Nucleoporin-mediated regulation of the Kcnq1ot1 imprinted domain

Saqib Sachani
The University of Western Ontario

Supervisor
Mellissa Mann
The University of Western Ontario

Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
© Saqib Sachani 2016

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Cell Biology Commons, Developmental Biology Commons, Genetics Commons, and the Molecular Genetics Commons

Recommended Citation
https://ir.lib.uwo.ca/etd/3962

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.
Genomic imprinting is an epigenetic phenomenon that restricts gene expression to one parental allele while the other copy is silent. How this duality is regulated is not fully understood. Using the Kcnq1ot1 imprinted domain as a model, previous work in the laboratory identified nucleoporin 107 as a candidate regulator of imprinted domain regulation. Within the Kcnq1ot1 domain resides the imprinting control region, the paternally expressed Kcnq1ot1 (Kcnq1 opposite transcript 1) noncoding RNA, nine maternal-expressed protein-coding genes, as well as genes that escape imprint regulation. On the maternal allele, the Kcnq1ot1 imprinting control region is methylated, silencing the embedded Kcnq1ot1 promoter and its transcription, and thereby permitting expression of neighbouring genes. On the paternal allele, the Kcnq1ot1 imprinting control region is unmethylated, allowing Kcnq1ot1 noncoding RNA transcription, which results in neighbouring protein-coding gene silencing. In the present study, I showed that depletion of nucleoporin 107, nucleoporin 62 and nucleoporin 153 in mouse extraembryonic endoderm stem cells reduced Kcnq1ot1 noncoding RNA expression and volume, decreased Kcnq1ot1 imprinted domain positioning at the nuclear rim and reactivated normally silent paternal alleles of a core group of protein-coding genes in the domain. Paternal alleles of additional protein-coding genes were also reactivated but in a nucleoporin-specific manner. While DNA methylation at the Kcnq1ot1 imprinting control region was not changed, nucleoporin 107, nucleoporin 62 and nucleoporin 153 depletion led to alterations in active and repressive histone modifications and a reduction in cohesin complex protein interactions at the Kcnq1ot1 imprinting control region. Therefore, I identified a novel mechanism of imprinted domain regulation, namely nucleoporin-mediated imprinted domain regulation at the Kcnq1ot1 domain in extraembryonic endoderm stem cells. Next, I demonstrated that this novel nucleoporin-mediated mechanism also regulated the Kcnq1ot1 imprinted domain in embryonic stem and trophoblast stem cells, albeit for a different subset of genes. While CTCF and the cohesin complex interacted at the same sites within the paternal Kcnq1ot1 imprinting control region in a NUP107, NUP62 and NUP153 dependent manner in embryonic stem cells, trophoblast stem cells lacked CTCF and cohesin binding at the Kcnq1ot1 imprinting control region. My results establish an important role for nucleoporins NUP107, NUP62 and NUP153 in mediating imprinted domain regulation in all three cell lineages.
Co-Authorship Statement


   All experimental work, which is presented in Chapter 3, was carried out by myself except for:

   Dr. Michael Golding derived XEN cells.

   Liyue Zhang performed Western blot analysis, helped with immunoprecipitation, gel shift, reverse biotin ChIP experiments and provided technical assistance for the development of ChIP assay.

   Carlee White performed bisulfite mutagenesis assays.

   William MacDonald helped with bioinformatics analysis

   Lauren Landschoot performed the shRNA screen, which identified NUP107 as a candidate factor.

   Dr. Mellissa Mann and Saqib Sachani conceived the project, experiments and wrote the manuscript.


   All experimental work, which is presented in Chapter 4, was carried out by myself except for:

   Dr. Michael Golding derived ES and TS cells.

   Carlee White performed bisulfite mutagenesis assays.

   Dr. Mellissa Mann and Saqib Sachani conceived the project, experiments and wrote the manuscript.
Acknowledgments

I would like to express my sincere gratitude to all those who have helped and inspired me during my graduate studies. First and foremost, I would like to thank my advisor, Dr. Mellissa Mann for giving me the opportunity to work in her lab. She has been a wonderful mentor and role model for the past five years. Her passion for science has always been motivational and her support and advice have been vital. Her constant encouragement and mentorship have been instrumental throughout my graduate career. I would also like to thank members of my advisory committee Drs. Nathalie Berube, Chris Pin and Fred Dick for their constructive and continuous guidance over the years and suggestions to my thesis and manuscript.

I would like to thank all members of the Mann lab, Dr. William MacDonald, Liyue Zhang, Dr. Lauren Landschoot, Carlee White, Dr. Michelle Denomme, Taylor Smith, Alisha Bester and Josef Ianni for their advice and support throughout my project. In particular, I would specially like to thank Liyue Zhang for his technical expertise and assistance in development of protocols during my work. Dr. William MacDonald for his help in bioinformatics and stimulating discussions. Carlee White for help with bisulfite mutagenesis assay and Dr. Lauren Landschoot for initial help with techniques. I would also like to thank Dr. Rashid Mehmood for providing the E47-RFP plasmid for transport experiments. I am also thankful to Yan Jiang (Berube lab) for providing technical assistance with the CTCF, SMC1 and SMC3 antibodies.

Lastly, I would like to thank my family for all their support. I can’t thank enough my parents for their continuous support and encouragement. Finally, I would like to thank my wife, Naazish, for her patience, and for all the love and support she provided during my studies.
Table of Contents

Abstract ........................................................................................................................................... i
Co-Authorship Statement .............................................................................................................. ii
Acknowledgments ........................................................................................................................... iii
Table of Contents ........................................................................................................................... iv
List of Tables ................................................................................................................................... viii
List of Figures ................................................................................................................................. ix
List of Abbreviations ...................................................................................................................... xiii
Chapter 1 .......................................................................................................................................... 1
  1 Introduction .................................................................................................................................. 1
    1.1 Epigenetics ............................................................................................................................. 1
    1.2 Epigenetic Mechanisms ......................................................................................................... 2
      1.2.1 DNA Methylation .......................................................................................................... 2
      1.2.2 Histone Modifications .................................................................................................... 4
      1.2.3 Noncoding RNAs .......................................................................................................... 6
    1.3 Chromatin Organization ......................................................................................................... 8
      1.3.1 Topologically Associated Domains .............................................................................. 8
      1.3.2 CTCF and the Cohesin Complex .................................................................................... 9
      1.3.3 Scaffold/matrix Attachment Regions .............................................................................. 11
      1.3.4 Nucleolar Associated Domains .................................................................................... 11
      1.3.5 Lamin Associated Domains .......................................................................................... 12
      1.3.6 Nuclear pore complex .................................................................................................... 13
    1.4 Genomic imprinting .............................................................................................................. 16
      1.4.1 H19 domain .................................................................................................................... 18
      1.4.2 Airn domain .................................................................................................................... 22
1.4.3  *Kcnqlot1* imprinted domain ......................................................... 24

1.5  Beckwith-Wiedemann Syndrome .......................................................... 31

1.6  Functional screen for epigenetic factors regulating the *Kcnqlot1* domain ........ 31

1.7  Rationale .................................................................................................. 32

1.8  Hypothesis .............................................................................................. 32

1.8.1  Objectives ............................................................................................ 33

Chapter 2 ........................................................................................................ 34

2  Methods ....................................................................................................... 34

2.1  Cell culture, treatments, transfection and transduction ...................... 34

2.2  RNA Isolation, cDNA preparation and PCR amplification .............. 34

2.3  Quantitative PCR analysis ................................................................. 35

2.4  Allelic expression analysis ................................................................. 35

2.5  RNA/DNA fluorescence in situ hybridization, immunocytochemistry and confocal microscopy ........................................... 35

2.6  Bisulfite mutagenesis and sequencing ................................................. 36

2.7  RNA stability assay .............................................................................. 36

2.8  Chromatin Immunoprecipitation (ChIP) assay ................................ 37

2.9  Western Blot Analysis and Immunoprecipitation Assay ..................... 37

2.10  Biotin Immunoprecipitation Assay .................................................... 37

2.11  Electromobility Shift Assay ................................................................. 37

2.12  Statistical analysis .............................................................................. 38

Chapter 3 ........................................................................................................ 43

3  Results ........................................................................................................ 43

3.1  Nucleoporins regulate *Kcnqlot1* ncRNA expression in XEN cells ........ 43

3.2  Nucleoporins regulate *Kcnqlot1* ncRNA volume in XEN cells ........... 46

3.3  Nucleoporins regulate *Kcnqlot1* domain positioning in XEN cells .... 50
3.4 Nucleoporins physically interact with the \textit{Kcnq1ot1} domain in XEN cells \ldots.. 55

3.5 NUP107, NUP62 and NUP153 regulate paternal allele silencing in XEN cells \ldots.. 65

3.6 Loss of \textit{Kcnq1ot1} domain regulation is not a consequence of abrogated nuclear-cytoplasmic transport \ldots.. 68

3.7 Nucleoporin depletion does not alter \textit{Kcnq1ot1} ICR DNA methylation in XEN cells \ldots.. 73

3.8 Nucleoporin interaction with the \textit{Kcnq1ot1} domain regulates histone modifications at the domain in XEN cells \ldots.. 73

3.9 Nucleoporins regulate cohesin complex interactions at the \textit{Kcnq1ot1} ICR in XEN cells \ldots.. 80

Chapter 4 \ldots.. 85

4 Results \ldots.. 85

4.1 \textit{Nup107, Nup62 and Nup153} depletion alters \textit{Kcnq1ot1} ncRNA expression in ES and TS cells \ldots.. 85

4.2 NUP107, NUP62 and NUP153 regulate \textit{Kcnq1ot1} ncRNA volume in ES and TS cells \ldots.. 91

4.3 NUP107, NUP62 and NUP153 regulate \textit{Kcnq1ot1} domain positioning in ES and TS cells \ldots.. 94

4.4 Nucleoporins physically interact with the \textit{Kcnq1ot1} domain in ES and TS cells \ldots.. 99

4.5 Nucleoporin proteins regulate paternal allele silencing in ES and TS cells \ldots.. 106

4.6 Loss of \textit{Kcnq1ot1} domain regulation is not a consequence of abrogated nuclear-cytoplasmic transport \ldots.. 109

4.7 Nucleoporin depletion does not alter \textit{Kcnq1ot1} ICR DNA methylation \ldots.. 113

4.8 Nucleoporins regulate histone modifications at the \textit{Kcnq1ot1} domain in ES and TS cells \ldots.. 116

4.9 Nucleoporins regulate CTCF/cohesin complex at the \textit{Kcnq1ot1} ICR in ES cells but not in TS cells \ldots.. 123

Chapter 5 \ldots.. 129

5 Discussion \ldots.. 129

5.1 Nucleoporin 107, 62 and 153 regulate \textit{Kcnq1ot1} domain both on- and off-pore \ldots.. 137
5.2 Requirement for the *Kcnq1ot1* noncoding RNA .............................................. 139
5.3 Nucleoporin 107, 62 and 153 in higher order chromatin structure ....................... 140
5.4 Future Directions ........................................................................................................ 144
References .......................................................................................................................... 147
Appendix 1: Copyright Release ......................................................................................... 155
Curriculum Vitae ................................................................................................................ 157
List of Tables

Table 2—1: List of siRNA for depletion studies................................................................. 39

Table 2—2: Primer list........................................................................................................ 40

Table 2—3: List of antibodies for Western blot analysis, Immunohistochemistry, RNA FISH, ChIP and IP. .................................................................................................................. 42
List of Figures

Figure 1—1: DNA methylation ........................................................................................................... 3

Figure 1—2: Histone Modifications .................................................................................................. 5

Figure 1—3: Mechanisms of long noncoding RNA-mediated gene silencing.............................. 7

Figure 1—4: Chromatin organization within the nucleus ................................................................. 10

Figure 1—5: Structure of the nuclear pore complex ....................................................................... 14

Figure 1—6: Representation of imprinted genes ............................................................................. 17

Figure 1—7: Structure and regulation of the *H19* imprinted domain ........................................... 20

Figure 1—8: ICR-mediated enhancer-blocking model for the *H19* imprinted domain.............. 21

Figure 1—9: Structure of the *Airn* imprinted domain ................................................................... 23

Figure 1—10: Structure of the *Kcnq1ot1* imprinted domain ......................................................... 26

Figure 1—11: Derivation of ES, TS and XEN stem cells from a blastocyst................................. 29

Figure 1—12: Schematic representation of *Kcnq1ot1* domain in XEN, ES and TS cells ...... 30

Figure 3—1: Nucleoporin depletion levels in XEN cells. ............................................................... 45

Figure 3—2: Nucleoporin depletion disrupts *Kcnq1ot1* ncRNA expression, domain volume and nuclear periphery localization. .......................................................................................... 48

Figure 3—3: *Kcnq1ot1* ncRNA stability is not altered upon nucleoporin depletion............... 49

Figure 3—4: Paternal and maternal *Kcnq1ot1* domain have distinct volumes in XEN cells. 52

Figure 3—5: Antibody validation in XEN cells. ............................................................................. 58

Figure 3—6: Antibody validation, and mAb414 and NUP153 enrichment at the *Kcnq1ot1* ICR and enhancer element in XEN cells. ........................................................................................................... 60
Figure 3—7: NUP107/62 and NUP153 interaction with the Kcnq1ot1 ICR, and the Oshpl5, Kcnq1, Cd81 promoters in control and Nup-depleted XEN cells. ................................. 62

Figure 3—8: NUP107, NUP62 and NUP153 bound to a Kcnq1ot1 ICR fragment ........... 64

Figure 3—9: Nup107, Nup62 and Nup153 depletion reactivates a subset of paternal alleles at the Kcnq1ot1 domain. .................................................................................................................. 67

Figure 3—10: Nuclear transport is not altered upon nucleoporin depletion. ............... 71

Figure 3—11: XEN cell growth rate is not altered upon nucleoporin depletion. .......... 72

Figure 3—12: DNA methylation is maintained upon nucleoporin depletion............... 74

Figure 3—13: Nucleoporin-RNAPII interactions, and validation of active and repressive chromatin modifications at the FoxA2 and MyoD genes. ................................................................. 77

Figure 3—14: Nucleoporin depletion disrupts histone modifications at the Kcnq1ot1 ICR and imprinted gene promoters. ........................................................................................................ 79

Figure 3—15: Nucleoporin-CTCF and cohesin complex protein interactions in XEN cells, and CTCF enrichment at the H19 ICR CTCF positives in ES cells but not at Kcnq1ot1 ICR CTCF positives sites in XEN cells................................................................. 82

Figure 3—16: SMC1 and SMC3 enrichment at the paternal Kcnq1ot1 ICR was reduced upon nucleoporin depletion........................................................................................................ 84

Figure 4—1: Nucleoporin depletion levels in ES and TS cells. ................................. 88

Figure 4—2: Nup107, Nup62 and Nup153 depletion disrupts Kcnq1ot1 ncRNA expression, domain volume and nuclear periphery localization ....................................................... 90

Figure 4—3: Kcnq1ot1 ncRNA stability is not altered upon nucleoporin depletion in ES and TS cells....................................................................................................................... 93

Figure 4—4: Nup107, Nup62 and Nup153 depletion disrupts Kcnq1ot1 domain nuclear periphery localization................................................................................................................... 96
Figure 4—5: Kcnq1ot1 ncRNA volume to distance correlation in control and nucleoporin-depleted ES and TS cells

Figure 4—6: NUP107/62 and NUP153 interact with the Kcnq1ot1 ICR, enhancer site, Cdkn1c and the Osbpl5, Cd81 and Th promoters in wildtype cells

Figure 4—7: NUP107/62 and NUP153 interaction with the Kcnq1ot1 domain in control and Nup-depleted ES and TS cells

Figure 4—8: NUP107, NUP62 and NUP153 bound to the Kcnq1ot1 ICR fragment

Figure 4—9: Nucleoporin depletion reactivates a subset of paternal alleles at the Kcnq1ot1 domain in ES and TS cells

Figure 4—10: Nuclear transport is not altered upon nucleoporin depletion

Figure 4—11: Nucleoporin depletion does not alter ES and TS cell growth rate

Figure 4—12: DNA methylation is maintained upon nucleoporin depletion

Figure 4—13: Validation of active and repressive chromatin modifications at the Oct4 and Cdx2 genes in ES and TS cells

Figure 4—14: Nucleoporin depletion disrupts histone modifications at the Kcnq1ot1 ICR and at reactivated imprinted gene promoters in ES and TS cells

Figure 4—15: Nucleoporin depletion did not alter histone modifications at imprinted gene promoters, where paternal allelic-silencing was maintained in ES and TS cells

Figure 4—16: CTCF, SMC1 and SMC3 localization at the H19 and Peg3 domains in ES and TS cells

Figure 4—17: CTCF, SMC1 and SMC3 enrichment at the Kcnq1ot1 ICR and enhancer site in ES and TS cells

Figure 4—18: CTCF, SMC1 and SMC3 enrichment at the paternal Kcnq1ot1 ICR is reduced upon nucleoporin depletion in ES cells
Figure 5—1: Summary of nucleoporin-mediated regulation of the *Kcnq1ot1* imprinted domain in XEN cells ................................................................. 132

Figure 5—2: Summary of nucleoporin-mediated regulation of the *Kcnq1ot1* imprinted domain in ES cells ........................................................................................................ 134

Figure 5—3: Summary of nucleoporin-mediated regulation of the *Kcnq1ot1* imprinted domain in TS cells ........................................................................................................ 136

Figure 5—4: Proposed model of topological organization of the paternal *Kcnq1ot1* imprinted domain orchestrated by nucleoporins in XEN, ES and TS cells ........................................ 143
List of Abbreviations

%  Percent
<  Less than
>  Greater than
°C  Degree Celsius
α  Alpha
β  Beta
µ  Micro
µg  Microgram
µL  Microliter
µM  Micro molar
3D  Three-dimensional
A  Adenine
Ac  Acetyl group
Aimn  Antisense Igf2 RNA
Ascl2  Aschaete-scute complex homolog 2
B6  C57BL/6
bp  Base pair
C  Cytosine
CAST  *Mus musculus* castaneus
Cd81  CD81 antigen
Cdkn1c  Cyclin-dependent kinase inhibitor IC (also known as p57)
cDNA  Complementary deoxyribonucleic acid
Cdx2  Caudal-related homeobox transcription factor family
CH3  Methyl group
ChIP  Chromatin Immuno-Precipitation
CpG  Phosphorylated cytosine followed by guanine
CTCF  CCCTC-binding factor
DMR  Differentially methylated region
DNA  Deoxyribonucleic acid
DNase  Deoxyribonuclease
DNMT  DNA methyltransferase
DNMT1  DNA methyltransferase 1
DNMT3A  DNA methyltransferase 3a
DNMT3B  DNA methyltransferase 3b
DNMT3L  DNA methyltransferase 3l
E  Enhancer element
En  Enhancer element
ES cells  Embryonic stem cells
E3.5  Embryonic day 3.5
EhMT2  Euchromatic histone lysine N-methyltransferase 2 (G9a)
FISH  Fluorescence *in situ* hybridization
G  Guanine
GFP  Green fluorescence protein
Gtl2  Gene trap locus 2
H1  Histone 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>H19</td>
<td>Hepatocyte 19 fetal liver mRNA</td>
</tr>
<tr>
<td>H2A</td>
<td>Histone 2A</td>
</tr>
<tr>
<td>H2B</td>
<td>Histone 2B</td>
</tr>
<tr>
<td>H3</td>
<td>Histone 3</td>
</tr>
<tr>
<td>H3K4me1</td>
<td>Histone 3 lysine 4 mono-methylation</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>Histone 3 lysine 4 di-methylation</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Histone 3 lysine 4 tri-methylation</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>Histone 3 lysine 9 di-methylation</td>
</tr>
<tr>
<td>H3K9me3</td>
<td>Histone 3 lysine 9 tri-methylation</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>Histone 3 lysine 27 tri-methylation</td>
</tr>
<tr>
<td>H4</td>
<td>Histone 4</td>
</tr>
<tr>
<td>HDM</td>
<td>Histone demethylase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDAC4</td>
<td>Histone deacetylase 4</td>
</tr>
<tr>
<td>HDAC9</td>
<td>Histone deacetylase 9</td>
</tr>
<tr>
<td>HeLa cells</td>
<td>Immortal cervical cancer cell line</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>ICR</td>
<td>Imprinting control region</td>
</tr>
<tr>
<td>Igf2</td>
<td>Insulin-like growth factor 2</td>
</tr>
<tr>
<td>Igf2r</td>
<td>Insulin-like growth factor 2 receptor</td>
</tr>
<tr>
<td>Ins2</td>
<td>Insulin 2 gene</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>Kcnq1</td>
<td>Potassium voltage-gated channel, KQT-like subfamily member</td>
</tr>
<tr>
<td>Kcnq1ot1</td>
<td>Kcnq1-overlapping transcript 1</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LAD</td>
<td>Lamin associated domain</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>Mat</td>
<td>Maternal</td>
</tr>
<tr>
<td>Mb</td>
<td>Megabase</td>
</tr>
<tr>
<td>Me</td>
<td>Methyl group</td>
</tr>
<tr>
<td>MDa</td>
<td>Mega dalton</td>
</tr>
<tr>
<td>mg/mL</td>
<td>Milligram per milliliter</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NAD</td>
<td>Nucleolar associated domain</td>
</tr>
<tr>
<td>ncRNA</td>
<td>Noncoding ribonucleic acid</td>
</tr>
<tr>
<td>Nup</td>
<td>Nucleoporin</td>
</tr>
<tr>
<td>Nup50</td>
<td>Nucleoporin 50</td>
</tr>
<tr>
<td>Nup62</td>
<td>Nucleoporin 62</td>
</tr>
<tr>
<td>Nup98/96</td>
<td>Nucleoporin 98/96</td>
</tr>
<tr>
<td>Nup107</td>
<td>Nucleoporin 107</td>
</tr>
<tr>
<td>Nup153</td>
<td>Nucleoporin 153</td>
</tr>
<tr>
<td>Nup214</td>
<td>Nucleoporin 214</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>Nup358</td>
<td>Nucleoporin 358</td>
</tr>
<tr>
<td>NUPAC</td>
<td>Nucleoporin associated chromatin</td>
</tr>
<tr>
<td>Oct4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>Osbp15</td>
<td>Oxysterol binding protein-like 5</td>
</tr>
<tr>
<td>Pat</td>
<td>Paternal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Peg3</td>
<td>Paternally expressed gene 3</td>
</tr>
<tr>
<td>Oct4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>Osbp15</td>
<td>Oxysterol binding protein-like 5</td>
</tr>
<tr>
<td>Pat</td>
<td>Paternal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Peg3</td>
<td>Paternally expressed gene 3</td>
</tr>
<tr>
<td>Phlda2</td>
<td>Pleckstrin homology-like domain, family A, member 2</td>
</tr>
<tr>
<td>RNAPII</td>
<td>RNA Polymerase II</td>
</tr>
<tr>
<td>PRC</td>
<td>Polycomb repressive complex</td>
</tr>
<tr>
<td>RAD21</td>
<td>Radiation mutant 21</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescence protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>Slc22a2</td>
<td>Solute carrier family 22 (organic cation transporter), member 2</td>
</tr>
<tr>
<td>Slc22a3</td>
<td>Solute carrier family 22 (organic cation transporter), member 3</td>
</tr>
<tr>
<td>Slc22a18</td>
<td>Solute carrier family 22 (organic cation transporter), member 18</td>
</tr>
<tr>
<td>SMC1</td>
<td>Structural maintenance of chromosomes 1</td>
</tr>
<tr>
<td>SMC3</td>
<td>Structural maintenance of chromosomes 3</td>
</tr>
<tr>
<td>Snrpn</td>
<td>Small nuclear ribonucleoprotein polypeptide N</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium saline citrate</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TAD</td>
<td>Topologically associated domain</td>
</tr>
<tr>
<td>Th</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TPR</td>
<td>Translocated promoter region</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TS cells</td>
<td>Trophoblast stem cells</td>
</tr>
<tr>
<td>Tssc4</td>
<td>Tumor-suppressing subchromosomal transferable fragment 4</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin group</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>XEN cells</td>
<td>Extraembryonic endoderm stem cells</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Epigenetics

