitted mice; 50% was expected according to Mendelian ratio. This showed initial evidence that *Nipbl FlEx/+* mice exhibit a significantly reduced rate of survival that is lower than that of the *Nipbl RRS564/+* line, which was approximately 25% (Kawauchi et al. 2009; Schostak 2011).

Nipbl FlEx/+ Line Phenocopies Original Nipbl RRS564/+ Line

As described above, *Nipbl FlEx/+* indicated a reduction in survival rate similar to that found in *Nipbl RRS564/+*. We hypothesized that *Nipbl FlEx/+* mice and *Nipbl RRS564/+* mice should show similar phenotypes since they are both heterozygous for *Nipbl*. To test this hypothesis, we collected data on *Nipbl* mRNA expression levels using QRT-PCR and body weight. QRT-PCR results showed that E17.5 *Nipbl FlEx/+* brain tissue had reduced levels of *Nipbl* expression compared to *Nipbl +/+* (wildtype) littermates (Schostak 2011). Similar results were observed in the *Nipbl RRS564/+* line. *Nipbl FlEx/+* mice showed an overall reduction in body size compared to wildtype littermates, as was observed in the *Nipbl RRS564/+* line (Figure 2). Furthermore, *Nipbl FlEx/+* mice were similar to *Nipbl RRS564/+* mice, not only in body size but also in reduced survival rates (6.9%), eye defects (n=11 out of 14 *Nipbl FlEx/+* mice observed) and behavioral defects (*e.g.* circling, n=1 out of 14 *Nipbl FlEx/+* thus far).

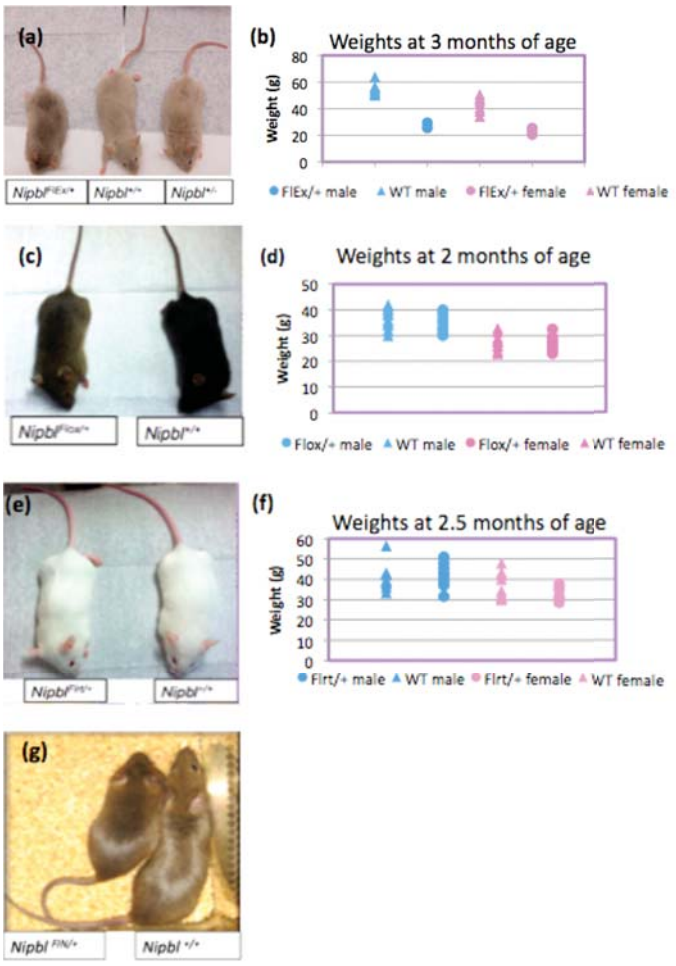

Figure 2

Body size and weight analysis. *Nipbl FlEx/+* mice are similar in size to *Nipbl RRS564/+* mice (a), but are leaner than wildtype (WT) littermates (b). *Nipbl Flox/+* mice are comparable in body size (c) and weight (d) to their wildtype littermates. *Nipbl Flrt/+* mice also show similar body sizes (e) and weights (f) to their wildtype littermates. *Nipbl FIN/+* mice have a small body size compared to wildtype *Nipbl +/+* littermates (g). For weights, n ≥ 3 per genotype. Adapted from unpublished data (R.Santos, A.D. Lander, A.L. Calof et al., 2012).