Gene expression and its regulation are essential for embryonic development and differentiation. Gene expression is orchestrated by sets of proteins that bind to gene regulatory elements. These elements include promoters, insulators, enhancers and silencers (Wallace and Felsenfeld, 2007). In eukaryotes, interaction between enhancer elements and gene promoters results in gene activation. These interactions can occur at a distance of a few hundred base pairs to 100 kb or more (Sanyal et al., 2012). The process of regulating gene expression through transcription factor binding and RNA polymerase II mediated transcription is well studied (Egecioglu and Brickner, 2011; Hakim et al., 2010; Meldi and Brickner, 2011). However, other mechanisms govern gene expression beyond DNA sequence information. These mechanisms are termed as epigenetic mechanisms. Epigenetics was originally described by Waddington in 1942 as the branch of biology that studies interactions between genes and their products, which bring the phenotype into being (Waddington, 2012). Today, epigenetics is defined as heritable changes of gene expression that are not caused by changes in primary nucleotide sequence of the DNA (Gibney and Nolan, 2010). To store the genetic information within eukaryotic cells, DNA is wrapped around histones to form nucleosomes that are the basic unit of chromatin. This structure allows efficient reading of the DNA as well as accessibility to the DNA template. Each nucleosome contains an octamer of histones that is comprised of two copies each of H2A, H2B, H3 and H4 (Bártová et al., 2008). The two central mechanisms that directly regulate how chromatin is packaged are DNA methylation and histone modifications (Jenuwein and Allis, 2001; Li et al., 1993). Chromatin packaging can also be regulated by chromatin remodeling proteins as well as noncoding RNAs. All together, these mechanisms are involved in regulating gene expression. Recent advances in high resolution microscopy have shown that the three-dimensional conformation of chromatin within the nucleus as well as nuclear architecture play key roles in gene expression and regulation (Cremer and Cremer, 2010; Smeets et al., 2014). Proteins such as CTCF and the cohesin complex are
involved in 3D chromatin conformation, acting to establishing boundaries that separate heterochromatin and euchromatin (Hark et al., 2000; Yusufzai et al., 2004). For nuclear architecture, sub-nuclear compartments such as the perinucleolar region and the nuclear membrane, comprised of nuclear lamina and nuclear pore complexes play significant roles in activating or silencing gene expression (Pombo and Dillon, 2015; Ptak and Wozniak, 2016). Below, I discuss the various epigenetic mechanisms in detail that regulate gene expression.

1.2 Epigenetic Mechanisms

1.2.1 DNA Methylation

DNA methylation is the addition of a methyl group to the 5'-position of a cytosine residue and is often correlated with transcriptional repression (Figure 1-1). In mammals, cytosine methylation most commonly occurs when its neighbor is a guanine, which is denoted as a CpG dinucleotide. DNA methylation is carried out by a group of proteins called DNA methyltransferases (DNMTs). DNMT3A, 3B and 3L are responsible for de novo DNA methylation (Li, 2002; Li et al., 1993). Once DNA methylation is acquired, it is maintained by DNMT1. During DNA replication, DNMT1 recognizes hemi-methylated DNA, adding methyl groups to the newly synthesized DNA strand (Okano et al., 1999). DNA methylation is a highly stable mark (Chodavarapu et al., 2010). As such, it is important for genomic stability, chromatin structure, genomic imprinting, and silencing of repetitive elements (reviewed in Rodenhisser and Mann, 2006). Aberrant DNA methylation can result in changes in gene expression, cancer and genomic imprinting disorders.
DNA methylation is the covalent addition of a methyl group (CH₃, red) to the 5' position of the pyrimidine ring of cytosine residues. Methyl groups are most commonly added to cytosine residues within CpG dinucleotides. This addition is catalyzed by DNA methyltransferases. DNA methylation at CpGs generally results in a more compact state of chromatin, inhibiting transcription. When CpGs are unmethylated, chromatin is in a more relaxed state, and can be transcribed. Open circles (white) represent unmethylated CpGs, while closed (red) circles represent methylated CpGs.
1.2.2 Histone Modifications

Histones are the protein components of chromatin. They are subjected to a wide variety of post-translational modifications, including acetylation, methylation, phosphorylation, and sumoylation. These modifications are heritable during cell division and play a significant role in gene expression (Bannister and Kouzarides, 2011; Brown et al., 2008; Desvoyes et al., 2010; Martens et al., 2005). Histone modifying enzymes specify different histone modifications that are involved in regulating gene expression. Histone methyltransferases (HMTs) catalyze the transfer of methyl groups to lysine and arginine residues of histones. Different HMTs modify specific methyl states, mono, di or tri, for example, H3K4me1, H3K4me2, and H3K4me3. Histone demethylases (HDMs) are proteins that have the catalytic ability to remove methyl groups from lysine and arginine residues of histones (Mozzetta et al., 2015; Zentner and Henikoff, 2013). An active or repressed state for chromatin depends on the histone modification conferred to the histone tails. For example, tri-methylation of lysine 4 of H3 (H3K4me3) has been associated with active chromatin, whereas di- and tri-methylation of lysine 9 (H3K9me2/3) and tri-methylation of lysine 27 of H3 (H3K27me3) have been correlated with inactive chromatin (Figure 1-2) (Tessarz and Kouzarides, 2014).

Active or repressed chromatin is also specified by the acetylation state of chromatin. Euchromatin is characterized by histone acetylation, which is catalyzed by histone acetyl transferases (HATs). Histone acetylation neutralizes the positive charge on histones, resulting in a more open chromatin state. Conversely, histone deacetylases (HDACs) remove acetyl groups, re-establishing the positive charge to histone tails, thereby enabling their interaction with negatively charged DNA and the formation of heterochromatin. Histone phosphorylation, ubiquitination and sumoylation are other histone modifications that are not as intensively studied but play a role in gene regulation (Bannister and Kouzarides, 2011). For example, during interphase phosphorylation of serine 10 in histone H3 is correlated to an active chromatin state, whereas H2A phosphorylation is associated with a repressed state (Fischle et al., 2005; Hirota et al., 2005). Histone modifications can co-exist and a particular histone profile can provide binding sites for other chromatin-binding proteins that promote active or repressed state.
Figure 1—2: Histone Modifications

Post-translational modifications of histone tails can modulate gene expression. Active modifications (green) represented by H3K4me3 and H3K9ac promote an active chromatin state, thereby enabling gene expression. Repressive histone modifications (red) represented by H3K9me2/3 and H3K27me3 are associated with inactive genes and promote a compact chromatin state. Histone methyltransferases (HMTs) confer methyl groups on histone tails, while histone demethylases (HDMs) remove these methyl groups. Histone acetyltransferases (HATs) add acetyl groups, while histone deacetylase (HDACs) remove acetyl groups. Through these histone modifications, chromatin remodeling proteins can bind, altering the structure of chromatin, thereby inducing transcriptional activation or repression.
1.2.3 Noncoding RNAs

In mammals, the majority of the genome (>85%) is composed of regions that are transcribed, although many of the resulting RNAs are not translated (Hangauer et al., 2013). These transcripts are known as noncoding RNAs (ncRNAs). Noncoding RNAs are classified into two groups based on size (Cheetham et al., 2013). Small noncoding RNAs are less than 200 nucleotides in length while long non-coding RNAs are greater than 200 nucleotides. Small ncRNAs, such as microRNAs, short interfering RNAs, PIWI-RNAs, and small nucleolar RNA, repress translation by binding to their target sequence, initiating the RNA interference pathway, where the RNA will be degraded. Long noncoding RNAs are transcribed both sense and antisense to coding sequences with their transcription start sites being either intronic or intergenic. Many long ncRNAs contain features similar to mRNAs, including a 5' 7-methyl guanosine cap and 3' polyA-tail (Meller et al., 2015). It is currently unclear how long ncRNAs mediate gene expression and overall chromatin structure. A common feature of most long ncRNAs is that transcripts are confined to the nucleus, suggesting the transcript itself may be functionally important in directing expression (Furuno et al. 2006; Derrien et al. 2012). Three mechanisms have been proposed for long ncRNAs cis function: transcriptional interference, coating of chromatin and recruitment of epigenetic repressors (Figure 1-3). Transcriptional interference occurs when transcription of the long ncRNA overlaps the promoter of the target gene, disrupting RNA polymerase recruitment. For chromatin coating, a long ncRNA spreads from the site of transcription to cover the neighbouring chromosomal region, inducing a repressed chromatin conformation. Finally, long ncRNAs, through a secondary conformation, may act as adaptors to recruit chromatin modifiers, such as histone methyltransferases, to chromosomal regions, inducing gene silencing. More studies are required to further understand how long noncoding RNAs regulate gene expression.
Long noncoding RNAs are proposed to regulate gene silencing by the following methods.

A: Transcriptional interference. Transcription of a long ncRNA through the promoter of a gene abrogates transcription factor (TF) and RNA polymerase II (RNAPII) binding at the promoter, thereby silencing genes.

B: Coating the domain in cis. Long ncRNAs spread in cis coating the domain, inducing a repressed chromatin conformation that silences neighbouring genes.

C: Recruitment of silencing factors. Long ncRNAs may function as adaptors, recruiting histone modifying enzymes to chromatin, thereby silencing neighbouring genes.

**Figure 1—3: Mechanisms of long noncoding RNA-mediated gene silencing**
1.3 Chromatin Organization

The mammalian genome is highly organized within the 3-dimensional (3D) space of the nucleus. On a macro scale, chromosomes are highly organized within the nucleus, forming distinct territories that do not overlap with other chromosomes, which are termed as chromosome territories (Cremer and Cremer, 2001; Cremer and Cremer, 2010; Smeets et al., 2014). Furthermore, contiguous regions along the chromosome are organized by chromatin topologies, which encompass local chromatin-chromatin interactions and their associations within the nucleus. Proteins such as CCCTC-binding factor protein CTCF and its binding partner the cohesin complex have key roles in chromatin-chromatin interactions, that give rise to chromosome territories. Formation of chromosome territories at different sub-nuclear compartments results in formation of active and silenced domains. These chromatin structures are facilitated by nuclear architecture, which comprises the scaffold on which chromosome territories and chromatin topologies are formed. Included are features such as the nuclear lamina, nuclear pore complex, nuclear scaffold/matrix and the nucleolus. Associations of chromatin with nuclear architecture have a direct influence on chromatin topology, indicating that nuclear positioning is key to gene expression.

1.3.1 Topologically Associated Domains

Over the years, various terms have been used to describe the organization of chromatin into domains depending on the method used for demarcation. One emerging methodology that utilizes chromatin capture revealed that the genome is organized into topologically associating domains (TADs). TADs represent ~1 Mb chromatin domains, that are enriched for chromatin-chromatin interactions while interactions between neighbouring domains are low (Figure 1-4) (Dixon et al., 2012; Nora et al., 2012; Pope et al., 2014). Remarkably, TADs are largely stable across cell types and conserved between species (Dixon et al. 2012; Nora et al. 2012). This suggests that generally TADs are not indicative of a particular transcriptional state, and that higher-order chromatin associations remain stable even with a transcriptional state change. Thus, TADs have been proposed to represent the linear segregation of the genome into regulatory neighbourhoods. By comparison, variations in subtopologies within TADs, and changes in DNA replication timing of a TAD, occur in distinct cell types (Phillips-Cremins et al., 2013; Pope et al., 2014). Generally, these
substructures are achieved by specific looping of chromatin within TADs (Jin et al., 2013; Phillips-Cremins et al., 2013; Rao et al., 2014).

### 1.3.2 CTCF and the Cohesin Complex

Two factors mediate local chromatin-chromatin interactions in mammals, CTCF and the cohesin complex. CTCF is a multifunctional protein known for its role as an insulator protein. CTCF binds to its consensus sequence within chromatin, then forms homodimers that generate chromatin loops (Phillips and Corces, 2009). The cohesin complex consists of four subunits, which include RAD21/sister chromatid cohesin protein 1 (SSC1), SSC3, structural maintenance of chromosome 1 (SMC1) and SMC3. The cohesin complex forms a ring-like structure capturing and holding chromatin strands together, assisting in the chromatin loop formation (Nasmyth and Haering, 2009; Phillips and Corces, 2009). Cohesin binding frequently overlaps with that of CTCF to regulate chromatin topology (Figure 1-4) (Handoko et al., 2011; Schmidt et al., 2010). Recent high-resolution 3D mapping of chromatin places a conservative estimate of ~10,000 loops in the human genome, the majority of which are anchored by CTCF (Rao et al. 2014). Because of their role in loop formation, CTCF and cohesin demark regions between different chromatin loops, generating chromatin boundaries (Figure 1-4). While mostly enriched within TADs to form sub-TAD chromatin loops (Phillips-Cremins et al. 2013), CTCF binding sites are also enriched at TAD boundaries (Dixon et al. 2012). Interestingly, independent depletion of CTCF or cohesins subunits has distinct effects on chromatin interactions. CTCF depletion results in decreased interactions within TADs as well as increased interactions between TADs (Zuin et al., 2014). Depletion of RAD21 also reduced subtopological chromatin interactions within TADs, although the TADs themselves were not disrupted. These results support previous findings that chromatin interactions are not strictly dependent on the presence of CTCF or cohesin, but rather that 3D chromatin structure is supported by multiple factors (Phillips-Cremins et al. 2013).
Figure 1—4: Chromatin organization within the nucleus

The mammalian genome is highly organized into chromatin topologies via interactions with the nuclear architecture. Topologically associated domains (TADs) segregate the genome into regulatory neighbourhoods, which are enriched for chromatin-chromatin interactions that facilitate subTAD chromatin looping. CTCF and cohesin play a role in generating these subTAD chromatin interactions, as well as marking TAD boundaries. Lamin associated domains (LADs) are regions of chromatin that are bound to Lamin B1, a major component of the nuclear lamina, linking chromatin with low to no transcription to the inner nuclear membrane. Nucleolar associated domains (NADs) describe chromatin with low transcriptional activity that localize to the perinucleolar region. NADs partially overlap with LADs, with a possible shift of chromatin between LADs and NADs during the cell cycle or in different tissues. Scaffold/matrix attachment regions (S/MARs) are DNA loci that have a high affinity for the nuclear scaffold/matrix and have been associated with repressed and active chromatin. Nucleoporin-associated chromatin (NUPAC) describes nucleoporin/nuclear pore complex interactions with active chromatin, facilitating chromatin looping and demarcating chromatin boundaries.
1.3.3 Scaffold/matrix Attachment Regions

Chromosome territories are maintained in part through interactions with the nuclear scaffold/matrix. Scaffold/matrix attachment regions (S/MARs) are DNA loci that have a high affinity for the nuclear scaffold/matrix after the depletion of histones and other soluble factors (Mirkovitch et al. 1984; Cockerill and Garrard 1986). S/MARs are significant in that they provide a direct connection between chromatin and the nuclear scaffold/matrix that is independent from the histone state, indicating an important role in nuclear architecture (Figure 1-4). In human cell lines, disruption of the nuclear scaffold/matrix releases S/MAR associated proteins and leads to the disorganization of chromosomal territories (Ma et al. 1999). Almost 100 S/MAR-binding proteins have been identified in model organisms, including lamins, topoisomerases, AT-rich binding proteins and histone deacetylases (Liebich et al., 2002a; Mika and Rost, 2005). The DNA sequences of S/MARs are highly polymorphic, without any common motif enrichment, suggesting a dynamic role for S/MAR association with the nuclear scaffold/matrix (Liebich et al., 2002b). That being said, S/MARs are enriched for AT-rich elements but not for CTCF binding sites (Goetze et al., 2005). This suggests that S/MARs are unlikely to primarily serve as anchors for CTCF/cohesin-mediated chromatin loop formation but may serve specific roles for chromatin access and loop scaffolding.

1.3.4 Nucleolar Associated Domains

Besides the nuclear periphery, chromatin also associates with the nucleolus, in what are defined as nucleolar associated domains (NADs) (Figure 1-4) (Németh et al. 2010; van Koningsbruggen et al. 2010). The nucleolus is the largest subnuclear structure in the nucleus that is primarily responsible for the generation of ribosomal RNAs (Németh and Längst 2011). Recently, genome-wide mapping has revealed that all human chromosomes have NADs located at unique loci as well as at repetitive sequences (van Koningsbruggen et al. 2010; Németh and Längst 2011). NADs share common features of low gene density, low transcriptional activity and high AT-rich sequence elements density (van Koningsbruggen et al. 2010). Interestingly, following cell division, chromatin associated with NADs, either returned to the nucleolus or to the nuclear periphery, suggesting that
defined chromatin regions shuttle between the nuclear periphery and nucleolus depending on cell type or cell cycle (van Koningsbruggen et al. 2010; Kind et al. 2013).

1.3.5 Lamin Associated Domains

In general, higher order chromatin organization in the nucleus positions chromatin to the nuclear periphery, where genes are generally silenced or in the nuclear interior where genes are typically active. At the nuclear periphery resides the nuclear lamina, an architectural framework that links chromatin to the inner nuclear membrane (Gruenbaum et al. 2003). Lamin associated domains (LADs) are regions of chromatin that are bound to Lamin B1, a major component of the nuclear lamina (Figure 1-4), and frequently span ~1 Mb in size (Guelen et al. 2008). There is a close association of LADs with chromatin possessing low to no transcription. Mapping of LADs has shown that as much as 30% to 40% of the mouse and human genomes reside in LADs, respectively (Guelen et al. 2008; Wu and Yao 2013).

Unlike TADs that remain largely stable across cell and tissue types, LADs appear to fall into two groups, constitutive LADs and dynamic LADs. Constitutive LADs are stable across multiple undifferentiated and differentiated cell types and account for ~33% of the mouse genome, while dynamic LADs with distinct patterns depending on cell type represent ~29% of the genome (Meuleman et al. 2013). Generally, inactive genes within a dynamic LAD in one cell-type will move away from the nuclear periphery to become activated in another cell-type (Peric-Hupkes et al. 2010). In total, over 60% of chromatin within the genome is constituted by LADs (Meuleman et al. 2013). LADs are characterized by the repressive histone modification H3K9me2 and H3K27me3. H3K9me2 is catalyzed by the histone methyltransferase G9a (Wen et al. 2009). One role identified for G9a-dependent H3K9me2 is to help tether chromatin to the lamina, supporting the nuclear periphery association of LADs (Kind et al. 2013). By comparison, H3K27me3 is mostly excluded from the interior of LADs, instead demarcating LAD borders (Wen et al. 2009). These LADs borders typically interface with chromatin boundaries where lamin association markedly declines, and CTCF binding sites together with CpG islands are enriched (Guelen et al. 2008). In addition to Lamin B1, other components of the nuclear lamina are important for maintaining chromatin association with the nuclear matrix and gene silencing, including Lamin A/C (Harr et al. 2015) and the Lamin B receptor
(Polioudaki et al. 2001; Guarda et al. 2009). In a two-step process, Lamin A/C followed by Lamin B receptor tethers heterochromatin to the nuclear matrix (Namekawa et al. 2010; Solovei et al. 2013). Loss of both proteins relocates heterochromatin from the nuclear periphery into the nuclear interior.

1.3.6 Nuclear pore complex

The nuclear membrane, which encases the genetic material of the cell, is studded with numerous large aqueous transport channels called nuclear pore complexes (NPCs). Each NPC is made up of 30 different proteins termed as nucleoporins (Tran and Wente, 2006). The NPC is one of the largest macromolecules in the cell with an average molecular mass of 60-90 MDa (Rout et. al., 2000). The major components of the central scaffold of the NPC include the core scaffold NUP107-160 subcomplex, the inner ring (includes NUP98) and the central channel NUP62 complex (Figure 1-5). The NUP107-160 subcomplex is located on both nuclear and cytoplasmic sides as rings. On one side of the central scaffold are the cytoplasmic fibers, which include NUP358 and NUP214. On the nucleoplasmic side, NUP153, NUP50 and TPR form eight filament extensions and the distal ring, known as the nuclear basket. The fiber extensions on both sides create a permeability barrier, acting as gates for controlled import and export of macromolecules larger than 5 nm/40 kDa (Hullsman et. al. 2012, Mohr et. al. 2009). Molecules measuring less than 5 nm or smaller than 40 kDA can diffuse freely between the nucleus and cytoplasm.
The Nuclear Pore Complex (NPC) consists of 30 nucleoporin proteins arranged in an octahedral formation. The key structural components of the nuclear pore complex are the central scaffold, which consists of central channel NUP62 complex, the inner ring (includes NUP98) and the core scaffold NUP107-160 subcomplex. On either sides of the central scaffold are the cytoplasmic fibers, which include NUP358 (not shown) and NUP214, and the nuclear basket, which includes NUP153, NUP50 (not shown) and TPR (not shown). Arrows through the central scaffold indicate nuclear import and export.

**Figure 1—5: Structure of the nuclear pore complex**
1.3.6.1 Nucleoporin Associated Chromatin

One key function of NPCs is transport of cellular material between the cytoplasm and nucleus (Hoelz et al., 2011). In recent years, it has become apparent that nuclear transport is not the sole function of the NPC. Studies in mammalian cells, yeast and Drosophila have identified a role for NPCs in gene regulation, including chromosome organization, chromatin loop formation, chromatin boundary formation, and prevention of heterochromatin spreading into euchromatic regions (Figure 1-4) (Ishii et al. 2002; Dilworth et al. 2005; Capelson et al., 2010; Mendjan et al., 2006; Vaquerizas et al., 2010). In mammals, NUP107, NUP62, NUP98 and NUP153 have been identified as chromatin-interacting nucleoporins. NUP107-160 and NUP62 complex proteins also associate with spindles and kinetochores (Favreau et al., 1996), where they play a role in relocating and/or reorganizing chromatin upon nuclear membrane reformation at the end of mitosis (Kalverda et al., 2010). NUP98 plays a role during human embryonic stem (ES) cells differentiation to neural progenitor cells, altering chromatin structure to an active state with different binding targets in each cell type (Light et al. 2013). For example, induction with interferon gamma results in NUP98-dependent target gene activation accompanied by H3K4me2 and RNAPII enrichment at target gene promoters. NUP153 has documented roles in gene activation and repression. For example, in Drosophila NUP153 interacts with the dosage compensation complex for two-fold hyperactivation of the male X chromosome (Mendjan et. al., 2006). In mouse ES cells, NUP153 binds to and represses key developmental genes. Upon Nup153 depletion, these genes are reactivated, promoting differentiation (Jacinto et. al., 2015). Further studies are required to determine the precise function of nucleoporins in the regulation of gene expression as well as the organization of chromatin domains.
1.4 Genomic imprinting

Genomic imprinting is an epigenetic process that restricts expression of specific genes to the maternally or paternally inherited allele; whereas the opposite parental copy is silent (Figure 1-6). The first evidence of genomic imprinting in mouse came from nuclear transplantations (Barton et al., 1984; McGrath and Solter, 1983; McGrath and Solter, 1984; Surani et al., 1984). Embryos generated using two female pronuclei (gynogenetic embryos) exhibit normal embryonic development, but are defective in extraembryonic tissue development, whereas embryos generated using two male pronuclei (androgenetic embryos) have well-developed extraembryonic tissues but are defective in embryonic tissue development. These results indicate that both the maternal and paternal genomes are essential for development of the embryo and that the two parental genomes are not functional equivalents, expressing different set of genes. Currently, over 100 genes in mice and humans are known to be regulated by genomic imprinting (Bartolomei and Ferguson-Smith 2011). Often, these imprinted genes are located within imprinted domains that are controlled from a single germline differentially methylated region (gDMR). gDMRs reside within CG dinucleotide dense regions, known as CpG islands. Imprinted domains can range in size from single retrotransposed gene to large regions spanning up to ~3 Mb. In the mouse, 21 maternal and 3 paternal gDMRs have been identified. Imprinted gDMRs are referred to as imprinting control regions (ICRs) if gDMR deletion results in a domain-wide loss of imprinting (Spahn and Barlow 2003). Parental-specific DNA methylation of gDMRs/ICRs is established in the germline and maintained during preimplantation development (MacDonald and Mann 2014). Histone modification are also associated with gDMRs/ICRs, including the repressive marks, H3K9me3, H3K27me3 and H4K20me3, and active modifications, such as H3K4me2/3 (McEwen and Ferguson-Smith 2010). Thus, imprinted domains have bivalent chromatin, with the unmethylated gDMR possessing active euchromatic epigenetic modifications, while the silent methylated gDMR bears repressive epigenetic modifications of heterochromatin. Overall, an intriguing aspect of imprinted domains is how the allelic duality of chromatin states is regulated.
Biallelic expression from maternal and paternal alleles is a common characteristic for most genes within the genome. A small subset of genes display monoallelic parental-specific expression. They are either expressed from the maternal allele (red) and are paternally silent (grey), or are expressed from the paternal allele (blue) and are maternally silent (grey). This monoallelic expression may be due to repressive histone modifications or DNA methylation at the promoters of these genes only on the silent parental allele, while the active allele exhibits active histone modifications and no DNA methylation.

**Figure 1—6: Representation of imprinted genes**

Biallelic expression from maternal and paternal alleles is a common characteristic for most genes within the genome. A small subset of genes display monoallelic parental-specific expression. They are either expressed from the maternal allele (red) and are paternally silent (grey), or are expressed from the paternal allele (blue) and are maternally silent (grey). This monoallelic expression may be due to repressive histone modifications or DNA methylation at the promoters of these genes only on the silent parental allele, while the active allele exhibits active histone modifications and no DNA methylation.
Long noncoding RNAs are a key feature of imprinted domains and play a key role in regulating imprinted expression along with higher order chromatin structure. Imprinted long ncRNAs range in length from ~1.6 kb to ~1000 kb, and thus, many imprinted long ncRNAs are also classified as macro ncRNAs due to their extraordinary length (Guenzlh and Barlow 2012). Generally, long ncRNAs are associated with gene repression. To date, seven long ncRNAs have been identified that are regulated by imprinted gDMRs and are conserved between mice and humans; Airn, Gtl2, H19, Kcnq1ot1, Nespas, Peg13, and Ube3a-as (Brannan et al. 1990; Gray et al. 1999; Smilinich et al. 1999; Lyle et al. 2000; Paulsen et al. 2001; Coombes et al. 2003; Court et al. 2014). The proposed mechanisms by which ncRNAs act on imprinted domain include transcription interference, coating of the domain, and ncRNA-mediated silencing.

Currently, there are two models of imprinted domain regulation. The first is an ICR-mediated enhancer-blocking mechanism. The second is through a ncRNA-mediated mechanism. Two domains are presented as models below, the H19 domain and the Airn domain.

1.4.1 H19 domain

The H19 domain is a well-characterized example of an ICR-mediated enhancer-blocking mechanism. The H19 imprinted domain contains the paternally methylated ICR, two paternally methylated secondary somatic DMRs [DMR1 proximal to insulin-like growth factor 2 (Igf2) and DMR2 within Igf2], the maternally-expressed H19 long ncRNA gene, the paternally-expressed protein-coding genes, Igf2 and insulin II (Ins2), and the insulin-like growth factor 2 antisense (Igf2as) gene (Constancia et al. 2000; Murrell et al. 2001) (Figure 1-7). The H19 ncRNA is 2.3 kb in length. On the maternal allele, CTCF binds the unmethylated ICR, acting as an enhancer blocker, that directs maternal expression of H19 (Lopes et al. 2003; Fedoriw et al. 2004), and maintains the maternal ICR in an unmethylated state (Schoenherr et al. 2003). Together with cohesin, CTCF mediates chromatin looping such that the maternal ICR interacts with DMR1 to isolate Igf2 and Ins2 away from the enhancers, and protects somatic DMRs from becoming methylated on the maternal allele (Figure 1-8) (Lopes et al. 2003; Yoon et al. 2007; Li et al. 2008a; Nativio
et al. 2009). On the paternal allele, DNA methylation prevents CTCF binding the H19 ICR, leading to paternal expression of Igf2 and Ins2 (Leighton et al. 1995; Kurukuti et al. 2006; Yoon et al. 2007).

The H19 ICR is critical for mediating parental-specific chromatin loops and enhancer insulator function. Maternal inheritance of a H19 ICR deletion results in a loss of insulator function and biallelic Igf2 and Ins2 expression, while paternal inheritance of a H19 ICR deletion results in biallelic H19 expression and reduced Igf2 expression (Leighton et al. 1995; Thorvaldsen et al. 1998). Targeted deletion of CTCF binding sites and point mutations within the maternal H19 ICR abrogates CTCF binding, leads to a loss in H19 expression, as well as activation of the normally silent maternal Igf2 allele (Engel et al., 2006; Pant et al., 2003; Pant et al., 2004). However, CTCF depletion did not yield changes to imprinted regulation of the H19 ncRNA (Lin et al. 2011). Furthermore, depletion of cohesin proteins in mouse embryonic fibroblasts resulted in increased Igf2 expression, yet Igf2 remained paternally expressed, suggesting that additional mechanisms beyond chromatin looping are required for the regulation of allelic expression (Lin et al. 2011). In humans, chromatin topology changes occur at the H19 imprinted domain in Beckwith-Wiedemann Syndrome and Silver-Russell Syndrome patients with imprinting errors (Nativio et al. 2011). Beckwith-Wiedemann Syndrome patients with a gain of maternal H19 ICR DNA methylation have altered chromatin looping, bringing the downstream enhancer into proximity with the maternal Igf2 gene, while Silver-Russell Syndrome patients with a loss of paternal H19 ICR DNA methylation have a chromatin loop that sequesters the downstream enhancer away from paternal Igf2 and into proximity with H19 (Nativio et al. 2011). Such alterations to chromatin looping are likely the consequence of subtopological changes.
**Figure 1—7: Structure and regulation of the H19 imprinted domain**

The H19 domain is located on the mouse chromosome 7 and is a well-studied example of the ICR-mediated enhancer-block mechanism. On the maternal allele, the ICR is unmethylated (open circles), allowing the enhancers (E, enhancer) to access the H19 promoter, enabling H19 ncRNA is expressed. CTCF blocks the enhancers from accessing the Igf2 and Ins2 genes. On the paternal allele, the H19 ICR is methylated (closed circles), inhibiting CTCF binding, thereby silencing paternal H19 expression. The enhancers can now access the Igf2 and Ins2 promoters, activating these genes. DMR1 acts a silencer while DMR2 is an enhancer that is activated upon methylation. Igf2 and Ins2 are expressed.
On the maternal allele, CTCF binds to the unmethylated ICR and DMR2, thereby generating a loop that prevents *Igf2* from interacting with the downstream enhancer. This loop brings the enhancers in close proximity to the *H19* promoter, promoting H19. On the paternal allele, CTCF is unable to bind to the methylated *H19* ICR, allowing *Igf2* and *Ins2* to interact with the downstream enhancer, enabling their expression.

**Figure 1—8: ICR-mediated enhancer-blocking model for the *H19* imprinted domain**

On the maternal allele, CTCF binds to the unmethylated ICR and DMR2, thereby generating a loop that prevents *Igf2* from interacting with the downstream enhancer. This loop brings the enhancers in close proximity to the *H19* promoter, promoting H19. On the paternal allele, CTCF is unable to bind to the methylated *H19* ICR, allowing *Igf2* and *Ins2* to interact with the downstream enhancer, enabling their expression.
1.4.2  *Airn* domain

The antisense to *Igf2* receptor ncRNA domain (*Airn*) imprinted domain is localized on mouse chromosome 17 and serves as an example of noncoding RNA-mediated silencing. The *Airn* domain consists of a maternally methylated ICR, paternally expressed 118 kb *Airn* macro ncRNA and maternally expressed protein-coding genes which include insulin-like growth factor 2 receptor (*Igf2r*), solute carrier family 22, member 2 (*Slc22a2*) and solute carrier family 22, member 3 (*Slc22a3*) (Figure 1-9). The *Airn* ICR is located in intron 2 of the *Igf2r* gene. On the maternal allele, the *Airn* ICR is methylated, repressing *Airn* transcription, and *Igf2r*, *Slc22a2* and *Slc22a3* are expressed. On the paternal allele, the *Airn* ICR is unmethylated, and the *Airn* ncRNA is expressed. *Airn* is transcribed antisense to *Igf2r* through its promoter, thereby interfering with RNA polymerase II binding (Stricker 2008) and silencing *Igf2r* in the embryo and placenta. *Slc22a2* and *Slc22a3* are positioned upstream of of *Airn*. *Airn* coats the domain and recruits silencing factors, repressing *Slc22a2* and *Slc22a3* transcription in the placenta. Paternal deletion of the *Airn* ICR, including the embedded *Airn* promoter, results in paternal allelic reactivation of the three imprinted genes (Sleutels et al., 2002). Similarly, truncation of the paternal *Airn* transcript by insertion of a polyadenylation signal results in reactivates the paternal *Igf2r*, *Slc22a2* and *Slc22a3* alleles, pointing to the requirement of the *Airn* ncRNA in mediating silencing (Sleutels et al., 2002). Three mechanisms have been proposed for *Airn* ncRNA function. First, *Airn* transcription through the *Igf2r* promoter interferes with *Igf2r* transcription. Second, *Airn* coats the paternal allele, promoting heterochromatin formation. Lastly, the *Airn* ncRNA act as adaptors to recruit silencing factors, inducing repression of neighbouring genes. Biotin-based labeling and purification studies have shown that *Airn* interacts with the paternal *Slc22a3* promoter, hundreds of kilobases away from the *Airn* transcription start site, and further recruits G9a, a histone H3K9 methyltransferase to the paternal *Slc22a3* promoter, thereby silencing upstream genes (Nagano et al., 2008). Thus, all three mechanisms for the *Airn* ncRNA may be operating at this domain.
Figure 1—9: Structure of the Airn imprinted domain

Schematic representation of the Airn domain in mouse. On the paternal allele, the Airn ICR is unmethylated, allowing expression of the Airn ncRNA (blue wavy arrow), which results in neighbouring protein-coding gene silencing (black boxes). On the maternal, the Airn ICR is methylated, silencing the embedded Airn promoter and its transcription, and thereby permitting maternal expression of Ig2r, Slc22a2 and Slc22a3. Igf2r is maternally expressed in the embryo and placenta, while Slc22a2 and Slc22a3 are maternally expressed only in placenta and are biallelic expressed in the embryo. Arrows indicate direction of transcription.
1.4.3 *Kcnq1ot1* imprinted domain

The *Kcnq1ot1* imprinted domain spans a 1 Mb-region in mice and is characterized by a maternally methylated ICR (also known as the KvDMR), paternally expressed *Kcnq1ot1* (*Kcnq1* opposite transcript 1) long ncRNA, nine maternally expressed protein-coding genes and five genes that escape imprinted regulation (Figure 1-10) (Paulsen et al., 1998; Smilinich et al., 1999; Umlauf et al., 2004). The maternally expressed, protein-coding genes within this domain include oxysterol binding protein-like 5 (*Osbpl5*), pleckstrin homology-like domain, family A, member 2 (*Phlda2*), solute carrier family 22, member 18 (*Slc22a18*), cyclin-dependent kinase inhibitor 1C (*Cdkn1c*), potassium voltage-gated channel, subfamily Q, member 1 (*Kcnq1*), tumor-suppressing subchromosomal transferable fragment 4 (*Tssc4*), CD81 antigen (*Cd81*), achaete-scute complex homolog 2 (*Ascl2*) and tyrosine hydroxylase (*Th*). On the maternal allele, the *Kcnq1ot1* ICR is methylated, silencing the embedded *Kcnq1ot1* promoter and its transcription, and thereby permitting expression of neighbouring genes. On the paternal allele, the *Kcnq1ot1* ICR is unmethylated, allowing *Kcnq1ot1* ncRNA transcription, which results in neighbouring protein-coding gene silencing. Paternal inheritance of *Kcnq1ot1* ICR deletions lead to paternal reactivation of imprinted genes within the domain (Fitzpatrick et al., 2002; Mancini-Dinardo et al., 2006). Similarly, paternal transmission of a *Kcnq1ot1* truncation, which was generated by insertion of a polyadenylation site 2.6 kb from the transcription start site, leaving the *Kcnq1ot1* ICR intact, results in paternal allelic reactivation (Shin et al., 2008). However, when the deletions or truncation are maternally transmitted, there is no effect on *Kcnq1ot1* domain regulation (Fitzpatrick et al., 2002; Mancini-Dinardo et al., 2006; Shin et al., 2008). These results suggest that elements within the ICR and the *Kcnq1ot1* ncRNA and/or its transcription are essential for paternal allelic silencing of imprinted genes within the *Kcnq1ot1* domain. Consistent with its DNA methylated status, the maternal *Kcnq1ot1* ICR also possesses repressive histone modifications (H3K9me2/3 and H3K27me3) (Lewis et al., 2004). By comparison, the unmethylated paternal ICR, which harbours the active *Kcnq1ot1* ncRNA promoter, is marked by H3K4me3. The paternally silent protein-coding genes also carry repressive histone modifications (H3K9me2/3 and H3K27me3).
A number of mechanisms have been proposed to regulate the \textit{Kcnq1ot1} imprinted domain. One mechanism, which is similar to the \textit{H19} domain, is the ICR-mediated enhancer blocking mechanism (Fitzpatrick et al., 2007; Lin et al., 2011). Using reporter assays, the \textit{Kcnq1ot1} ICR has been shown to act as an insulator when introduced between a promoter and enhancer for a gene (Mohammad et al., 2008). In addition, in an episomal system, a region within the \textit{Kcnq1ot1} ICR has been identified to have silencer activity, bidirectionally repressing adjacent genes in a position independent manner. Point mutations in this minimal repressive region result in loss of bidirectional silencing (Kanduri et al., 2002; Thakur et al., 2003; Thakur et al., 2004). At the paternal \textit{Kcnq1ot1} domain, CTCF binds to the unmethylated \textit{Kcnq1ot1} ICR at two CTCF recognition sites, thereby restricting access of the enhancer element to upstream genes, for example \textit{Cdkn1c}, thereby inducing their silencing (Fitzpatrick et al., 2007; Lin et al., 2011; Shin et al., 2008). However, it is unknown if this enhancer functions to promote \textit{Kcnq1ot1} expression (Fitzpatrick et al., 2007). In contrast, other studies have found that CTCF binding occurs at both the maternal and paternal \textit{Kcnq1ot1} ICRs and is dispensable for enhancer-blocking mechanisms (Lin et al., 2011). Similar to the \textit{Airn} domain, a second model proposes that the \textit{Kcnq1ot1} ncRNA has the ability to interact, bind and recruit various polycomb repressive complex proteins to chromatin within the domain, thereby inducing silencing via repressive histone modifications (Mancini-Dinardo et al., 2006). In support of this, the \textit{Kcnq1ot1} ncRNA has been reported to interact with promoters of the imprinted genes within the domain, leading to the incorporation of repressive histone modifications (Pandey et al., 2008). Several epigenetic factors, including the polycomb repressive complex 2, histone methyltransferase G9a, and DNA methyltransferase 1, have been shown to interact with \textit{Kcnq1ot1} ncRNA (Kaneko et al., 2014; Mohammad et al., 2008; Pandey, 2008; Terranova et al., 2008; Zhao et al., 2010). Finally, it has been proposed that active transcription of the \textit{Kcnq1ot1} gene leads to paternal allelic silencing of neighboring genes in the domain rather than the \textit{Kcnq1ot1} transcript itself (Golding et al., 2011). In the case of all three proposed mechanisms, it is still unclear how the \textit{Kcnq1ot1} ICR performs its function and how the \textit{Kcnq1ot1} domain is fully regulated.
Figure 1—10: Structure of the Kcnq1ot1 imprinted domain

Schematic representation of the Kcnq1ot1 domain in mouse. On the paternal allele, the Kcnq1ot1 ICR is unmethylated, allowing expression of the Kcnq1ot1 ncRNA, which results in neighbouring protein-coding gene silencing. On the maternal allele, Kcnq1ot1 ICR is methylated, silencing the embedded Kcnq1ot1 promoter and its transcription, and thereby permitting expression of neighbouring genes. Arrows indicate the direction of transcription.
1.4.3.1 Tissue-specific regulation of the *Kcnq1ot1* domain

One characteristic of imprinted genes is that they display tissue-specific and developmental stage-specific imprinted expression. The *Kcnq1ot1* domain exhibits both tissue-specific and developmental stage-specific regulation. Between embryonic and extraembryonic lineages, there is a clear difference between imprinted regulation of the protein-coding genes. At the *Kcnq1ot1* domain, genes are classified by their expression during midgestation development as inner/ubiquitously imprinted genes (i.e. imprinted expression in embryonic and placental tissue), *Phlda2*, *Sle22a18*, *Cdkn1c*, *Kcnq1* and *Kcnq1ot1*, and outer placental-specific imprinted genes, *Osbpl5*, *Nap1l4*, *Tssc4*, *Cd81*, *Ascl2* and *Th* (Lewis et al. 2004; Umlauf et al. 2004; Golding et al. 2011; Mohammad et al. 2012). Several mechanism have been identified that may account for this differential tissue-specific regulation. In the placenta, the *Kcnq1ot1* long ncRNA transcript interact with the polycomb complex PRC2 and histone methyltransferase G9a (Pandey et al. 2008). This interaction is not observed in embryonic liver. Furthermore, in the placenta, the *Kcnq1ot1* long ncRNA interacts with the *Osbpl5*, *Cdkn1c*, *Kcnq1*, *Cd81* and *Ascl2* promoters, providing a functional role in directing the repressive epigenetic marks H3K27me3 and H3K9me2 to paternally silent genes (Pandey et al. 2008; Terranova 2008; Umlauf 2004). Interestingly, on a *Dnmt1*-deficient background, the maternal *Kcnq1ot1* ICR becomes unmethylated (Lewis 2004; Tanaka 1999), resulting in reduced maternal expression as well as reactivation of paternal alleles of the *Phlda2*, *Sle22a18*, and *Cdkn1c* genes in the both placenta and embryo. By comparison, *Osbpl5*, *Kcnq1*, *Tssc4*, *Cd81* and *Ascl2* maintain maternal allelic expression and paternal allelic silencing, indicating that in the placenta, these genes are regulated in a DNA methylation-independent manner. Finally, the paternal *Kcnq1ot1* domain is larger than the maternal *Kcnq1ot1* domain (Redrup et al., 2009; Terranova et al., 2008). The size of paternal *Kcnq1ot1* domain differs between lineages, such that in the placenta, the paternal *Kcnq1ot1* domain is larger compared to the paternal *Kcnq1ot1* domain in the embryo (Redrup et al., 2009). This is possibly due to the larger subset of genes with paternal allelic silencing in the placenta. All together, these data point to differential regulation of the *Kcnq1ot1* domain in embryonic and extraembryonic lineages. Currently, it is unclear when embryonic and extraembryonic lineages acquired
differential imprinted domain regulation and how this differential regulation is regulated during early stages of development.