Confirming whether FlEx Technology Works in Vivo

A specific breeding path using FlEx technology was used to create *Nipbl Flox/+* animals (Figure 1). This was done by crossing *Nipbl FlEx/+* males with *Actin-FlpE* homozygous females. LacZ PCR was performed on the offspring of this cross to identify which animals carried the gene trap. FlpE PCR was performed to ensure the transmission of the *FlpE* gene. FlEx PCRs were performed to identify the conformation of the allele (*FlEx* or *Flox*). If the original gene trap (*FlEx* conformation) is inverted correctly to the *Flox* conformation, PCR results should show a band at 782 base pairs, which can be distinguished from the *FlEx* conformation shown at 652 base pairs. Of the 110 mice obtained from this cross, all contained the *FlpE* allele; 39 were positive for *LacZ*, and these showed FlEx PCR results with both *FlEx* and *Flox* bands. This was hypothesized to be due to a mosaic expression or activity of the FlpE recombinase in different cells of the body, resulting in some cells maintaining the *FlEx* conformation and some cells inverting to the *Flox* conformation.

To generate a line of animals that was purely *Nipbl Flox/+* and isolate the *Nipbl Flox* allele from the *Actin FlpE* allele, the *Nipbl FlEx-Flox* mosaic mice were mated with CD1 mice (Schostak 2011). Four pure *Nipbl Flox/+* animals were obtained and crossed with CD1 mice to establish the *Nipbl Flox/+* line. *Nipbl Flox/+* animals have a body size that is comparable to wildtype animals and an increased survival rate (37%) compared to the *Nipbl FlEx/+* line (Figure 2a and b), indicating that *Nipbl* function is restored (Figure 2c and d). QRT-PCR on embryonic brain tissue showed comparable levels of *Nipbl* expression between *Nipbl Flox/+* and wildtype littermates (data not shown). Mating *Nipbl Flox/+* animals with Nanog-Cre mice gave rise to *Nipbl FIN/+* mice, which showed a mutant small body phenotype compared to wildtype littermates (Figure 2g) and reduced *Nipbl* levels (data not shown).

Nipbl FlEx/+ mice were also crossed with Nanog-Cre animals to give rise to the *Nipbl Flrt/+* line. *Nipbl Flrt/+* mice were phenotypically wildtype (Figure 2e and f), and expressed wildtype levels of *Nipbl*, with an increase in survival rate (44%) similar to the results seen in the *Nipbl Flox/+* line.

Reducing Nipbl Levels in Cardiogeneic Mesoderm Results in Heart Defects

Since 58.3% of *Nipbl RRS564/+* late stage embryos exhibit large ASDs (Kawauchi et al. 2009), we set out to determine if *Nipbl FlEx/+* mice also exhibit ASDs at late stages of development. We examined E17.5 heart sections and found that more than 50% of *Nipbl FlEx/+* embryos exhibit large ASDs (Figure 3b), demonstrating further evidence that the *Nipbl FlEx/+* line phenocopies the *Nipbl RRS564/+* line.

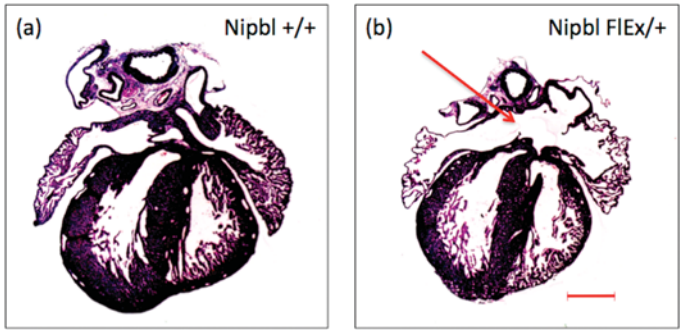

Figure 3

Atrial septal defects in *Nipbl FlEx/+* embryos. Hematoxylin and Eosin stained sections through E17.5 hearts showing ASD in *Nipbl FlEx/+* (red arrow). Scale bar: 500μm

To determine which tissues are responsible for the heart defects observed when *Nipbl* is reduced, we lowered *Nipbl* levels specifically in cardiogenic mesoderm, which develops into heart tissue (Nkx2.5 expressing domain). To achieve this, we crossed *Nipbl Flox/+* mice with *Nkx2.5Cre/+* mice (Moses et al. 2001). From this cross we expected to obtain wildtype, *Nipbl Flox/+* (phenotypically wildtype), *Nkx2.5Cre/+*, and conditional mutant (*Nipbl Flox/+; Nkx2.5Cre*) mice. Conditional mutant mice display wildtype phenotype when exposed to specific recombinases under certain conditions and a mutant phenotype when exposed to different recombinases under other specific conditions.

Nipbl Flox/+; Nkx2.5Cre conditional mutant animals should have reduced levels of *Nipbl* only in heart tissue and wildtype levels of *Nipbl* in all other tissues. We studied mice with these genotypes to determine if heart defects seen in *Nipbl* mutants are due to reduced *Nipbl* levels in only the heart tissue. We found that 65.31% of conditional mutant embryos had large ASDs, whereas only 12.24% wildtype, 20.41% *Nipbl Flox/+* and 22.45% of *Nkx2.5Cre/+* displayed large ASDs. VSDs were also seen in 50% of conditional mutants, but not observed in controls (Figure 4). This result was unexpected as VSDs had not been observed in *Nipbl FlEx/+* or *Nipbl RRS/+* mutant mice. Our results indicate that reduced levels of *Nipbl* in the cardiogenic mesoderm are sufficient to create ASDs. This may be more detrimental than having reduced levels of *Nipbl* globally as *Nipbl* conditional mutants not only exhibit large ASDs, but also VSDs. Further investigation is underway.