During early development, three distinct cell lineages emerge, such that at the early blastocyst stage, there are epiblast precursor, trophectoderm and primitive endoderm cells (Figure 1-11). These cells will give rise to the fetus, placenta and yolk sac, respectively. Pluripotent stem cells can be derived from these three lineages to produce embryonic (ES), trophoblast (TS) and extraembryonic endoderm (XEN) stem cells (Figure 1-11). At this early developmental time point, XEN, ES and TS cells have distinct imprinted expression patterns for genes in the Kcnq1ot1 imprinted domain (Golding et al., 2011; Lewis et al., 2004; Lewis et al., 2006; Terranova et al., 2008; Umlauf et al., 2004) (Figure 1-12). For example, Osbpl5 is maternally expressed in XEN cells, but biallelically expressed in ES and TS cells. These expression patterns suggest that the Kcnq1ot1 imprinted domain must be differential regulated even at the early stage of lineage differentiation. However, the mechanisms responsible for this differential imprinted expression between the three lineages are still not clear.
The blastocyst is composed of three distinct cell lineages. Stem cells can be derived from these lineages, such that epiblast precursor cells (inner cell mass) gives rise to embryonic stem cells (ES), the trophectoderm lineage gives rise to trophoblast stem cells (TS) and the primitive endoderm gives rise to extraembryonic endoderm stem cells (XEN). ES, TS and XEN cells can be cultured in vitro and represent characteristic features of the lineage from which they are derived.

**Figure 1—11: Derivation of ES, TS and XEN stem cells from a blastocyst**
Parental-specific expression patterns differ for genes in the Kcnq1ot1 domain in XEN, ES and TS cells. For example, Osbpl5 is maternally expressed in XEN cells, but biallelically expressed in ES and TS cells, while Acsl2 is maternally expressed in TS cells, but biallelically expressed in ES and XEN cells. Red boxes, maternal expression; blue boxes, paternal expression; black boxes, silent; green boxes, biallelic expression; light blue box, maternal biased expression with less than 30% expression from the paternal allele; blue wavy line represents the Kcnq1ot1 ncRNA; black circle, methylated CpGs; white circle, unmethylated CpGs.
1.5 Beckwith-Wiedemann Syndrome

Perturbations in genomic imprinting can lead to imprinting defects that cause misregulation of imprinted domains and can lead to imprinting disorders (Plasschaert and Bartolomei, 2014). One such disorder is Beckwith-Wiedemann Syndrome (BWS) which occurs at an incidence of 1 in 13,700 (Weksberg et al., 2001). BWS is characterized by macroglossia, neonatal hypoglycemia, abdominal wall defects, and postnatal growth above the ninetieth percentile (Weksberg et al., 2005). BWS result from genetic and epigenetic defects at the \( KCNQ1OT1 \) and \( H19 \) imprinting domains. Of these patients, 50% exhibit a loss of DNA methylation on the maternal \( KCNQ1OT1 \) ICR resulting in bialleic expression of the \( KCNQ1OT1 \) ncRNA along with silencing of maternal-expressed genes within the domain, including \( CDKN1C \) and \( KCNQ1 \). By comparison, 5% of BWS patients have a gain of methylation at the \( H19 \) ICR that results in loss of maternal \( H19 \) ncRNA expression and reactivation of the maternally silent \( IGF2 \) allele. Therefore, it is critical to understand the mechanisms that regulate imprinted domains, including at the \( Kcnq1ot1 \) domain.

1.6 Functional screen for epigenetic factors regulating the \( Kcnq1ot1 \) domain

To identify epigenetic factors that regulate imprinted domains, we performed a novel loss-of-function, positive-selection, RNA interference screen using the \( Kcnq1ot1 \) imprinted domain as a model domain (Landschoot, 2014). For the screen, we used a short-hairpin RNA library for 250 epigenetic factors. XEN cells carrying a paternal-targeted mutation of the \( Cdkn1c \) gene, where exons 1 and 2 were replaced by the PGK-neomycin resistance cassette (\( Cdkn1c^{+/\Delta neoR} \)), were used for the screen (Zhang et al., 1997). Reactivation of the silent \( Cdkn1c^{+/\Delta neoR} \) allele following depletion of epigenetic factors allowed for survival and selection of colonies in the presence of neomycin. Thus, the screen was unbiased with respect to the type of epigenetic factors involved. In total, 41 different epigenetic factors were identified. The screen identified a few factors (PRC2 proteins, EZH2 and RNF2, histone 4 lysine 20 methyltransferase, SUV420H1, and DNMT1) that play a role in the \( Kcnq1ot1 \) domain regulation (Mohammad et al., 2010; Pandey et al., 2008; Pannetier et al.,
2008; Terranova et al., 2008), which attests to the validity of the screen. However, they were recovered at a lower frequency than other candidates. Among of the top four candidates from the screen was nucleoporin 107 (NUP107). The role of nucleoporins in imprinted domain regulation is unknown.

### 1.7 Rationale

The mechanism by which the *Kcnq1ot1* imprinted domain is regulated is poorly understood. More specifically, it is still unclear what regulates paternal *Kcnq1ot1* ncRNA expression as well as paternal allelic silencing of the neighbouring protein-coding genes. Nucleoporin 107 was an exciting candidate that emerged from our screen. Currently, it is not known whether NUP107 and other chromatin-binding nucleoporins (NUP62, NUP98 and NUP153) play a role in regulating the *Kcnq1ot1* domain imprinting in XEN cells. Moreover, the mechanisms responsible for regulating the *Kcnq1ot1* domain in the three lineages of the preimplantation embryo are poorly understood. ES, TS and XEN stem cells serve as an excellent model to study these three lineages. They are free from maternal contamination and allow us to perform genetic manipulations. The work presented here is the first investigation of nucleoporins as an epigenetic factor regulating the *Kcnq1ot1* imprinted domain. It is also the first to investigate whether nucleoporins have a role in regulating the *Kcnq1ot1* imprinted domain in different lineages from the blastocyst stage embryo.

### 1.8 Hypothesis

I hypothesize that nucleoporins interact with the paternal *Kcnq1ot1* ICR at the nuclear periphery allowing for *Kcnq1ot1* ncRNA transcription. This in turn will enable silencing of neighbouring genes in the domain-wide manner in extraembryonic endoderm stem cells. Given that the imprinted genes within the *Kcnq1ot1* domain exhibit differential paternal allelic silencing in embryonic, extraembryonic endoderm and trophoblast stem cells, I further hypothesize that nucleoporins will have different regulatory roles or may act on different sets of genes within the *Kcnq1ot1* imprinted domain in ES, TS and XEN cells.
1.8.1 Objectives

1. Determine whether NUP107 and other nucleoporins regulate imprinting at the \textit{Kcnq1ot1} domain in extraembryonic endoderm stem cells.

2. Determine the role of nucleoporins in regulation of imprinting at the \textit{Kcnq1ot1} domain in embryonic and trophoblast stem cells.

For both objectives, I will determine whether NUP107 as well as other NUPs

a. regulate paternal \textit{Kcnq1ot1} ncRNA expression.

b. play a role in \textit{Kcnq1ot1} domain positioning at the nuclear periphery.

c. bind within the \textit{Kcnq1ot1} domain.

d. regulate paternal allelic silencing of imprinted genes within the \textit{Kcnq1ot1} domain.

e. regulate the DNA methylation status of the \textit{Kcnq1ot1} ICR and histone modifications at the \textit{Kcnq1ot1} ICR and promoters of imprinted genes.

f. interact with CTCF and cohesin proteins at the \textit{Kcnq1ot1} domain.
Chapter 2

2 Methods

2.1 Cell culture, treatments, transfection and transduction

B6XCAST XEN, ES and TS cells were generated and cultured as described (Golding et al., 2010). XEN, ES and TS cells were cultured on a gelatin-coated feeder-free environment prior to experiments to avoid feeder contamination (Golding et al., 2011). For biological replicates, ES, TS and XEN cells derived from different embryos were used. Three technical replicates from each sample were performed. Where specified, cells were synchronized in G1 phase by treatment with 2 mM hydroxyurea (Sigma) followed by siRNA transfection. Cells were transfected with siRNAs using siPepMute (SignaGen). siRNA sequences are listed in Table 2-1. For nuclear import experiments, cells were transfected with E47-RFPNLS construct using Lipofectamine2000 (Invitrogen), followed by siRNA transfection. As a positive control, cells were treated with 10 µM of ivermectin (Sigma) for 48 hours. Recombinant lentiviral particles with shRNA targeting Luciferase with a green fluorescent protein (GFP) and puromycin resistance gene were generated in HEK293 cells (Golding et al., 2010). Following transduction and successful rounds of selection with puromycin, GFP-positive XEN cell populations were collected. To assess growth rates, ~25 000 cells were seeded, and then transfected 12 hours later with siRNAs. Direct cell counts were performed every 12 hours using a hemacytometer (VWR). Three replicates each were performed for control and Nup-depleted cells.

2.2 RNA Isolation, cDNA preparation and PCR amplification

RNA was isolated using PureLink® RNA Mini Kit (Life Technologies) and QuickRNA™ MicroPrep (Zymo Research) according to manufacturers’ instructions. Before cDNA preparation, total RNA was subjected to DNase I (Life Technologies) treatment as described (Golding et al., 2011). cDNA was prepared using ProtoScript® II Reverse Transcriptase (NEB) as per instructions with oligodT (Sigma) and Random Primers (Life Technologies). cDNA was treated with RNaseA (Sigma) after preparation to remove residual RNA. PCR was performed on C1000 and MJ Research Thermocyclers (BioRad).
2.3 Quantitative PCR analysis

Quantitative (q) PCR was performed as described (Golding et al., 2011). Briefly, qPCR was performed using iQ SYBR Green Supermix (BioRad) and SensiFAST™ SYBR® No-ROX Kit (Bioline) on a MJ Thermocycler Chromo4 Real-time PCR system. For gene expression analysis, data were analyzed using the ΔΔCT method (Schmittgen and Livak, 2008). For ChIP-qPCR data analysis was performed as described (Kernohan et al., 2010). Table 2-3 lists primers, annealing temperatures and amplicon sizes.

2.4 Allelic expression analysis

Allelic expression analysis to differentiate between maternal (B6) and paternal (CAST) allele was performed by restriction digestion of PCR product followed by separation on an 8% acrylamide (29:1, Bio-Shop) gel. Densitometry was performed to quantify band intensity using QuantityOne 1-D Analysis Software (BioRad). Parental allele-specific expression was determined as a percent of B6 or CAST expression relative to the total expression. Table 2-2 lists SNPs, enzymes and fragment sizes.

2.5 RNA/DNA fluorescence in situ hybridization, immunocytochemistry and confocal microscopy

*Kcnq1ot1* RNA/DNA FISH probe was generated using fosmid W11-2505B3 in the *Kcnq1* intronic region (CHORI) using the BioPrime DNA labeling System (Invitrogen) with fluorescein-12-dUTP (Roche) and Biotin-12-dUTP (Roche) for RNA FISH, and Cy5-UTP (GE Healthcare) for DNA FISH as described (Golding et al., 2011). For RNA FISH, cells seeded on coverslips in 12-well plates were fixed, dehydrated followed by hybridization with RNA-FISH probes as described (Golding et al., 2011). Briefly, following overnight incubation at 37°C, coverslips were carefully washed in 4X saline-sodium citrate (SSC) and 2X SSC (Ambion) at 37°C for 5 minutes 3 times each. Coverslips were incubated with primary antibody in blocking buffer (20X SSC, 10% Tween-20, 10% Skim Milk) at 37°C for 1 hour in the dark, then washed 3 times with 4X SSC (37°C) with agitation. Next, coverslips were incubated with the secondary antibody in blocking buffer at 37°C in the dark for 1 hour, then washed 3 times with 4X SSC and 2 times with 2X SSC buffers at
37°C with continuous agitation, 5 minutes each. The final wash was done in 1X SSC for 5 minutes. Coverslips were mounted on glass slides with Vectashield DAPI (antifade mounting medium, Vector labs, H-1000) and stored in the dark for a few hours or overnight at 4°C. For DNA/RNA FISH, DNA FISH was first performed as described (Korostowski et al., 2011) followed by RNA FISH. Biotin-labeled oligo-dT-50 (Life Technologies) was used for polyA-mRNA FISH. Coverslips were imaged using z-stacks on a FluoView FV1000 coupled to an IX81 motorized inverted system (Olympus). Fluorescence signal volume, nuclear periphery distance from the signal centroid and fluorescence levels were measured using Volocity (PerkinElmer) and ImageJ. Table 2-3 lists antibody dilutions.

2.6 Bisulfite mutagenesis and sequencing

Control and siRNA treated cells (20% confluent) were seeded on gelatin-coated 6-well dishes. Forty-eight hours after transfection, cells were washed once with 1X PBS (Sigma) followed by a 5-minute incubation with 1X Trypsin-EDTA (Sigma) in PBS. Trypsin was inactivated by addition of RPMI medium. Detached cells were collected and pelleted gently, washed and re-suspended in 1X PBS. One percent of cells (~10,000 cells) were embedded into a 2:1 3% LMP agarose (Sigma) and lysis solution as described (Denomme et al., 2011). Bisulfite mutagenesis was performed as described for Kcnq1ot1 amplification in XEN cells (Denomme et al., 2011). For first round PCR amplification, the XEN cell agarose bead with bisulfite converted DNA (10 µL) was added to Hot Start Ready-To-Go PCR bead (GE Healthcare) containing 0.2 µM Kcnq1ot1 external primers, 9.6 ng/mL transfer RNA with a mineral oil overlay. First round PCR product (5 µL) was added to PCR beads containing 0.2 µM Kcnq1ot1 internal primers. See Table 2-2 for primers.

2.7 RNA stability assay

Kcnq1ot1 half-life studies were performed by addition of 2 µg/mL of actinomycin D (Sigma) to growth medium according to Hazan-Halevy et al. (2010) followed by collection at time-intervals up to 12 hours. RNA was extracted and cDNA was synthesized as described above. Kcnq1ot1 ncRNA levels were normalized to time 0 hours.
2.8 Chromatin Immunoprecipitation (ChIP) assay

ChIP was performed as described with modifications for siRNA depletions (Kernohan et al., 2010). Equal numbers of cells were collected from control and siRNA treatment groups (~0.5-1.2 million cells). DNA extraction was performed using Chelex beads (BioRad). Table 2-3 lists antibody dilutions. Data are represented as total enrichment (percent input) or allelic enrichment (total enrichment x allelic enrichment ratio).

2.9 Western Blot Analysis and Immunoprecipitation Assay

Cytoplasm and nuclear protein extracts were isolated using cytoplasm extraction buffer and nuclear extraction buffer followed by Western blot analysis as described (Baldwin, 1996; Golding et al., 2010). For list of antibodies and concentrations see Table S2. Protein immunoprecipitation was performed using Dynabeads Protein G (Novex, Life Technologies) as per manufacturers’ instructions. Immunoprecipitated protein samples were subjected to Western blot analysis. Antibodies that were used for Western blot analysis and IP assays are listed in Table 2-3.

2.10 Biotin Immunoprecipitation Assay

Biotin immunoprecipitation assay was performed as described (Hazan-Halevy et al., 2010) with modifications. Briefly, biotin-labeled DNA was incubated with nuclear lysate for two hours at 37°C. Nuclear lysate with biotin-labeled DNA was then incubated with streptavidin antibody (Abcam) conjugated beads (Invitrogen) at 4°C for three hours. Following washes, IP samples were mixed with Western blot loading buffer and subjected to Western blot analysis. See Table 2-3 for list of antibodies.

2.11 Electromobility Shift Assay

Electromobility shift assay was performed as per manufacturers’ instructions (LightShift™ Chemiluminescent EMSA Kit, ThermoFisher Scientific). Briefly, 20-30 µg of nuclear extracted protein was incubated with biotin-labeled DNA and specific antibodies (Table 2-3) for 2 hours at 37°C. Antibody-protein-DNA complexes were resolved on a 8% non-denaturing polyacrylamide gel, transferred onto a nylon membrane and then UV cross-
linked. Shifts and super-shifts were determined using a streptavidin-horseradish peroxidase conjugate and LightShift chemiluminescent substrate.

2.12 Statistical analysis

For statistical analysis, one-tailed Student’s t-test was performed on mean values. Treatment samples were compared to control (Vehicle or siNT). A p-value less than 0.05 was considered to be significant.
Table 2—1: List of siRNA for depletion studies

<table>
<thead>
<tr>
<th>siRNA Target</th>
<th>Type</th>
<th>Supplier</th>
<th>Catalog</th>
<th>Concentration</th>
<th>Transfection Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nup107-A</td>
<td>SMARTpool ON-TARGETplus</td>
<td>Dharmacon</td>
<td>L-065221-01-0005</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup62-A</td>
<td>SMARTpool ON-TARGETplus</td>
<td>Dharmacon</td>
<td>L-064100-00-0005</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup98-A</td>
<td>SMARTpool ON-TARGETplus</td>
<td>Dharmacon</td>
<td>L-060137-01-0005</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>siNT-A</td>
<td>SMARTpool ON-TARGETplus</td>
<td>Dharmacon</td>
<td>D-001810-01-20</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup107-B1</td>
<td>Silencer Select siRNA</td>
<td>Life Technologies</td>
<td>s98083</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup107-B2</td>
<td>Silencer Select siRNA</td>
<td>Life Technologies</td>
<td>s98085</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup62-B1</td>
<td>Silencer Select siRNA</td>
<td>Life Technologies</td>
<td>s70887</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup62-B2</td>
<td>Silencer Select siRNA</td>
<td>Life Technologies</td>
<td>s70885</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup98-B1</td>
<td>Silencer Select siRNA</td>
<td>Life Technologies</td>
<td>s114479</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup98-B2</td>
<td>Silencer Select siRNA</td>
<td>Life Technologies</td>
<td>s114477</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup153-A</td>
<td>Silencer Select siRNA</td>
<td>Life Technologies</td>
<td>s104224</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Nup153-B</td>
<td>Silencer Select siRNA</td>
<td>Life Technologies</td>
<td>s104225</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>siNT-B</td>
<td>Silencer Select siRNA</td>
<td>Life Technologies</td>
<td>4390847</td>
<td>10 nmol</td>
<td>48 h</td>
</tr>
<tr>
<td>Locus</td>
<td>Primers</td>
<td>Annealing Temp (°C)</td>
<td>Amplicon (bp)</td>
<td>SNP Enzyme</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>---------------------</td>
<td>---------------</td>
<td>------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Gene Expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osbpl5</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phlda2</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sicc22a18</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdkn1c</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcnq1t1</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tssc4</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromatin Immunoprecipitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osbpl5 Promoter 1 (Or1)</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phlda2 Promoter 1 (Ph1)</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sicc22a18 Promoter 1 (Sl1)</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solution mutagenesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BIS Outer</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BIS Inner</td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td>57</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 5'CAAGGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'GAGAGTGGTCTTCTTCAACATGGAAGATATG3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2—2: Primer list
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Annealing Temperature</th>
<th>SNP Digestion Enzyme</th>
<th>Amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vim Promoter</td>
<td>F 5' GGTTCCTTGTCCCGACTTGT 3'</td>
<td>58</td>
<td>MluCl</td>
<td>169</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5' CCTCCCTCCCCCTTTGGTCTCTCTT 3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vim Exon 3</td>
<td>F 5' GCCCTGGGAAACTAGTGAGCAT 3'</td>
<td>58</td>
<td></td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>Vim 3'UTR</td>
<td>F 5' GGGGCTGTTTGGGAGATGAGCTT 3'</td>
<td>60</td>
<td></td>
<td>167</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5' GAGAAGTAAGAGGGGTTGGTACAG 3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orai2 Promoter</td>
<td>F 5' GGTTCTGCGACGTCCAGAGAT 3'</td>
<td>60</td>
<td>MluCl</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td>Orai2 Exon 2</td>
<td>F 5' GAGATCTGCTCTGAGCAATGTA 3'</td>
<td>60</td>
<td></td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Orai2 3'UTR</td>
<td>F 5' GTGCGAGGGAGGAGGTGAAGT 3'</td>
<td>60</td>
<td></td>
<td>189</td>
<td></td>
</tr>
<tr>
<td>Shank2 Promoter</td>
<td>F 5' GAGCTTACAGGAGGATGGTCC 3'</td>
<td>60</td>
<td>Bfl</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>Shank2 Exon 4</td>
<td>F 5' GAGACGCCAGCTGCACGTCTCGT 3'</td>
<td>60</td>
<td></td>
<td>179</td>
<td></td>
</tr>
<tr>
<td>Dhc7 promoter</td>
<td>F 5' GGCATTGCTTTGGGAGGAGGATG 3'</td>
<td>60</td>
<td>Bgl</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>Dhc7 Exon3</td>
<td>F 5' GAGGAGGGAGGAGGAGGAGGATG 3'</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dhc7 3'UTR</td>
<td>F 5' CTGCTGGGTGACGTCTGTGAC 3'</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR1</td>
<td>F 5' AAGGACATCACCCACCTCCTCA 3'</td>
<td>58</td>
<td></td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR2</td>
<td>F 5' GAGGTGGCTGGGTCTAACCCTC 3'</td>
<td>60</td>
<td></td>
<td>223</td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR5</td>
<td>F 5' ACAACAAATGGAGGATGAGGCTGA 3'</td>
<td>58</td>
<td></td>
<td>169</td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR6</td>
<td>F 5' GGATCTGCTCCAGCAGTACCACAC 3'</td>
<td>58</td>
<td></td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR7</td>
<td>F 5' AGCATATGGCAGTTGAGTAAGG 3'</td>
<td>58</td>
<td></td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR8</td>
<td>F 5' GAAAGAAGATCTCCCATCCTTATTTT 3'</td>
<td>60</td>
<td></td>
<td>199</td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR9</td>
<td>F 5' ATTGGCTGGGAGGAGGTCTTAACCCTC 3'</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR10</td>
<td>F 5' CCAAAAGAGTTGAGATAGGAGGCTGA 3'</td>
<td>60</td>
<td></td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR11</td>
<td>F 5' GCAGGTGCTGACGCAAAAGGAGGATG 3'</td>
<td>60</td>
<td></td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR12</td>
<td>F 5' GGTAGCTGGGAGGAGGAGGATG 3'</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR13</td>
<td>F 5' ATCTGCTGGGAGGAGGAGGATG 3'</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR14</td>
<td>F 5' GTATCTGCGTCTGCTGCTGCT 3'</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1 ICR15</td>
<td>F 5' TTTTCTATACGGCTGGGACTGCT 3'</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enhancer element 3</td>
<td>F 5' CCAAAACCAAAGGAGGAGGAGGATG 3'</td>
<td>60</td>
<td></td>
<td>193</td>
<td></td>
</tr>
<tr>
<td>Enhancer element 4</td>
<td>F 5' ACAAGGAAGAGGAGGAGGATG 3'</td>
<td>58</td>
<td></td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Enhancer element 5</td>
<td>F 5' GCAAGGAGGAGGAGGATG 3'</td>
<td>58</td>
<td></td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>Enhancer element 6</td>
<td>F 5' GGAGGAGGAGGAGGATG 3'</td>
<td>58</td>
<td></td>
<td>194</td>
<td></td>
</tr>
<tr>
<td>H19 ICR</td>
<td>R 5' CAGGACTCAAAAGGAAGAGGATGCTAC 3'</td>
<td>58</td>
<td>DpnII</td>
<td>398</td>
<td>Verona et. al. 2008</td>
</tr>
<tr>
<td>H19 exon 5</td>
<td>R 5' GCAACTGGTTTGGGAGGAGGATG 3'</td>
<td>58</td>
<td></td>
<td>150</td>
<td>Verona et. al. 2008</td>
</tr>
<tr>
<td>FoxA2 promoter</td>
<td>R 5' TTCTAGAGATCTCAGGGGACTG 3'</td>
<td>58</td>
<td></td>
<td>279</td>
<td></td>
</tr>
<tr>
<td>MyoD promoter</td>
<td>R 5' TGCTGGCTGGGAGGAGATG 3'</td>
<td>58</td>
<td></td>
<td>196</td>
<td></td>
</tr>
</tbody>
</table>
Table 2—3: List of antibodies for Western blot analysis, Immunohistochemistry, RNA FISH, ChIP and IP.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalogue Number</th>
<th>Supplier</th>
<th>Concentration/Dilution</th>
<th>Duration</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Western</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mAb414 - Nuclear Pore Complex</td>
<td>ab24609</td>
<td>Abcam</td>
<td>1:1000</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>NUP98</td>
<td>ab50610</td>
<td>Abcam</td>
<td>1:1000</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>NUP107</td>
<td>ab73290</td>
<td>Abcam</td>
<td>1:1000</td>
<td>1 hr</td>
<td>RT</td>
</tr>
<tr>
<td>NUP153</td>
<td>sc-101545</td>
<td>Santa Cruz Biotech</td>
<td>1:1000</td>
<td>1 hr</td>
<td>RT</td>
</tr>
<tr>
<td>H3</td>
<td>ab1791</td>
<td>Abcam</td>
<td>1:5000</td>
<td>1 hr</td>
<td>RT</td>
</tr>
<tr>
<td>INCENP</td>
<td>IS283</td>
<td>Sigma-Aldrich</td>
<td>1:10,000</td>
<td>1 hr</td>
<td>RT</td>
</tr>
<tr>
<td>GFP</td>
<td>G46-66M</td>
<td>SignalChem</td>
<td>1:3000</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>SMC1</td>
<td>A300-055A</td>
<td>Bethyl Laboratories</td>
<td>1:3000</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>SMC3</td>
<td>A300-060A</td>
<td>Bethyl Laboratories</td>
<td>1:3000</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>α-TUBULIN</td>
<td>sc-8035</td>
<td>Santa Cruz Biotech</td>
<td>1:7000</td>
<td>1 hr</td>
<td>RT</td>
</tr>
<tr>
<td>Anti-Mouse-HRP Secondary</td>
<td>SC-2314</td>
<td>Santa Cruz Biotech</td>
<td>1:5000</td>
<td>1 hr</td>
<td>RT</td>
</tr>
<tr>
<td>Anti-Rat-HRP Secondary</td>
<td>SC-2956</td>
<td>Santa Cruz Biotech</td>
<td>1:4000</td>
<td>1 hr</td>
<td>RT</td>
</tr>
<tr>
<td>Anti-Rabbit-HRP Secondary</td>
<td>G33-62G</td>
<td>SignalChem</td>
<td>1:6000</td>
<td>1 hr</td>
<td>RT</td>
</tr>
<tr>
<td><strong>Immunohistochemistry/RNA FISH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LaminB1 (S-20)</td>
<td>sc-30264</td>
<td>Santa Cruz Biotech</td>
<td>1:1000</td>
<td>1 hr</td>
<td>37</td>
</tr>
<tr>
<td>Anti-Biotin</td>
<td>ab1227</td>
<td>Abcam</td>
<td>1:1000</td>
<td>1 hr</td>
<td>37</td>
</tr>
<tr>
<td>Anti-Rabbit Alexa Fluor® 488</td>
<td>A-1034</td>
<td>Life Technologies</td>
<td>1:1000</td>
<td>1 hr</td>
<td>37</td>
</tr>
<tr>
<td>Anti-Goat Alexa Fluor® 594</td>
<td>A-11080</td>
<td>Life Technologies</td>
<td>1:1000</td>
<td>1 hr</td>
<td>37</td>
</tr>
<tr>
<td><strong>Chromatin Immunoprecipitation / Immunoprecipitation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>ab1791</td>
<td>Abcam</td>
<td>2 µg</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>39159</td>
<td>Active Motif</td>
<td>3 µg</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>ab1220</td>
<td>Abcam</td>
<td>3 µg</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>07-449</td>
<td>Millipore</td>
<td>3 µg</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>RNAPII</td>
<td>sc-889</td>
<td>Millipore</td>
<td>3 µg</td>
<td>3 hr</td>
<td>4</td>
</tr>
<tr>
<td>mAb414 - Nuclear Pore Complex</td>
<td>ab24609</td>
<td>Abcam</td>
<td>4 µg</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>NUP153</td>
<td>sc-101545</td>
<td>Santa Cruz Biotech</td>
<td>4 µg</td>
<td>overnight</td>
<td>RT</td>
</tr>
<tr>
<td>SMC1</td>
<td>A300-055A</td>
<td>Bethyl Laboratories</td>
<td>1.5 µg</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>SMC3</td>
<td>A300-060A</td>
<td>Bethyl Laboratories</td>
<td>1.5 µg</td>
<td>overnight</td>
<td>4</td>
</tr>
<tr>
<td>Anti-Mouse IgG</td>
<td>sc-2029</td>
<td>Santa Cruz Biotech</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Anti-Rabbit IgG</td>
<td>2729s</td>
<td>Cell Signalling</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Anti-Goat IgG</td>
<td>sc-2028</td>
<td>Santa Cruz Biotech</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

* same as corresponding antibody used
Chapter 3

3 Results

The mechanism by which the \textit{Kcnq1ot1} imprinted domain is regulated is poorly understood. More specifically, how paternal \textit{Kcnq1ot1} ncRNA expression and paternal allelic silencing of the neighbouring protein-coding genes are regulated. Nucleoporin 107 was an exciting candidate that emerged from our screen. Given that roles for nuclear pore complex proteins in gene regulation have been reported, I hypothesized that nucleoporins interact with the paternal \textit{Kcnq1ot1} ICR at the nuclear periphery, allowing for \textit{Kcnq1ot1} ncRNA transcription. This in turn will enable silencing of neighbouring genes in the domain-wide manner in extraembryonic endoderm stem cells.

3.1 Nucleoporins regulate \textit{Kcnq1ot1} ncRNA expression in XEN cells

NUP107 was investigated for its role in regulating imprinted gene regulation at the \textit{Kcnq1ot1} domain. In addition, to determine whether imprinted gene regulation was specific to NUP107, three additional nucleoporins, NUP62, NUP98 and NUP153, with documented chromatin association (Jacinto et al., 2015; Kalverda et al., 2010; Light et al., 2013), were also investigated for their role in regulating the \textit{Kcnq1ot1} domain. Wildtype C57BL6 X \textit{Mus musculus castaneus} (B6XCAST) XEN cells were transfected with two sets of \textit{Nup107}, \textit{Nup62}, \textit{Nup98/96} and \textit{Nup153} siRNAs to produce RNA and protein depletion (Figures 3-1), and cells were then assessed for total and allelic-specific \textit{Kcnq1ot1} ncRNA expression. Compared to controls, \textit{Nup107}, \textit{Nup62} and \textit{Nup153} depletion significantly reduced \textit{Kcnq1ot1} ncRNA levels to 0.25, 0.45 and 0.76 times, respectively (Figure 3-2A). By comparison, \textit{Nup98/96} depletion produced a 1.88 times increase in \textit{Kcnq1ot1} ncRNA levels. For all nucleoporin depletions, except for \textit{Nup153}, the \textit{Kcnq1ot1} ncRNA maintained paternal-specific expression, indicating that nucleoporin function is specific to the paternal allele (Figure 3-2B). Interestingly, for \textit{Nup153} depletion, the normally silent maternal \textit{Kcnq1ot1} ncRNA was reactivated (24%), suggesting that NUP153 functions on both parental alleles.
A

- Relative Nup107 expression level
- Relative Nup62 expression level
- Relative Nup98 expression level
- Relative Nup153 expression level

B

- NUP107 levels
- NUP62 levels
- NUP98 levels
- NUP153 levels

* = p < 0.05

Hours after actinomycin D treatment

WT Vehicle siNT siNup107

WT Vehicle siNT siNup62

WT Vehicle siNT siNup98

WT Vehicle siNT siNup153

Kcnq1ot1 ncRNA levels
Figure 3—1: Nucleoporin depletion levels in XEN cells.

(A) Nucleoporin RNA depletion levels. Quantitative real-time PCR analysis for Nup107, Nup62, Nup98/96 and Nup153 relative to Gapdh expression 48 hours after transfection. Transfections were performed using the two different sets of siRNAs (A and B; n=3). The Nup98 gene is a bicistronic gene that encodes for two separate nucleoporins, NUP98 and NUP96, from one mRNA. Since the siRNAs target the mRNA that will produce both proteins, the siRNAs have been designated si98/96. (B) Nucleoporin protein depletion levels. Western blot analysis for nucleoporins was performed 48 hours after transfection. The NUP98 antibody specifically recognized NUP98; no commercial antibody was available for NUP96. Histone 3 (H3) was used as loading control. Transfections were performed using the two different sets of siRNAs (n=6). Error bars, s.e.m.; *, significance p < 0.05 compared to the WT control; Veh, vehicle; siNT, non-targeting siRNA; si107, Nup107 siRNA; si62, Nup62 siRNA; si98/96, Nup98/96 siRNA; si153, Nup153 siRNA.
3.2 Nucleoporins regulate Kcnq1ot1 ncRNA volume in XEN cells

The Kcnq1ot1 ncRNA localizes to the nuclear compartment as a strong signal that overlaps the paternal Kcnq1ot1 domain (Fedoriw et al., 2012; Pandey et al., 2008; Redrup et al., 2009). To determine whether reduced Kcnq1ot1 ncRNA abundance altered nuclear Kcnq1ot1 ncRNA volume, 3D RNA/DNA FISH for Kcnq1ot1 was performed on G1-synchronized control and nucleoporin-depleted XEN cells, and Kcnq1ot1 ncRNA volume was calculated. Consistent with the expression analysis (Figure 3-2B), Kcnq1ot1 ncRNA localization was restricted to the paternal Kcnq1ot1 domain in control and Nup107-, Nup62- and Nup98-depleted XEN cells, while the Kcnq1ot1 ncRNA localized to both parental domains in Nup153-depleted XEN cells (Figure 3-2C,D). In controls, the majority of cells possessed medium Kcnq1ot1 ncRNA volumes (0.7-1.4 µm³, 74-76%), with a minority having volumes in low (<0.7 µm³, 7-10%), high (1.4-2.1 µm³, 15-17%) to very high (>2.1 µm³, 0-1%) ranges (Figure 3-2E). By comparison, Nup107, Nup62 and Nup153 depletion generated a significant increase in percentage of cells, 78%, 69%, 26%, with low Kcnq1ot1 ncRNA volumes, respectively, while Nup98/96 depletion significantly increased the percentage of cells with high (39%) or very high (17%) volumes. In addition, upon Nup153 depletion, 75% of cells acquired low Kcnq1ot1 ncRNA volumes at the maternal Kcnq1ot1 domain, suggesting that NUP153 acts on both maternal and paternal alleles. To determine whether nucleoporins altered Kcnq1ot1 ncRNA stability, the Kcnq1ot1 ncRNA half-life was measured in control and Nup-depleted XEN cells after blocking transcription with actinomycin D. No difference in Kcnq1ot1 ncRNA half-life (5-6 hours) was found between control and Nup-depleted cells (Figure 3-3), indicating that alterations in Kcnq1ot1 ncRNA abundance and volume were unrelated to Kcnq1ot1 transcript stability. All together, these results demonstrate that nucleoporins facilitate paternal Kcnq1ot1 ncRNA expression and localization at the Kcnq1ot1 domain.
The image contains a graph and several images.

**A** Graph showing the relative expression levels of a gene across different treatments.

**B** Graph showing the percentage of Kcnf1 allelic expression across different genotypes and treatments.

**C** Images showing nuclear localization of DAPI, Kcnf1, and RNA in different conditions.

**D** Graph showing the percentage of allelic RNA signal in different treatments.

**E** Histogram showing the percentage of signal volume in different conditions.

**F** Histogram showing the percentage of DNA signal in different conditions.
Figure 3—2: Nucleoporin depletion disrupts Kcnq1ot1 ncRNA expression, domain volume and nuclear periphery localization.

(A) Kcnq1ot1 ncRNA expression levels normalized to Gapdh. (B) Allelic Kcnq1ot1 ncRNA expression in control and Nup-depleted XEN cells (n=4). (C) Representative confocal nuclear images displaying Kcnq1ot1 DNA (red), Kcnq1ot1 ncRNA (green) and DAPI staining (blue) for G1-synchronized control and Nup-depleted XEN cells (n=111); upper panel, DNA FISH; middle panel, RNA FISH; lower panel, merge; m, maternal domain; p, paternal domain. (D) Percent of cells with paternal or maternal Kcnq1ot1 ncRNA signals. (E) Percent of cells with Kcnq1ot1 ncRNA signal volume; low, 0-0.7 µm³; medium, 0.7-1.4 µm³; high, 1.4-2.1 µm³; very high, >2.1 µm³. (F) Distance of the Kcnq1ot1 ncRNA signal from the nuclear membrane in G1-synchronized control and Nup-depleted XEN cells; nuclear periphery, 0-0.5 µm; sub-nuclear periphery, 0.6-1.5 µm; nuclear interior, 1.6-4 µm; LAMINB1, red; Kcnq1ot1 ncRNA, green; DAPI, blue. B6, maternal (red); CAST; paternal (blue); error bars, s.e.m; *, significance p < 0.05 compared to vehicle control; WT, wildtype; Veh, vehicle; siNT, non-targeting siRNA; si107, Nup107 siRNA; si62, Nup62 siRNA; si98/96, Nup98/96 siRNA, si153, Nup153 siRNA, scale bar, 1 µm.
Figure 3—3: *Kcnq1ot1* ncRNA stability is not altered upon nucleoporin depletion. Control and *Nup*-depleted XEN cells were treated with actinomycin D for 1 hour, after which cells were collected up to 12 hours after release from treatment. *Kcnq1ot1* expression levels were normalized to 0 hours. No significant changes in *Kcnq1ot1* ncRNA levels were seen at different time intervals after treatment between samples compared to the WT control, indicating that there was no difference in *Kcnq1ot1* ncRNA half-life in control and *Nup*-depleted XEN cells (n=3). WT, wildtype; Veh, vehicle; siNT, non-targeting siRNA; si107, *Nup107* siRNA; si62, *Nup62* siRNA; si98/96, *Nup98/96* siRNA, si153, *Nup153* siRNA.
3.3 Nucleoporins regulate Kcnq1ot1 domain positioning in XEN cells

Previous studies have shown that the Kcnq1ot1-coated domain is situated at the nuclear periphery, or perinucleolar region during S-phase (Fedoriw et. al. 2012; Pandey et. al., 2008). To examine the position of the Kcnq1ot1-coated domain in XEN cells, DNA/RNA FISH was performed on G1-synchronized control and Nup-depleted XEN cells and the distance of the Kcnq1ot1 DNA centroid from the nuclear rim was calculated from confocal images (Figure 3-2F). Previous studies found that the distance between distal probes within the Kcnq1ot1 domain differed for the maternal and paternal alleles (Terranova et al. 2008; Redrup et al. 2009). Here, the maternal (0.1-0.8 \( \mu m^3 \)) and paternal (0.9-1.3 \( \mu m^3 \)) Kcnq1ot1 domains were differentiated by domain volume using a centrally located probe (Figure 3-4). In control cells, the Kcnq1ot1 ncRNA was stationed at the nuclear periphery (0-0.5 \( \mu m \)), sub-nuclear periphery (0.5-1.5 \( \mu m \)) and nuclear interior (> 1.5 \( \mu m \)) in 91-92%, 4-6% and 3-4% of cells, respectively (Figure 3-2G). In Nup107-, Nup62 and Nup153-depleted XEN cells, nuclear periphery positioning of the Kcnq1ot1 ncRNA was significantly reduced to 54%, 61% and 64% of the cells, shifting to sub-nuclear periphery and nuclear interior locales. Furthermore, in Nup153-depleted XEN cells, Kcnq1ot1 ncRNA positioning was similar for the paternal and maternal domains. Nup98/96 depletion also produced a significant shift in Kcnq1ot1 ncRNA nuclear peripheral (84%) positioning, albeit to a lesser extent than other nucleoporin depletions. Next, I examined the relationship between Kcnq1ot1 ncRNA volume and nuclear positioning. In control cells, the majority of cells had medium Kcnq1ot1 ncRNA volume that resided at the nuclear periphery. Nup107-, Nup62- and Nup153-depleted cells with low Kcnq1ot1 ncRNA volume shifted away from the nuclear periphery, while Nup98/96-depleted cells with increased ncRNA volumes primarily retained nuclear periphery positioning (Figure 3-5). By comparison, the maternal Kcnq1ot1 domain was randomly positioned within the nucleus. Upon Nup153 depletion, those cells with primarily low Kcnq1ot1 ncRNA expression had a shift in maternal Kcnq1ot1 domain positioning toward the nuclear periphery. These results indicate that NUP107, NUP62 and NUP153 are required to anchor the Kcnq1ot1-coated allele to the nuclear periphery.
Figure 3—4: Paternal and maternal $Kcnq1ot1$ domain have distinct volumes in XEN cells

Paternal (blue, identified by $Kcnq1ot1$ ncRNA expression) and maternal (red) $Kcnq1ot1$ domain volume were plotted on the Y-axis with the number of G1-synchronized control and $Nup$-depleted XEN cells plotted on the X-axis. Paternal $Kcnq1ot1$ DNA domain volume had a range of 0.9 to 1.3 $\mu m^3$ while maternal $Kcnq1ot1$ DNA domain volume ranged from 0.1 to 0.8 $\mu m^3$, except for the siNup153 treatment group, where the maternal $Kcnq1ot1$ DNA domain volume ranged from 0.14 to 1.1 $\mu m^3$ (black bar, 0.85 $\mu m^3$). For those cells with a maternal $Kcnq1ot1$ DNA domain volume greater than 0.9 $\mu m^3$, the majority (92%) possessed an equal or larger paternal $Kcnq1ot1$ DNA domain volume; n=109-123.
Figure 3—5: *Kcnq1ot1* ncRNA volume to distance correlation in control and nucleoporin-depleted XEN cells.

*Kcnq1ot1* ncRNA volume correlated with distance from nuclear periphery. *Kcnq1ot1* ncRNA volume and distance from nuclear periphery were plotted on X- and Y-axes, respectively, for G1-synchronized control and Nup-depleted XEN cells. Upon *Nup107*, *Nup62* and *Nup153* depletion, cells with low *Kcnq1ot1* ncRNA volume shifted to sub-nuclear peripheral and nuclear interior positions. The maternal *Kcnq1ot1* domain was randomly positioned within the nucleus (expected NP 15%, SP 30%, NI 60%; observed NP 13-16%, 21-30; NI, 58-66), except for *Nup153*-depleted cells. Upon *Nup153* depletion, those cells with maternal *Kcnq1ot1* ncRNA expression (primarily low) had a shift in maternal *Kcnq1ot1* domain positioning toward the nuclear periphery (NP 60%, SP 21%, NI, 19%). NP, Nuclear Periphery; SP, Sub-nuclear Periphery; NI, Nuclear Interior; Veh, vehicle; siNT, non-targeting siRNA; si107, *Nup107* siRNA; si62, *Nup62* siRNA; si98/96, *Nup98/96* siRNA; si153, *Nup153* siRNA; P, paternal; M, maternal; n=109-123.
3.4 Nucleoporins physically interact with the Kcnq1ot1 domain in XEN cells

I next investigated nucleoporin interactions with the Kcnq1ot1 imprinted domain. Quantitative chromatin immunoprecipitation (ChIP) was performed using the NUP107 antibody (Figure 3-5A), the mAb414 antibody, which primarily interacts with NUP62, NUP107 and NUP160 (Figure 3-5B), and the NUP153 antibody (Figure 3-5C) in XEN cells; ChIP-grade NUP107 and NUP62 antibodies were not available. The identity of NUP107 and NUP62 following mAb414 IP were verified with the NUP107 antibody and in Nup62-depleted cells, respectively (Figure 3-5A). Antibodies were first validated at positive and neighbouring negative sites identified from mouse ES cells NUP153 DNA adenine methyltransferase identification (DamID) (Jacinto et al., 2015) (Figure 3-6A). Of the four NUP153 positive sites, Vim, Orai2, and Shank2 promoters displayed NUP153 enrichment in XEN cells. mAb414 (NUP107/62) enrichment was observed at the Orai2 and Shank2 promoters (Figure 3-6B). Curiously, Shank2, which resides upstream of the Kcnq1ot1 imprinted domain, showed preferential paternal mAb414 (NUP107/62) and NUP153 enrichment (Figure 3-6B). At the Kcnq1ot1 domain, 21 sites extending across the Kcnq1ot1 ICR to the reported H3K4me1-enriched enhancer elements (Korostowski et al., 2011; Schultz et al., 2015), and 1-2 sites at imprinted gene promoters were assessed for total and allele-specific binding in WT XEN cells (Figure 3-6C, Figure 3-7A). Significant mAb414 (NUP107/62) enrichment was observed at 2 sites within the Kcnq1ot1 ICR (IC3, 100 bp upstream, IC4, 1.7 kb downstream of the Kcnq1ot1 transcription start site), as well as at the Kcnq1ot1 enhancer element (E1 and E2) and the Ospbl5 promoter (Os1 and Os2) on the paternal allele (Figure 3-6C, Figure 3-7B,D). Significant NUP153 enrichment was also observed at the Kcnq1ot1 ICR (IC3, IC4), where both parental alleles were equally enriched, and at the paternal Kcnq1 (Kc1, Kc2) and Cd81 (Cd1) promoters (Figure 3-7C,E). Next, I investigated whether nucleoporin-chromatin interactions were lost upon Nup depletion. Since the mAb414 antibody recognizes both NUP62 and NUP107, double depletion was performed. I found a significant decrease in mAb414 (NUP107/62) occupancy at the Kcnq1ot1 ICR and putative enhancer element in double-depleted cells (Figure 3-7D). Upon Nup153 depletion, NUP153 binding on the paternal and maternal Kcnq1ot1 ICR, and the paternal Kcnq1 and Cd81 promoters was significantly reduced (Fig
These results indicate that NUP107/NUP62 and NUP153 localize at the Kcnq1ot1 domain. To determine whether there was interdependency between NUP107/62 and NUP153 binding, Nup153-depleted cells were examined for mAb414 enrichment, and Nup107- and Nup62-depleted cells were assessed for NUP153 enrichment. Nup153 depletion significantly decreased mAb414 (NUP107/62) enrichment at the Kcnq1ot1 ICR region but not at the Oshpl5 promoter and enhancer sites (Figure 3-7F). Conversely, Nup107 and Nup62 depletion significantly decreased NUP153 enrichment at the Kcnq1ot1 ICR and Kcnq1 promoter, while no change in enrichment was observed at the Cds81 promoter (Figure 3-7G). These results indicate that nucleoporin interactions at the paternal Kcnq1ot1 ICR are cooperatively mediate through NUP107, NUP62 and NUP153.