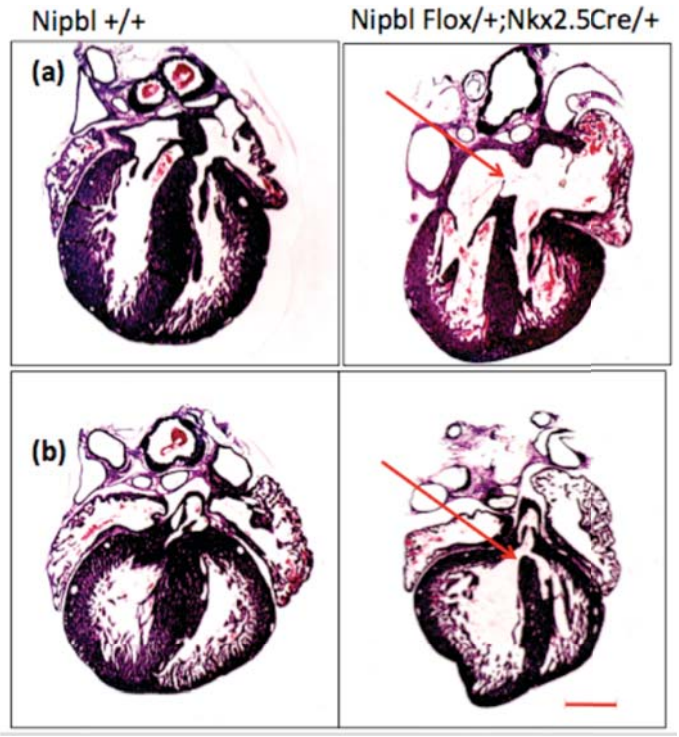

Figure 4

Nipbl conditional mutants develop atrial septal defects (ASD) and ventricular septal defects (VSD). Hematoxylin and Eosin stained sections through E17.5 hearts showing ASD (a, arrow) and VSD (b, arrow) in *Nipbl Flox/+;Nkx2.5Cre/+* embryos. Scale bar: 500μm

Discussion

The primary goal of our research was to establish a conditional mutant mouse model for *Nipbl* using FlEx technology to generate mice with reduced *Nipbl* only in specific tissues. We were able to manipulate the *Nipbl* gene from mutant (*FlEx*) to wildtype form (*Flox* or *Flrt*) and back to mutant (*FIN*) in mice by crossing multiple generations of FlEx mice with mice carrying specific recombinase enzymes (Figure 1). Evidence from our research also showed that *the Nipbl FlEx/+* line phenocopies the previously reported *Nipbl RRS564/+* line. The body weight data and QRT-PCR results showed that *Nipbl* is significantly downregulated in both lines. However, the lower survival rate of *Nipbl FlEx/+* mice as compared to *Nipbl RRS564/+* mice suggests that the severity of phenotypes varies between the two lines, which could be attributed to different gene trap efficiencies (Schostak 2011).

Continuous rounds of crossing animals with CD1 mice to obtain pure *Nipbl Flox/+* offspring successfully established the *Nipbl Flox/+* line. The body size and *Nipbl* levels of these offspring were comparable to wildtype littermates, demonstrating that the gene-trap cassette yielded a functional allele. Crossing *Nipbl Flox/+* animals with Nanog-Cre mice resulted in the final *Nipbl FIN/+*mice with a mutant phenotype. Although a principle of FlEx technology has been available and shown to work in bacteria and *in vitro* cell culture systems, our results were successful in showing the FlEx system is reliable *in vivo* using a mammalian model.

Using tissue specific recombinases, we have also created conditional mutants to study the developmental heart defects associated with reduced levels of *Nipbl*. We found that reducing levels of *Nipbl* in cardiogenic tissues results in heart defects found in *Nipbl* mutant animals (Figure 4). Current experiments aim to test the efficiency of recombination in conditional mutant animals. Further research is being conducted to rescue levels of *Nipbl* in cardiogenic mesoderm in an attempt to recover allele function and determine if we can rescue heart defects in *Nipbl* mutant mice. Together, these studies aim to identify which genes are being misregulated during heart development when *Nipbl* levels are reduced and, ultimately, their effects on congenital heart defects. Selecting candidate genes that are known to cause heart defects and conducting a microarray to monitor differential gene expression can help identify new potential genes of interest and identify specific genes that are being misregulated. These observations are important in understanding how congenital heart defects develop in people with CdLS.

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