I further investigated whether nucleoporins can directly interact with Kcnq1ot1 ICR using biotin-labelled DNA fragments and nuclear lysates from control and Nup-depleted XEN cells. Electromobility shift assays were performed using the biotin-labelled Kcnq1ot1 ICR and control fragments (Figure 3-8A). Unlike the control fragment, supershifts were observed for the Kcnq1ot1 ICR fragment with mAb414 (NUP107/62), NUP107 and NUP153 antibodies, pointing to a direct interaction of these proteins with the Kcnq1ot1 ICR. No supershift was produced using the NUP98 antibody. Next, reverse ChIP was performed to isolate proteins bound to a biotin-labelled Kcnq1ot1 ICR fragment containing the IC3 enrichment site, as well as a negative control fragment located 2-kb upstream of the Kcnq1ot1 ICR. This was followed by Western blot analysis using mAb414, NUP107, NUP98 and NUP153 antibodies. NUP107 bound to the Kcnq1ot1 ICR fragment but not the control fragment (Figure 3-8B). Nup107-depleted lysates abrogated NUP107 binding to levels observed for the control fragment. Furthermore, NUP62 (detected by the mAb414 antibody) and NUP153 binding was also reduced, indicating that NUP62 and NUP153 binding at the Kcnq1ot1 ICR required NUP107 binding. NUP153 also bound directly to the IC3-containing Kcnq1ot1 ICR fragment but not the control fragment. Upon Nup153 depletion, binding was lost. However, NUP107 and NUP62 binding to the Kcnq1ot1 ICR fragment was not altered, perhaps indicating that there was sequential binding of NUP107/NUP62 and NUP153 in this in vitro system.
Figure 3—5: Antibody validation in XEN cells.

(A) NUP107 IP was performed followed by Western blot analysis using the same NUP107 antibody. (B) mAb414 IP was performed followed by Western blot analysis using the same mAb414 antibody. The most prominent nucleoporins detected with the mAb414 antibody in XEN cells were NUP62, NUP107 and NUP160. NUP62, NUP107 and NUP160 were identified by size. NUP107 identity was verified by mAb414 IP followed by Western blot analysis using the NUP107 antibody. NUP62 identity was verified using Nup62-depleted XEN cells. (C) NUP153 IP was performed followed by Western blot analysis using the same NUP153 antibody.
Figure 3—6: Antibody validation, and mAb414 and NUP153 enrichment at the Kcnq1ot1 ICR and enhancer element in XEN cells.

(A) NUP153 enrichment regions were mapped from ES cell DamID-seq data (Jacinto et. al, 2015) at the Vim, Orai2 and Shank2 promoters (orange bar). These genes were not upregulated upon Nup153 depletion in ES cells and/or in ES cells differentiated into NeuP cells (Jacinto et. al, 2015). Positive sites within the enriched region as well as negative control sites within Vim, Orai2 and Shank2 exonic and 3’UTR sites (magenta bars) were selected for analysis in XEN cells. Additional negative control sites were selected from the Dhcr7 gene, which is located 80 kb upstream of the Osbpl5 gene. (B, C) NUP153 and mAb414 ChIP validation in XEN cells. NUP153 and mAb414 ChIP were performed at NUP153 positive sites, Vim, Orai2, Shank2 and Dhcr7 promoters, identified from ES cell data (Jacinto et. al, 2015), and at negative control Vim, Orai2, Shank2 and Dhcr7 exonic and 3’UTR sites. Significant NUP153 enrichment was observed at Vim, Orai2, Shank2, with mAb414 enrichment at Orai2 and Shank2 promoters in XEN cells. NUP153 and mAb414 were enriched at both parental Vim and Orai2 promoters, and at the paternal Shank2 promoter (n=3). (D) mAb414 and NUP153 enrichment at the Kcnq1ot1 ICR. Of 21 sites (arrowheads) examined across the Kcnq1ot1 ICR and enhancer element, significant enrichment was observed at regions IC3, IC4, E1 and E2 for mAb414, and regions IC3 and IC4 for NUP153. ICR, imprinting control region; En, enhancer element; TSS, transcription start site (n=3). Error bars, s.e.m; *, significance p < 0.05 compared to the siNT or IgG controls.
Figure 3—7: NUP107/62 and NUP153 interaction with the Kcnq1ot1 ICR, and the Osbpl5, Kcnq1, Cd81 promoters in control and Nup-depleted XEN cells.

(A) The Kcnq1ot1 domain with regions of analysis (arrowheads); Os1, Os2, Osbpl5 promoter; Ph1, Ph2, Phlda2 promoter; Sl1, Sl2, Slc22a18 promoter; Ck1, Cdkn1c promoter; IC3, IC4, Kcnq1ot1 ICR; E1, E2, putative enhancer element; Kc1, Kc2, Kcnq1 promoter; Ts1, Ts2, Tssc4 promoter; Cd1, Cd81 promoter; Th1, Th promoter; Ctrl1, Ctrl2: control negative sites. (B) Quantitative ChIP analysis using mAb414 antibodies in wild type XEN cells at regions across the domain (n=4). (C) Quantitative ChIP analysis using NUP153 antibodies in wild type XEN cells at regions across the domain (n=4). (D) Quantitative allelic analysis for mAb414 in siNT and nucleoporin depleted XEN cells. Allelic proportions are represented as percent of the total enrichment levels (n=4). (E) Quantitative allelic analysis for NUP153 in siNT and nucleoporin depleted XEN cells. Allelic proportions are represented as percent of the total enrichment levels (n=4). (F) NUP107/NUP62 enrichment upon Nup153 depletion was significantly reduced at the Kcnq1ot1 ICR. Quantitative ChIP analysis using mAb414 antibodies was performed in control and Nup153-depleted XEN cells at sites of NUP153 enrichment (n=3). (G) NUP153 enrichment upon Nup107 and Nup62 depletion were significantly reduced at the Kcnq1ot1 ICR. Quantitative ChIP analysis using NUP153 antibodies was performed in control and Nup107- and Nup62-depleted cells at sites of mAb414 (NUP107/62) enrichment (n=3). * indicates significance p < 0.05 compared to the IgG and siNT controls.
**Figure 3—8:** NUP107, NUP62 and NUP153 bound to a *Kcnq1ot1* ICR fragment.

(A) NUP107, NUP62 and NUP153 supershifted a *Kcnq1ot1* ICR fragment but not to a control fragment. Electromobility shift assays were performed using biotin-labeled probes along with NPC, NUP107, NUP98 and NUP153 antibodies. Supershifts were observed for the *Kcnq1ot1* ICR biotin-labelled probe and NPC (NUP62/107), NUP107 and NUP153 but not NUP98 antibodies and not for a biotin-labelled control region 2 kb upstream of the ICR, (n=2-3). (B) NUP107, NUP62 and NUP153 bound to a *Kcnq1ot1* ICR fragment but not to a control fragment. Biotin-labeled fragments were incubated with control and Nup107- and Nup153-depleted XEN cell lysates, and then were immunoprecipitated using a streptavidin antibody attached to paramagnetic beads. Following IP washes, samples were subjected to Western blot analysis using NUP107, mAb414 (NUP62), NUP98 and NUP153 antibodies. NUP62 and NUP153 binding was reduced at the *Kcnq1ot1* ICR in Nup107-depleted samples, suggesting that NUP107 was required for NUP62 and NUP153 binding in vitro. However, Nup153 depletion showed continued NUP107 and NUP62 binding at the *Kcnq1ot1* ICR. By comparison, NUP98 did not bind the *Kcnq1ot1* ICR.
3.5 NUP107, NUP62 and NUP153 regulate paternal allele silencing in XEN cells

Given that aberrant Kcnq1ot1 ncRNA regulation could have domain-wide effects on imprinted gene regulation, allelic expression of imprinted protein coding genes in the Kcnq1ot1 domain was assessed (Figure 3-9A). Less than 15% expression from the normally silent paternal allele was considered repressed, given that controls had 12% paternal expression. Nup107 depletion resulted in paternal Osbpl5 (27%), Phlda2 (19%), Slc22a18 (47%), Cdkn1c (45%), Kcnq1 (32%) and Tssc4 (27%) reactivation but had no effect on paternal Cd81 and Th expression (Figure 3-9B). Nup62 depletion reactivated paternal alleles of the same genes as Nup107 depletion, Osbpl5 (23%), Slc22a18 (31%), Cdkn1c (32%), Kcnq1 (36%) and Tssc4 (23%), except for Phlda2 (Figure 3-8B). Nup153 depletion resulted in the same paternal reactivation of the core group of genes, Slc22a18 (16%), Cdkn1c (39%), Kcnq1 (34%), as well as Cd81 (31%). By comparison, Nup98/96 depletion had no effect on paternal allelic expression (Figure 3-9B). These data indicate that specific nucleoporins play a role in paternal allelic silencing of genes in the Kcnq1ot1 domain, although not in a domain-wide manner.
Figure 3—9: *Nup107, Nup62 and Nup153 depletion reactivates a subset of paternal alleles at the Kcnq1ot1 domain.*

(A) The *Kcnq1ot1* imprinted domain. The paternal *Kcnq1ot1* ICR (yellow), containing the *Kcnq1ot1* ncRNA promoter is unmethylated (white circles). The maternal *Kcnq1ot1* ICR is methylated (black circles). Arrows, direction of transcription. (B) Allelic expression analysis of imprinted genes in control and *Nup*-depleted XEN cells. Veh, vehicle; siNT, non-targeting siRNA; si107, *Nup107* siRNA; si62, *Nup62* siRNA; si98/96, *Nup98/96* siRNA; si153, *Nup153* siRNA (n=3-5). Error bars, s.e.m; *, significance p < 0.05 compared to the WT control.
3.6 Loss of Kcnq1ot1 domain regulation is not a consequence of abrogated nuclear-cytoplasmic transport

One explanation for altered regulation of the Kcnq1ot1 domain upon nucleoporin depletion is that aberrant nuclear pore function impaired nuclear-cytoplasmic transport. Previous studies have shown that import of cargo containing a classical bipartite nuclear localization signal (NLS) was impaired in NUP153-depleted HeLa cells, while transportin cargo was not affected (Vollmer et al., 2015). As such, import of exogenous and endogenous NLS-containing proteins was investigated in control and Nup-depleted XEN cells. For the former, XEN cells were transfected with a transcription factor 3 (E47)-red fluorescence protein (RFP)-nuclear localization signal (NLS) construct (Mehmood et al., 2011). E47-RFP^NLS XEN cells were in turn transfected with siRNAs. Wildtype XEN cells were treated with ivermectin, as a positive control for import inhibition. In contrast to ivermectin-treated cells where RFP import was inhibited, control and Nup-depleted XEN cells possessed similar nuclear RFP levels (Figure 3-10A). For endogenous nuclear NLS-containing cargo, RNAPII, SMC1, SMC3 and INCENP levels were quantified in control and Nup-depleted XEN nuclear extracts. No significant differences in protein levels were observed between control and Nup-depleted XEN nuclei (Figure 3-10B). Localization of the NLS-containing LAMINB1 protein at the internal nuclear membrane was also examined. LAMINB1 showed normal nuclear rim localization in control and Nup-depleted cells (Figure 3-10C).

With respect to export, previous studies found aberrant nuclear mRNA export in NUP107-depleted HeLa cells, with abnormal accumulation of polyA-mRNA in depleted nuclei (Boehmer et al., 2003). To assess the levels of mRNA retention upon nucleoporin depletion, RNA FISH was performed using a biotin-labeled oligodT probe. No significant difference in nuclear polyA mRNA retention levels was observed between control and Nup-depleted XEN cells (Figure 3-10D). To address whether export of a single mRNA species was compromised and whether passive protein diffusion was disrupted by nucleoporin depletion, transport of GFP mRNA and protein was investigated. GFP is a small molecule that undergoes bidirectional diffusion though nuclear pores (Seibel et al., 2007; Wei et al., 2003). XEN cells were transduced with a shRNA targeting the Luciferase gene tagged with a GFP reporter protein (shLucGFP) (Golding et al., 2010), and then...
transfected with siRNAs to examine GFP mRNA nuclear export and GFP protein nuclear diffusion. Nup-depleted cells exhibited comparable levels of nuclear and cytoplasmic GFP mRNA as well as nuclear GFP protein (Figure 3-10E). To examine endogenous mRNA transport, the cytoplasmic localized protein, α-TUBULIN, was quantified in cytoplasmic extracts, where the mRNA would have been translated. No significant difference in α-TUBULIN levels was observed between control and Nup-depleted cells (Figure 3-10B). As a final test of NPC transport upon nucleoporin depletion, XEN cell growth rate was measured since aberrant import/export may be expected to compromise cellular function (Boehmer et al., 2003). No significant change in XEN cell growth rate was observed in control and nucleoporin-depleted XEN cells in a direct cell counting assay (Figure 3-11), which was similar to what was observed for NUP107 depletion in HeLa cells (Boehmer et al., 2003). Overall, these results indicate that nuclear-cytoplasmic transport in XENs cells was not affected by nucleoporin depletion.
Figure 3—10: Nuclear transport is not altered upon nucleoporin depletion.

(A) Endogenous E47-RFP^NLS protein transport was not disrupted upon nucleoporin depletion, compared to the ivermectin control where nuclear import was blocked. Percent nuclear E47-RFP^NLS localization was similar in control and nucleoporin-depleted XEN cells, compared to ivermectin control. Scale bar, 1 µm; (n=60). (B) Endogenous protein transport was not disrupted upon nucleoporin depletion. RNAPII, SMC1, SMC3, and INCENP protein levels were the same in control and Nup-depleted XEN nuclear extracts, and α-TUBULIN protein levels were unchanged in control and Nup-depleted cytoplasmic extracts. Histone 3 (H3) was used as a loading control (n=2-3). (C) LAMINB1 protein transport was not altered upon nucleoporin depletion. Control and Nup-depleted XEN cells displayed LAMINB1 localization at the nuclear periphery. Scale bar, 1 µM µm; (n=30). (D) Nuclear polyA-mRNA retention levels were not changed upon nucleoporin depletion. Compared to controls, nucleoporin-depleted cells did not accumulate polyA-mRNA, showing similar biotin-labelleled oligodT fluorescence levels corrected for background levels. Scale bar, 1 µm; error bars, s.e.m; *, significance p < 0.05 compared to the siNT control; (n=30-45). (E) GFP mRNA and protein nuclear transport were not disrupted upon nucleoporin depletion. Nuclear and cytoplasmic GFP mRNA abundance relative to Gapdh expression and nuclear GFP protein levels using H3 as a loading control were similar in control and nucleoporin-depleted XEN cells, error bars, s.e.m; *, significance p < 0.05 compared to the siNT control; (n=3).
Figure 3—11: XEN cell growth rate is not altered upon nucleoporin depletion.

Approximately 25,000 cells were seeded and then transfected 12 hours later with siRNAs. Control and Nup-depleted XEN cells were monitored for 60 hours. Direct cell counts were performed every 12 hours in triplicate (n=3). No significant changes in cell growth rate were observed at different time intervals between samples compared to the WT control.
3.7 Nucleoporin depletion does not alter *Kcnq1ot1* ICR DNA methylation in XEN cells

Mechanistically, one explanation for a reduction in paternal *Kcnq1ot1* ncRNA levels and subsequent paternal allelic reactivation upon Nup107, Nup62 and Nup153 depletion could be a gain of DNA methylation at the normal unmethylated paternal ICR. In addition, reactivation of maternal *Kcnq1ot1* in Nup153-depleted XEN cells could be explained by a loss of DNA methylation at the methylated maternal ICR. To assess this, the bisulfite mutagenesis and sequencing assay was used to determine parental-specific methylation levels at the *Kcnq1ot1* ICR in control and Nup-depleted XEN cells. In control cells, the maternal *Kcnq1ot1* ICR was hypermethylated while the paternal ICR was hypomethylated (Figure 3-12). Surprisingly, no gain or loss in DNA methylation was seen at the paternal or maternal ICR in Nup-depleted XEN cells (Figure 3-12). Thus, changes in NUP107, NUP62 and NUP153 regulation at the *Kcnq1ot1* imprinted domain were not through alterations in *Kcnq1ot1* allelic methylation.

3.8 Nucleoporin interaction with the *Kcnq1ot1* domain regulates histone modifications at the domain in XEN cells

Another mechanism that may alter *Kcnq1ot1* ncRNA and paternal allelic silencing at the *Kcnq1ot1* imprinted domain is chromatin state. A gain of repressive modifications at the *Kcnq1ot1* ICR could account for reduced *Kcnq1ot1* ncRNA abundance and a gain of active modifications at promoters of imprinted genes could explain paternal allelic reactivation. Interaction between mAb414 (NUP107/NUP62), NUP107 and NUP153 and RNA polymerase II (RNAPII) provide support for facilitating *Kcnq1ot1* gene transcription (Figure 3-13A,B). ChIP assays were performed on control and depleted XEN cells using antibodies directed against RNAPII and histone 3 lysine 4 trimethylation (H3K4me3) as marks for active chromatin, and H3K9me2 and H3K27me3 for repressed chromatin. Antibodies were first validated at the XEN cell expressed FoxA2 gene and XEN cell repressed MyoD gene (Figure 3-13C) (Golding et al., 2010; Lim et al., 2008). Active FoxA2 and repressive MyoD modifications were observed as expected and were
**Figure 3—12: DNA methylation is maintained upon nucleoporin depletion.**

Methylation status of the *Kcnq1ot1* ICR in control and *Nup*-depleted XEN cells (n=2). Black circles, methylated CpGs; white circles, unmethylated CpGs. Each line represents an individual DNA strand. Total methylation percent is represented above each set of DNA strands (n=2). WT, wildtype; Veh, vehicle; siNT, non-targeting siRNA; si107, *Nup107* siRNA; si62, *Nup62* siRNA; si98/96, *Nup98/96* siRNA, si153, *Nup153* siRNA.
not altered upon nucleoporin depletion (Figure 3-13C). Compared to control cells and Nup98-depleted cells, there was decreased RNAPII and H3K4me3 enrichment and increased H3K9me2 and H3K27me3 enrichment on the paternal Kcnq1ot1 ICR in Nup107-, Nup62- and Nup153-depleted cells (Figure 3-14), which would account for reduced paternal Kcnq1ot1 ncRNA levels. In addition, we observed increased H3K4me3 and RNAPII enrichment along with decreased repressive histone modifications on the maternal Kcnq1ot1 ICR upon Nup153 depletion, which could account for reactivation of the maternal transcript (Figure 3-14). Compared to control cells, we also observed significantly increased enrichment of RNAPII and H3K4me3 at the paternal Slc22a18, Cdkn1c and Kcnq1 alleles upon Nup107, Nup62 and Nup153 depletion, H3K4me3 at the paternal Osbpl5 promoter upon Nup107 and Nup62 depletion, and RNAPII and H3K4me3 at the paternal Cd81 promoter upon Nup153 depletion, thereby accounting for their paternal reactivation (Figure 3-14). Conversely, there were significantly reduced levels of H3K9me2 and/or H3K27me3 at the paternal Slc22a18, Cdkn1c and Kcnq1 promoters upon Nup107, Nup62 and Nup153 depletion, and at the paternal Cd81 promoter upon Nup153 depletion. Additional decreases in H3K9me2 and/or H3K27me3 at the paternal Tssc4 and Th promoters upon Nup107 and Nup153 depletion, although this did not correlate with any change in paternal allele expression. Consistent with maintenance of paternal allelic repression, no significant changes in histone modifications were observed upon Nup98 depletion, except for at the Th promoter, where a significant decrease in paternal H3K27me3 enrichment was seen (Figure 3-14). These results demonstrate that NUP107, NUP62 and NUP153 act to regulate histone modifications at the Kcnq1ot1 ICR and specific imprinted gene promoters.
A

RNAPII

B

mAb414

NUP107

NUP153

C

RNAPII Enrichment (% input)

FoxA2 promoter  MyoD promoter

H3K4me3 Enrichment (% input)

FoxA2 promoter  MyoD promoter

H3K27me3 Enrichment (% input)

FoxA2 promoter  MyoD promoter
Figure 3—13: Nucleoporin-RNAPII interactions, and validation of active and repressive chromatin modifications at the FoxA2 and MyoD genes.

(A) RNAPII interacted with nucleoporins in XEN cells. mAb414 IP was performed followed by Western blot analysis using an RNAPII antibody, which showed positive interactions (n=2). (B) Nucleoporins interacted with RNAPII in XEN cells. RNAPII IP was performed followed by Western analysis using mAb414 (NUP62), NUP107 and NUP153 antibodies, which demonstrated positive interactions (n=2-3). (C) Active and repressive chromatin ChIP validation at the FoxA2 and MyoD genes in XEN cells. ChIP analysis using RNAPII, H3K4me3, H3K9me2 and H3K27me3 antibodies at FoxA2 and MyoD promoters. The expressed FoxA2 promoter (Golding et al., 2010) harbored active chromatin modification, RNAPII and H3K4me3, and low levels of repressive modifications, H3K9me2 and H3K27me3. Conversely, the repressed MyoD promoter (Lim et. al. 2008) was enriched for repressive modifications but lacked active chromatin modification. No significant change in enrichment levels was observed upon nucleoporin depletion compared to the siNT control (n=3).
Figure 3—14: Nucleoporin depletion disrupts histone modifications at the Kcnq1ot1 ICR and imprinted gene promoters.

(A) RNAPII and H3K4me3 ChIP at the maternal and paternal Kcnq1ot1 ICR and imprinted gene promoters in control and Nup-depleted XEN cells (n=3). Allelic proportions are represented as a percent of the total enrichment level. (B) H3K9me2 and H3K27me3 ChIP at the maternal and paternal Kcnq1ot1 ICR and imprinted gene promoters in control and Nup-depleted XEN cells (n=3). Allelic proportions are represented as a percent of the total enrichment level. WT, wildtype; Veh, vehicle; siNT, non-targeting siRNA; si107, Nup107 siRNA; si62, Nup62 siRNA; si98/96, Nup98/96 siRNA, si153, Nup153 siRNA; error bars, s.e.m; *, significance p < 0.05 compared to the siNT control.
3.9 Nucleoporins regulate cohesin complex interactions at the Kcnq1ot1 ICR in XEN cells

Another mechanism that may alter Kcnq1ot1 imprinted domain regulation is CTCF/cohesin complex binding. Previous studies have identified two CTCF-binding site within the Kcnq1ot1 ICR that were bound by CTCF and the cohesin complex in mouse embryonic fibroblasts (Fitzpatrick et al., 2007; Hark et al., 2000). While one study found that CTCF-binding at the Kcnq1ot1 ICR was paternal-specific, the other study observed that CTCF and cohesin bound with equal affinity to the maternal and paternal Kcnq1ot1 ICRs (Fitzpatrick et al., 2007). Regardless of allelic binding, CTCF and the cohesin complex may have regulatory roles at the Kcnq1ot1 imprinted domain. To determine whether CTCF and the cohesin complex interact with the nucleoporins, co-IP assays were performed. We observed positive interaction of mAb414, NUP107 and NUP153 with CTCF and the cohesin complex proteins, SMC1 and SMC3 (Figure 3-15A,B). To determine whether CTCF, SMC1 and SMC3 localized at the mAb414-positive enrichment sites, ChIP was performed using CTCF, SMC1 and SMC3 antibodies in XEN cells at the Kcnq1ot1 ICR, and for CTCF in embryonic stem cells at the maternal H19 ICR, as a positive control (Fitzpatrick et al., 2007; Hark et al., 2000; Prickett et al., 2013). As expected, strong CTCF enrichment was observed on the H19 maternal allele, whereas no CTCF enrichment was observed at the Kcnq1ot1 ICR and putative enhancer region (Figure 3-15C), indicating that CTCF does not bind to these sites in XEN cells. By comparison, SMC1 and SMC3 binding was significantly enriched at IC3 and IC4, but not at other tested sites, with preferential cohesin binding at the paternal IC3 and IC4 sites (Figure 3-15A,B). Upon Nup107- and Nup62 co-depletion and Nup153 depletion, SMC1 and SMC3 enrichment was significantly decreased at the paternal IC3 and IC4 sites (Figure 3-16A,B). These results indicate that NUP107, NUP62 and/or NUP153 interact with the cohesin complex at the paternal Kcnq1ot1 ICR.
Figure 3—15: Nucleoporin-CTCF and cohesin complex protein interactions in XEN cells, and CTCF enrichment at the *H19* ICR CTCF positives in ES cells but not at *Kcnq1ot1* ICR CTCF positives sites in XEN cells.

(A) CTCF and cohesin complex proteins were interacting partners of nucleoporins in XEN cells. mAb414 IP was performed followed by Western blot analysis using CTCF, SMC1, SMC3, NUP107 and NUP153 antibodies, which showed positive interactions (n=2-3). (B) CTCF, SMC1, and SMC3 IP was performed followed by Western analysis using mAb414, NUP107 and NUP153 antibodies, which demonstrated positive interactions (n=2-3). (C) CTCF was enriched at the H19 ICR in ES cells but not at the Kcnq1ot1 ICR in XEN cells. As a control for the CTCF antibody, one positive site at the H19 ICR and one negative site within H19 exon 5 (Ex5) (arrowheads) were were examined for CTCF enrichment in ES cells. Significant CTCF enrichment was observed at the maternal H19 ICR as a positive control but not at the negative control exon 5 (Ex5) site (n=3). (D) Seven sites (arrowheads) with mAb414 and/or NUP153 enrichment at the Kcnq1ot1 ICR and enhancer element were examined for CTCF enrichment). No significant CTCF enrichment was observed at the Kcnq1ot1 ICR and enhancer element in XEN cells (n=3). Error bars, s.e.m; *, significance p < 0.05 compared to IgG control.
Figure 3—16: SMC1 and SMC3 enrichment at the paternal Kcnq1ot1 ICR was reduced upon nucleoporin depletion.

(A) Quantitative ChIP analysis and allelic analysis for (A) SMC1 and (B) SMC3 at positive mAb414 and NUP153 enrichment sites in control and Nup107/Nup62-double-depleted and Nup153-depleted XEN cells (n=3). Allelic proportions are represented as a percent of the total enrichment level. Error bars, s.e.m; *, significance p < 0.05 compared to the siNT control.
Chapter 4

4 Results

The *Kcnq1ot1* domain exhibits both tissue-specific and developmental stage-specific regulation. Between embryonic and extraembryonic lineages, there is a clear difference between imprinted regulation of the protein-coding genes. At the *Kcnq1ot1* domain, genes are classified by their expression during mid-gestation development as inner/ubiquitously imprinted genes (i.e. imprinted expression in embryonic and placental tissue), *Phlda2, Sloc22a18, Cdkn1c, Kcnq1 and Kcnq1ot1*, and outer placental-specific imprinted genes, *Osbpl5, Nap1l4, Tssc4, Cd81, Ascl2 and Th* (Lewis et al. 2004; Umlauf et al. 2004; Golding et al. 2011; Mohammad et al. 2012). Currently, it is unclear when embryonic and extraembryonic lineages acquired differential imprinted domain regulation and how this differential regulation is regulated. For my second aim, I hypothesized that nucleoporins will have different regulatory roles or may act on different sets of genes within the *Kcnq1ot1* imprinted domain in ES, TS and XEN cells. To test this hypothesis, I evaluated the role of NUP107, NUP62, NUP98 and NUP153 in the regulation of the *Kcnq1ot1* imprinted domain in ES and TS stem cells.

4.1 *Nup107, Nup62 and Nup153* depletion alters *Kcnq1ot1* ncRNA expression in ES and TS cells

Wild type C57BL6 x *Mus musculus castaneus* ES and TS cells were transfected with two sets of siRNA’s targeting *Nup107, Nup62, Nup98/96* and *Nup153*. Compared to controls, RNA and protein levels were depleted to less than 30% of control levels in ES and TS cells (Figure 4-1). To determine the role on nucleoporins in regulating *Kcnq1ot1* ncRNA expression, total and allele-specific expression levels for the *Kcnq1ot1* ncRNA were assessed in control and Nup-depleted ES and TS cells. *Nup107* and *Nup62* depletion produced a significant decrease in *Kcnq1ot1* ncRNA levels in both ES (0.34 and 0.42) and TS (0.40 and 0.49) cells with expression restricted to the paternal allele (Figure 4-2). By comparison, no significant difference was observed between control and *Nup98*- or *Nup153*-depleted ES and TS cells. *Nup98*-depleted ES and TS cells maintained paternal
Kcnq1ot1 expression, while Nup153 depletion in ES and TS cells resulted in reactivation of the maternal Kcnq1ot1 allele to 22.3% and 25.9% (Figure 4-2B). This suggests that NUP153 depletion resulted in reduced paternal Kcnq1ot1 ncRNA expression levels by at least 20-25% of control cells. These results are similar to our previous finding in XEN cells where a 24% decrease in Kcnq1ot1 expression levels was observed upon Nup153 depletion.
Figure 4—1: Nucleoporin depletion levels in ES and TS cells.

Nucleoporin RNA (left) and protein (right) depletion levels in ES and TS cells. Quantitative real-time PCR analysis for Nup107, Nup62, Nup98/96 and Nup153 relative to Gapdh expression 48 hours after transfection (left). Transfections were performed using the two different sets of siRNAs (A and B; n=3). The Nup98 gene is a bicistronic gene that encodes for two separate nucleoporins, NUP98 and NUP96, from one mRNA. Since the siRNAs target the mRNA that will produce both proteins, the siRNAs have been designated si98/96. The NUP98 antibody specifically recognized NUP98; no commercial antibody was available for NUP96. Western blot analysis for nucleoporins was performed 48 hours after transfection. Histone 3 (H3) was used as loading control. Transfections were performed using the two different sets of siRNAs (n=6). Error bars indicate s.e.m. *, significance p < 0.05 compared to the WT control; Veh, vehicle; siNT, non-targeting siRNA; si107, Nup107 siRNA; si62, Nup62 siRNA; si98/96, Nup98/96 siRNA; si153, Nup153 siRNA.
Figure 4—2: *Nup107, Nup62 and Nup153* depletion disrupts *Kcnq1ot1* ncRNA expression, domain volume and nuclear periphery localization.

(A) Real-time *Kcnq1ot1* ncRNA expression levels normalized to *Gapdh*. (B) Allelic *Kcnq1ot1* ncRNA expression in control and *Nup*-depleted ES and TS cells (n=4). (C) Representative nuclear images displaying *Kcnq1ot1* ncRNA (green), LAMINB1 (red) and DAPI staining (blue) for G1-synchronized control and *Nup*-depleted ES and TS cells (n=3). (D) Percent of cells with paternal or maternal *Kcnq1ot1* ncRNA signals. (E) Percent of cells with *Kcnq1ot1* ncRNA signal volume; low, 0-0.7 µm$^3$; medium, 0.7-1.4 µm$^3$; high, 1.4-2.1 µm$^3$; very high, >2.1 µm$^3$. B6, maternal (red); CAST; paternal (blue); error bars, s.e.m; *, significance p < 0.05 compared to the vehicle control; WT, wildtype; Veh, vehicle; siNT, non-targeting siRNA; si107, *Nup107* siRNA; si62, *Nup62* siRNA; si98/96, *Nup98/96* siRNA, si153, *Nup153* siRNA, scale bar, 1 µm; n=4; cell count number=100.
4.2 NUP107, NUP62 and NUP153 regulate Kcnq1ot1 ncRNA volume in ES and TS cells

To determine whether reduced paternal Kcnq1ot1 ncRNA abundance and reactivation of the maternal Kcnq1ot1 transcript altered Kcnq1ot1 volume, 3D RNA FISH was performed for Kcnq1ot1 along with immunofluorescence for nuclear periphery marker LAMINB1. Consistent with the expression analysis (Figure 4-2B), Kcnq1ot1 ncRNA localization was restricted to the paternal Kcnq1ot1 domain in control and Nup107- and Nup62-depleted ES and TS cells, while the Kcnq1ot1 ncRNA localized to both parental domains in Nup153-depleted ES and TS cells (Figure 4-2C,D). In control ES and TS cells, 68-71% and 69-78% of cells possessed a Kcnq1ot1 ncRNA volume in the medium volume range (0.7-1.4 \( \mu m^3 \)) with the remaining cells in the low (<0.7 \( \mu m^3 \), 3-7% and 10-12%) and the high (1.4-2.1 \( \mu m^3 \), 12-21 and 7-15%) to very high (1.4-2.1 \( \mu m^3 \), 8-10 and 3-6%) volume ranges, respectively (Figure 4-2E). In ES and TS cells, a significant increase was observed in cells with low Kcnq1ot1 ncRNA volumes upon Nup107 depletion (66% and 71%, respectively) and Nup62 depletion (73% and 62%, respectively). For Nup153 depletion, a significant increase was also found for ES and TS cells in the low volume range for the paternal Kcnq1ot1 ncRNA (32% and 41%, respectively). While the maternal Kcnq1ot1 allele was not expressed in control and Nup107-, Nup62- and Nup98/96-depleted ES and TS, 92% and 83% of Nup153 depletion ES and TS cells, respectively, displayed maternal Kcnq1ot1 ncRNA volumes in the lower range. Nup98/96 depletion did not alter the Kcnq1ot1 ncRNA volume in ES or TS cells in contrast to XEN cells where I saw a significant increase in Kcnq1ot1 ncRNA volume. To determine whether decreased transcript abundance and volume was correlated with altered stability of the Kcnq1ot1 ncRNA, control and Nup-depleted ES and TS cells were treated with actinomycin D to block transcription and transcript half-life was measured. Kcnq1ot1 ncRNA was not altered between control and Nup-depleted ES and TS cells (Figure 4-3), suggesting the reduced Kcnq1ot1 ncRNA volumes were a result of altered Kcnq1ot1 ncRNA expression.
Figure 4—3: Kcnq1ot1 ncRNA stability is not altered upon nucleoporin depletion in ES and TS cells.

Control and Nup-depleted ES and TS cells were treated with actinomycin D for 1 hour, after which cells were collected up to 12 hours after release from treatment. Kcnq1ot1 expression levels were normalized to 0 hours. No significant changes in Kcnq1ot1 ncRNA levels were seen at different time intervals between samples and the WT control, indicating that there was no difference in Kcnq1ot1 ncRNA half-life in control and Nup-depleted XEN cells (n=3). Error bars indicate s.e.m. *, significance p < 0.05; Veh, vehicle; siNT, non-targeting siRNA; si107, Nup107 siRNA; si62, Nup62 siRNA; si98/96, Nup98/96 siRNA; si153, Nup153 siRNA.
4.3 NUP107, NUP62 and NUP153 regulate Kcnq1ot1 domain positioning in ES and TS cells

I previously showed that the Kcnq1ot1 ncRNA-coated domain was localized at the nuclear periphery in XEN cells. Upon Nup107, Nup62, and Nup153 depletion, nuclear periphery positioning was shifted to the sub-nuclear periphery and nuclear interior. Comparatively, while the volume increased, nuclear periphery positioning was mostly maintained in Nup98/96-depleted XEN cells. To test whether the nuclear periphery position of the Kcnq1ot1-coated domain was similarly altered upon Nup-depletion in ES and TS cells, DNA/RNA FISH was performed in G1-synchronized control and Nup-depleted ES and TS cells. In control and Nup-depleted cells, there was complete overlap of Kcnq1ot1 ncRNA with the Kcnq1ot1 domain (Figure 4-4A,B). Positioning within the nucleus was categorized as nuclear periphery, sub-nuclear periphery and nuclear interior. The larger domain with Kcnq1ot1 ncRNA signal (where visible) and the smaller domain without Kcnq1ot1 ncRNA signal were designated as the paternal and maternal domains, respectively. In the controls, the Kcnq1ot1 domain was positioned at the nuclear periphery in 86-92% and 83-88%, the sub-nuclear periphery in 5-9% and 4-15%, and the nuclear interior in 3-5% and 2-3% of ES and TS cells, respectively. Nup107 depletion resulted in decreased positioning of the Kcnq1ot1 domain at the nuclear periphery in 64% and 52%, and increased positioning at the sub-nuclear periphery in 27% and 34%, and nuclear interior in 9% and 14% of ES and TS cells, respectively. A similar shift to the sub-nuclear periphery and nuclear interior was observed in Nup62- and Nup153-depleted ES and TS cells. No significant change in positioning was observed upon Nup98/96 depletion. To determine whether there was any correlation between Kcnq1ot1 ncRNA volume and Kcnq1ot1 domain positioning, ncRNA volume and distance from nuclear periphery was correlated for both ES and TS cells. In G1-synchronized control ES and TS cells, the Kcnq1ot1 ncRNA primarily localized at the nuclear periphery with medium Kcnq1ot1 ncRNA volumes (Figure 4-5). Upon Nup107, Nup62 and Nup153 depletion, ES and TS cells with lower Kcnq1ot1 ncRNA volumes were situated away from the nuclear periphery. Nup98/96-depleted cells displayed similar volumes and positioning as control cells. These results suggest that NUP107, NUP62, and NUP153 play a role in directing the Kcnq1ot1 domain to the nuclear periphery in ES and TS cells, similar to XEN cells.
Figure 4—4: Nup107, Nup62 and Nup153 depletion disrupts Kcnq1ot1 domain nuclear periphery localization.

Nucleoporin depletion disrupted Kcnq1ot1 DNA nuclear periphery localization in ES (A) and TS (B) cells. Representative DNA/RNA FISH images are shown for control Nup-depleted ES and TS cells. Graph represents quantification of the distance of the DNA FISH signal from the nuclear periphery. Upon nucleoporin depletion, Kcnq1ot1 DNA localization shifts away from the nuclear periphery. Nuclear periphery, 0-0.5 µm; sub-nuclear periphery, 0.6-1.5 µm; nuclear interior, 1.6-4 µm; scale bar, 1 µM; m, maternal domain; p, paternal domain; *, significance p < 0.05 compared to the vehicle control; n=4; cell count number=100.
Figure 4—5: *Kcnq1ot1* ncRNA volume to distance correlation in control and nucleoporin-depleted ES and TS cells

*Kcnq1ot1* ncRNA volume correlated with distance from nuclear periphery in ES (A) and TS (B) cells. *Kcnq1ot1* ncRNA volume and distance from nuclear periphery were plotted on X- and Y-axes, respectively, for G1-synchronized control and *Nup*-depleted XEN cells. Upon *Nup107, Nup62* and *Nup153* depletion, cells with low *Kcnq1ot1* ncRNA volume shifted to sub-nuclear peripheral and nuclear interior positions. Nuclear Periphery; SP, Sub-nuclear Periphery; NI, Nuclear Interior; Veh, vehicle; siNT, non-targeting siRNA; si107, *Nup107* siRNA; si62, *Nup62* siRNA; si98/96, *Nup98/96* siRNA; si153, *Nup153* siRNA; P, paternal; M, maternal; n=100.
4.4 Nucleoporins physically interact with the \textit{Kcnq1ot1} domain in ES and TS cells

In the previous chapter on XEN cells, I observed that NUP107/NUP62 (mAb414) were enriched at the \textit{Kcnq1ot1} ICR, the enhancer element and the Osbpl5 promoter on the paternal allele, whereas NUP153 was enriched at the \textit{Kcnq1ot1} ICR, and the \textit{Kcnq1} and \textit{Cd81} promoters. To investigate nucleoporin proteins interactions at the \textit{Kcnq1ot1} imprinted domain in ES and TS cells (Figure 4-6), quantitative ChIP was performed using the mAb414 antibody that primarily interacts with NUP62, NUP107 and NUP160, as shown before in mouse XEN cells, as well as the NUP153 antibody; ChIP-grade NUP107 and NUP62 antibodies were not available. In ES cells, significant NUP107/NUP62 (mAb414) occupancy was observed at the \textit{Cdkn1c} promoter (Ck1), the \textit{Kcnq1ot1} ICR (IC3, 100 bp upstream; IC4, 1.7kb downstream of \textit{Kcnq1ot1} TSS), and the \textit{Kcnq1ot1} enhancer element (E1 and E2) (Fig 4-6). By comparison, NUP153 binds to the \textit{Kcnq1ot1} ICR (IC3, IC4), and the \textit{Cd81} (Cd1) and \textit{Th} (Th1) promoters. In TS cells, NUP107/NUP62 (mAb414) enrichment was restricted to the \textit{Kcnq1ot1} ICR (IC3, IC4) and enhancer element (E1, E2), while NUP153 was enriched only at the \textit{Kcnq1ot1} ICR (IC3, IC4) (Fig 4-6B). To examine whether nucleoporin-chromatin interactions were lost upon Nup depletion, ChIP was performed using the mAb414 and NUP153 antibodies at the positive enrichment and negative (Ctrl2) sites in siNT control and Nup107/Nup62 double-depleted, and Nup153-depleted ES and TS cells. Compared to control ES cells, NUP107/NUP62 enrichment was significantly decreased at the \textit{Cdkn1c} promoter, \textit{Kcnq1ot1} ICR and enhancer element sites upon Nup107/Nup62 double-depletion (Figure 4-7). NUP153 depletion in ES cells similarly produced a significant reduction in NUP153 enrichment at the enhancer element and \textit{Cd81} and \textit{Th} sites (Fig 4-7B). In TS cells, NUP107/NUP62 enrichment was significantly reduced at the \textit{Kcnq1ot1} ICR and enhancer element sites in Nup107/Nup62 double-depleted cells compared to the control (Fig 4-7C), while there was a significant reduction of NUP153 enrichment at the \textit{Kcnq1ot1} ICR sites upon Nup153 depletion (Fig 4-7D). For all interactions, reduced NUP107/NUP62 and NUP153 enrichment was found at the paternal allele in \textit{Nup}-depleted cells compared to the control cells, except for NUP153 enrichment which was decreased at both the maternal and paternal \textit{Cd81} sites in
$Nup153$-depleted ES cells. This result contrasts with those obtained in XEN cells, where NUP153 enrichment was found at both the maternal and paternal $Kcnq1ot1$ ICR sites. In XEN cells, I found that nucleoporin interactions at the paternal $Kcnq1ot1$ ICR were cooperatively mediated through NUP107, NUP62 and NUP153. I next investigated their interdependency in ES and TS cells. In both ES and TS cells, upon $Nup153$ depletion, NUP107/NUP62 enrichment at the $Kcnq1ot1$ ICR and enhancer element sites was significantly reduced compared to controls (Fig 4-7E,F). Likewise, $Nup107/Nup62$ double-depletion in ES and TS cells resulted in a significant decrease in NUP153 enrichment at the $Kcnq1ot1$ ICR sites (Fig 4-7E,F). These results indicate that NUP107, NUP62 and NUP153 interactions at the paternal $Kcnq1ot1$ ICR were cooperatively mediated in XEN, ES and TS cells.

I next investigated whether nucleoporins can directly interact with $Kcnq1ot1$ ICR using biotin-labelled DNA fragments and nuclear lysates from control and $Nup$-depleted XEN cells. Reverse ChIP was performed to isolate proteins bound to a biotin-labelled $Kcnq1ot1$ ICR fragment containing the IC3 enrichment site, as well as a negative control fragment located 2-kb upstream of the $Kcnq1ot1$ ICR. This was followed by Western blot analysis using mAb414, NUP107 and NUP153 antibodies. NUP107 bound directly to the $Kcnq1ot1$ ICR fragment but not the control fragment (Figure 4-8). $Nup107$-depleted lysates abrogated NUP107 binding to levels observed for the control fragment. Furthermore, NUP62 (detected by the mAb414 antibody) and NUP153 binding was also reduced, indicating that NUP62 and NUP153 binding at the $Kcnq1ot1$ ICR required NUP107 binding. NUP153 also bound directly to the IC3-containing $Kcnq1ot1$ ICR fragment but not the control fragment. Upon $Nup153$ depletion, binding was lost. However, NUP107 and NUP62 binding to the $Kcnq1ot1$ ICR fragment was not altered, perhaps indicating that there was sequential binding of NUP107/NUP62 and NUP153 in this $in vitro$ system.
A

MAT

PAT

Osbpl5
Phlda2
Cdkn1c
ICF
Kcnq1
Tsc2
Cd81
Ascl2
Th

Os1
Os2
Ph1
Ph2
Sl1
Sl2
Ctrl1
IC1
IC2
E1
E2
Ctrl2
Kc1
Kc2
Ts1
Ts2
Cd1
Th1

B

ES cells

Enrichment (% Input)

0 2 4 6 8

Os1 Os2 Ph1 Ph2 Sl1 Sl2 Ctrl1 IC3 IC4 E1 E2 Ctrl2 Kc1 Kc2 Ts1 Ts2 Cd1 Th1

mAb414
IgG

C

TS cells

Enrichment (% Input)

0 2 4 6

Os1 Os2 Ph1 Ph2 Sl1 Sl2 Ctrl1 IC3 IC4 E1 E2 Ctrl2 Kc1 Kc2 Ts1 Ts2 Cd1 Th1

NPC
IgG

NUP153
IgG
Figure 4—6: NUP107/62 and NUP153 interact with the Kcnq1ot1 ICR, enhancer site, Cdkn1c and the Osbpl5, Cd81 and Th promoters in wildtype cells.

(A) The Kcnq1ot1 domain with regions of analysis (arrowheads); Os1, Os2, Osbpl5 promoter; Ph1, Ph2, Phlda2 promoter; Sl1, Sl2, Slc22a18 promoter; Ck1, Cdkn1c promoter; IC3, IC4, Kcnq1ot1 ICR; E1, E2, putative enhancer element; Kc1, Kc2, Kcnq1 promoter; Ts1, Ts2, Tssc4 promoter; Cd1, Cd81 promoter; Th1, Th promoter; Ctrl1, Ctrl2: control negative sites. Partial blue genes represent maternal bias expression in ES cells. (B) Quantitative ChIP analysis using mAb414 and NUP153 antibodies in wild type ES cells at regions across the domain (n=4). (C) Quantitative ChIP analysis using mAb414 and NUP153 antibodies in wild type TS at regions across the domain (n=4). Error bars, s.e.m; *, significance p < 0.05 compared to the IgG control.
Figure 4—7: NUP107/62 and NUP153 interaction with the Kcnq1ot1 domain in control and Nup-depleted ES and TS cells

(A, B) Total enrichment and quantitative allelic analysis at positive and negative sites for mAb414 in siNT and Nup107/Nup62- and Nup153-depleted ES (A) and TS (B) cells. Allelic proportions are represented as percent of the total enrichment levels (n=4). (C, D) Total enrichment and quantitative allelic analysis for NUP153 in siNT and Nup107/Nup62- and Nup153-depleted ES (C) and TS (D) cells. Allelic proportions are represented as percent of the total enrichment levels (n=4). (E, F) NUP107/NUP62 enrichment was significantly reduced at the Kcnq1ot1 ICR and enhancer site upon Nup153 depletion in ES and TS cells. Quantitative ChIP analysis using mAb414 antibodies was performed in control and Nup153-depleted ES and TS cells at sites of mAb414 enrichment (n=3). NUP153 enrichment was significantly reduced at the Kcnq1ot1 ICR upon Nup107/Nup62 depletion in ES and TS cells. Quantitative ChIP analysis using NUP153 antibodies was performed in control and Nup107/62-depleted cells at sites of NUP153 enrichment in ES and TS cells (n=3). Error bars, s.e.m; *, significance p < 0.05 compared to the siNT control.
Figure 4—8: NUP107, NUP62 and NUP153 bound to the *Kcnq1ot1* ICR fragment.

(A) NUP107, NUP62 and NUP153 bound to a *Kcnq1ot1* ICR fragment but not to a control fragment. Biotin-labeled fragments were incubated with control and *Nup107* and *Nup153*-depleted ES cell lysates, and then were immunoprecipitated using a streptavidin antibody attached to paramagnetic beads. Following IP washes, samples were subjected to Western blot analysis using NUP107, NUP62 (mAb414) and NUP153 antibodies. NUP62 and NUP153 binding was reduced at the *Kcnq1ot1* ICR in *Nup107*-depleted samples, suggesting that NUP107 was required for NUP62 and NUP153 binding *in vitro*. However, *Nup153* depletion showed continued NUP107 and NUP62 binding at the *Kcnq1ot1* ICR in this in vitro system.
4.5 Nucleoporin proteins regulate paternal allele silencing in ES and TS cells

Given the changes in NUP107, NUP62 and NUP153 interactions at the Kcnq1ot1 domain, I next determined whether these alterations exerted any effect on domain-wide imprinted gene expression. The effects of Nup-depletion on expression of imprinted genes were assessed in ES and TS cells. Expression less than 15% from the normally silent paternal allele was considered repressed. Cd81 was considered to have maternal-biased expression, with less than 25% paternal expression. In ES cells, Osbp15, Slc22a18, Ascl2 and Th were biallelically expressed, with no change in paternal expression upon Nup depletion (Figure 4-9). Similar to XEN cells, Nup depletion had no effect on paternal Phlda2 and Tssc4 repression. Compared to controls, paternal Cdkn1c was reactivated upon Nup107, Nup62 and Nup153 depletion but not Nup98/96 depletion, similar to XEN cells. Unlike XEN cells, depletion of Nup107, Nup62 and Nup153 in ES cells produced no change in paternal Kcnq1 expression. Instead, Nup98/96 depletion in ES cells resulted in reactivation of the paternal Kcnq1 allele. Finally, similar to XEN cells, Nup153 depletion reactivated Cd81 paternal expression in ES cells.

In TS cells, Osbp15, Tssc4, Cd81, Ascl2 and Th were biallelically expressed, with no change in paternal expression upon Nup depletion (Figure 4-9). Similar to XEN cells, Nup depletion had no effect on paternal Phlda2 repression. Compared to controls, Nup107, Nup62 and Nup153 depletion in TS cells resulted in reactivation of the paternal Cdkn1c, Kcnq1 and Slc22a18, while no change in paternal repression was observed in Nup98-depleted TS cells, similar to XEN cells. These results indicate that nucleoporins play a role in paternal allelic silencing of multiple imprinted genes in the Kcnq1ot1 domain, although not in a domain-wide manner. They also highlight the differences in paternal allelic repression in ES, TS and XEN cells.
Figure 4—9: Nucleoporin depletion reactivates a subset of paternal alleles at the Kcnq1ot1 domain in ES and TS cells.

The Kcnq1ot1 imprinted domain in ES (A) and TS (B) cells. The paternal Kcnq1ot1 ICR (yellow), containing the Kcnq1ot1 ncRNA promoter is unmethylated (white circles). The maternal Kcnq1ot1 ICR is methylated (black circles). Arrows, direction of transcription. (C-D) Allelic expression analysis of imprinted genes in control and Nup-depleted ES (C) and TS (D) cells. Veh, vehicle; siNT, non-targeting siRNA; si107, Nup107 siRNA; si62, Nup62 siRNA; si98/96, Nup98/96 siRNA; si153, Nup153 siRNA (n=3-5). Error bars, s.e.m; *, significance p < 0.05 compared to the WT control.
4.6 Loss of Kcnq1ot1 domain regulation is not a consequence of abrogated nuclear-cytoplasmic transport

There are several explanations for altered regulation of the Kcnq1ot1 domain upon Nup depletion. This includes a change in nuclear transport, since one of the primary functions of the nucleoporin proteins is nuclear-cytoplasmic import and export. Previously, we showed that Nup107, Nup62, Nup98/96 and Nup153 depletion did not alter nuclear-cytoplasmic import or export mechanisms in XEN cells. Given the differences in stem cell profiles, we wanted to determine the status of nuclear-cytoplasmic transport in ES and TS cells upon nucleoporin depletion. First, I determined whether transport of NLS-containing proteins was altered in Nup-depleted ES and TS cells. No significant difference was observed in endogenous proteins with the classic NLS in nuclear import upon Nup depletion in both ES and TS nuclear extracts (Fig 4-10). Similarly, no significant difference was observed in the cytoplasmic retention of E47\textsuperscript{NLS}-Red fluorescent protein (RFP) tag in transfected ES and TS cells. This contrasts with cells treated with the nuclear import inhibitor, Ivermectin, which displayed cytoplasmic retention of the E47\textsuperscript{NLS}-RFP (Fig 4-10B). To determine whether Nup depletion altered nuclear export, RNA FISH was performed using a polyA-mRNA probe to determine nuclear mRNA retention levels in control and Nup-depleted samples. Nuclear polyA-mRNA retention levels remain unchanged upon Nup depletion (Fig 4-10C). As a final test of NPC transport function upon nucleoporin depletion, ES and TS cell growth rate was measured. No significant alteration in ES and TS cell growth rate was observed in control and nucleoporin-depleted ES and TS cells in a direct cell counting assay (Fig 4-11). Therefore, these results suggest that nuclear import and export mechanisms in ES and TS cells were not disrupted upon nucleoporin depletion.
Figure 4—10: Nuclear transport is not altered upon nucleoporin depletion.

(A) Endogenous protein transport was not disrupted upon nucleoporin depletion. CTCF, SMC1, SMC3, INCENP protein levels in nuclear extracts, and α-TUBULIN protein levels in cytoplasmic extracts were unchanged in control and Nup-depleted ES cells. In TS cells, CTCF, RNAPII, INCENP protein levels in nuclear extracts, and α-TUBULIN protein levels in cytoplasmic extracts were the same in control and Nup-depleted samples. Histone 3 (H3) was used as a loading control (n=2-3). (B) Endogenous E47-RFP^{NLS} protein transport was not disrupted upon nucleoporin depletion, compared to the ivermectin control where nuclear import was blocked in ES and TS cells. Percent nuclear E47-RFP^{NLS} localization was similar in control and nucleoporin-depleted ES and TS cells, compared to ivermectin control. Scale bar, 1 μm; error bars, s.e.m; *, significance p < 0.05 compared to the siNT control; (n=50). (C) Nuclear polyA-mRNA retention levels were not changed upon nucleoporin depletion. Compared to controls, nucleoporin-depleted ES and TS cells did not accumulate polyA-mRNA, showing similar biotin-labeled oligo-dT fluorescence levels corrected for background levels. Scale bar, 1 μm; error bars, s.e.m; *, significance p < 0.05 compared to the siNT control; (n=40).
Figure 4—11: Nucleoporin depletion does not alter ES and TS cell growth rate.
ES and TS cells were plated at 20% confluency and transfected with control or nucleoporin specific siRNAs. Cells were counted at intervals of every 12 hours. No significant change in ES or TS cell growth rate was observed at different time intervals between samples compared to the WT control.
4.7 Nucleoporin depletion does not alter \textit{Kcnq1ot1} ICR DNA methylation

A second explanation for altered regulation of the \textit{Kcnq1ot1} domain upon \textit{Nup} depletion was a change in DNA methylation at the \textit{Kcnq1ot1} ICR. More specifically, reduced paternal \textit{Kcnq1ot1} expression in \textit{Nup107}, \textit{Nup62} and \textit{Nup153}-depleted cells could be correlated with a gain in DNA methylation at the paternal \textit{Kcnq1ot1} ICR whereas reactivation of the normally-silent maternal \textit{Kcnq1ot1} in \textit{Nup153}-depleted cells could be due to a loss of DNA methylation at the ICR. Similar to XEN cells, the DNA methylation status of the \textit{Kcnq1ot1} ICR was unaltered in \textit{Nup}-depleted cells ES or TS cells (Fig 4-12).
Figure 4—12: DNA methylation is maintained upon nucleoporin depletion.
Methylation status of the Kcnq1ot1 ICR in control and Nup-depleted ES and TS cells (n=2). Black circles, methylated CpGs; white circles, unmethylated CpGs. Each line represents an individual DNA strand. Total methylation percent is represented above each set of DNA strands (n=2). WT, wildtype; Veh, vehicle; siNT, non-targeting siRNA; si107, Nup107 siRNA; si62, Nup62 siRNA; si98/96, Nup98/96 siRNA, si153, Nup153 siRNA
4.8 Nucleoporins regulate histone modifications at the 
*Kcnq1ot1* domain in ES and TS cells

Another explanation for altered regulation of the *Kcnq1ot1* domain upon Nup depletion is a change in histone modifications. Increased repressive modifications at the *Kcnq1ot1* ICR could explain the reduced levels of *Kcnq1ot1* ncRNA while an increase in active modifications at imprinted gene promoters could account for paternal allelic reactivation. ChIP assays were performed on control and *Nup*-depleted ES and TS cells using RNA polymerase II (RNAPII) and histone 3 lysine 4 trimethylation (H3K4me3) antibodies as marks for active transcription, and H3K9me2 and H3K27me3 antibodies as marks for repressed chromatin. As a control, active and repressive modifications were first validated at the ES cell expressed Oct4, and the TS cell expressed Cdx2 gene promoters (Lim *et al.* 2008). As expected, in ES cells, the Oct4 promoter, and in TS cells, the Cdx2 promoter showed H3K4me3 and RNAPII occupancy and lacked enrichment for H3K9me2 and H3K27me3 in control and *Nup*-depleted cells (Fig 4-13). Conversely, in ES cells, the Cdx2 promoter, and in TS cells, the Oct4 promoter exhibited enrichment of repressive but not active modifications in control and *Nup*-depleted-cells. Next, quantitative-allelic ChIP was performed at the *Kcnq1ot1* ICR and promoters of imprinted genes. Compared to control ES and TS cells, *Nup107*-, *Nup62*-, and *Nup153*-depleted cells had significantly decreased RNAPII and H3K4me3 enrichment at the paternal *Kcnq1ot1* ICR (Fig 4-14). Note that allelic enrichment was represented relative to total enrichment levels. This may account for the decreased *Kcnq1ot1* ncRNA expression levels upon *Nup107*, *Nup62*, and *Nup153* depletion. In addition, for *Nup153* depletion, a significant decrease in H3K9me2 and H3K27me3 enrichment was observed at the maternal *Kcnq1ot1* ICR in both ES and TS cells, offering an explanation for reactivation of the maternal *Kcnq1ot1* ncRNA. I also performed ChIP analysis at the promoters of imprinted genes. In ES cells, we observed a significant increase in RNAPII and H3K4me3 levels and a concurrent decrease in H3K9me2 and H3K27me3 at the paternal *Cdkn1c* promoter upon *Nup107*, *Nup62* and *Nup153* depletions compared to controls. Moreover, compared to ES cell controls, significantly increased RNAPII and H3K4me3 enrichment and significantly decreased H3K9me2 and H3K27me3 enrichment was observed at the paternal *Kcnq1* promoter upon
Nup98 depletion, and at the paternal Cd81 promoter in Nup153 depleted ES cells. Compared to TS cell controls, there was a significant increase in RNAPII and H3K4me3 levels along with a significant decrease in H3K9me2 and H3K27me3 levels at the paternal Slc22a18, Cdkn1c and Kcnq1 promoters upon Nup107, Nup62 and Nup153 depletion. Notably, no changes in RNAPII or histone modification enrichment was observed for the Tssc4 and/or Phlda2 promoters, which retained paternal allelic repression in Nup-depletion ES and TS cells (Fig 4-15). Similar to XEN cells, these results indicate that NUP107, NUP62 and NUP153 act to regulate histone modifications at the Kcnq1ot1 ICR and specific imprinted gene promoters.
RNAPII Enrichment (% input)

H3K4me3 Enrichment (% input)

H3K9me2 Enrichment (% input)

H327me3 Enrichment (% input)

ES cells

TS cells

Oct4 promoter

Cdx2 promoter

ES cells

TS cells

siNT

si107

si62

si98

si153

Oct4 promoter

Cdx2 promoter
Figure 4—13: Validation of active and repressive chromatin modifications at the *Oct4* and *Cdx2* genes in ES and TS cells.

ChIP analysis using RNAPII, H3K4me3, H3K9me2 and H3K27me3 antibodies at *Oct4* and *Cdx2* promoters. In ES cells, the expressed *Oct4* promoter harbored active chromatin modification, RNAPII and H3K4me3, and low levels of repressive modifications, H3K9me2 and H3K27me3. Conversely, the repressed *Cdx2* promoter was enriched for repressive modifications but lacked active chromatin modification in ES cells. In TS cells, the expressed *Cdx2* promoter harbored active chromatin modification, RNAPII and H3K4me3, and low levels of repressive modifications, H3K9me2 and H3K27me3. Conversely, the repressed *Oct4* promoter was enriched for repressive modifications but lacked active chromatin modification in ES cells. No significant change in enrichment levels was observed upon nucleoporin depletion (n=3). Error bars, s.e.m; *, significance p < 0.05 compared to the siNT control.
Allelic enrichment levels (Maternal, Paternal)

<table>
<thead>
<tr>
<th>Allelic enrichment levels</th>
<th>Kcnq1</th>
<th>Kcnq1ot1</th>
<th>Cdkn1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNAPII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K4me3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K9me2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K27me3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allelic enrichment levels</th>
<th>Kcnq1</th>
<th>Kcnq1ot1</th>
<th>Cdkn1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNAPII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K4me3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K9me2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K27me3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4—14: Nucleoporin depletion disrupts histone modifications at the **Kcnq1ot1** ICR and at reactivated imprinted gene promoters in ES and TS cells.

(A) RNAPII, H3K4me3, H3K9me2 and H3K27me3 ChIP at the maternal and paternal **Kcnq1ot1** ICR and imprinted gene promoters in control and *Nup*-depleted ES cells (n=3). Allelic proportions are represented as a percent of the total enrichment level. (B) RNAPII, H3K4me3, H3K9me2 and H3K27me3 ChIP at the maternal and paternal **Kcnq1ot1** ICR and imprinted gene promoters in control and *Nup*-depleted TS cells (n=3). Allelic proportions are represented as a percent of the total enrichment level. WT, wildtype; Veh, vehicle; siNT, non-targeting siRNA; si107, *Nup107* siRNA; si62, *Nup62* siRNA; si98/96, *Nup98/96* siRNA, si153, *Nup153* siRNA. Error bars, s.e.m; *, significance p < 0.05 compared to the siNT control.
Figure 4—15: Nucleoporin depletion did not alter histone modifications at imprinted gene promoters, where paternal allelic-silencing was maintained in ES and TS cells.

RNAPII, H3K4me3, H3K9me2 and H3K27me3 ChIP at the maternal and paternal Kcnq1ot1 ICR and imprinted gene promoters in control and Nup-depleted ES (Phlda2 and Tssc4) and TS (Phlda2) cells (n=3). Allelic proportions are represented as a percent of the total enrichment level. WT, wildtype; Veh, vehicle; siNT, non-targeting siRNA; si107, Nup107 siRNA; si62, Nup62 siRNA; si98/96, Nup98/96 siRNA, si153, Nup153 siRNA. Error bars, s.e.m; *, significance p < 0.05 compared to the siNT control.
4.9 Nucleoporins regulate CTCF/cohesin complex at the Kcnq1ot1 ICR in ES cells but not in TS cells

Another explanation for altered regulation of the Kcnq1ot1 domain upon Nup depletion is a change in CTCF and/or cohesin proteins interactions at the domain. Previously, I found that the cohesin proteins, SMC1 and SMC3, but not CTCF were enriched on the paternal Kcnq1ot1 ICR (IC3 and IC4) in XEN cells. Here, I determined the role of CTCF and the SMC1/SMC3 proteins in nucleoporin-mediated imprinted domain regulation. As a control, ChIP was performed using CTCF, SMC1 and SMC3 antibodies in ES and TS cells, and was assessed for binding at the H19 ICR and Peg3 differentially methylated region (DMR) as positive controls, and H19 exon 5 and Peg3 exon 2 as negative controls (Prickett et al., 2013). CTCF, SMC1 and SMC3 enrichment was observed at the H19 ICR preferentially on the maternal allele in ES cells but not at the H19 ICR in TS cells (Fig 4-16). CTCF, SMC1 and SMC3 were enriched at Peg3 DMR preferentially on the paternal allele in ES and TS cells, albeit at lower levels in the latter. Next, I assessed CTCF interactions at the Kcnq1ot1 ICR and enhancer element sites (Fig 4-17A). In ES cells, I observed CTCF enrichment at the two previously identified CTCF-binding sites (IC3, IC4) (Fitzpatrick et al., 2007; Hark et al., 2000) within the Kcnq1ot1 ICR (Fig 4-15B). SMC1 and SMC3 enrichment was also observed at the Kcnq1ot1 IC3 and IC4 sites (Fig 4-16B). Surprisingly, no enrichment of CTCF, SMC1 or SMC3 was observed at the Kcnq1ot1 ICR sites in TS cells (Fig 4-16). Thus, the effects of Nup depletion were only determined for ES cells. Compared to controls, a significant decrease in CTCF, SMC1 and/or SMC3 enrichment was found at the Kcnq1ot1 ICR sites (IC3, IC4) upon Nup107, Nup62 and Nup153 depletion. In control ES cells, this enrichment was biased toward the paternal allele. In Nup107-, Nup62- and Nup153-depleted ES cells, CTCF, SMC1 and SMC3 enrichment was significantly reduced at the paternal the Kcnq1ot1 ICR sites (Figure 4-17). These results indicate that NUP107, NUP62 and/or NUP153 play a role in CTCF and cohesin complex interaction on the paternal Kcnq1ot1 ICR in ES cells. By comparison, only the cohesin complex bound to the paternal Kcnq1ot1 ICR in XEN cells, while neither CTCF nor the cohesin complex bound the paternal Kcnq1ot1 ICR in TS cells.
**Figure 4—16:** CTCF, SMC1 and SMC3 localization at the *H19* and *Peg3* domains in ES and TS cells

CTCF, SMC1 and SMC3 ChIP at the *H19* ICR and *Peg3* DMR in control and *Nup*-depleted ES and TS cells (n=3). Allelic analysis indicated a maternal bias enrichment for CTCF, SMC1 and SMC3 at *H19* ICR and a paternal bias enrichment for CTCF, SMC1 and SMC3 at the *Peg3* DMR (n=3).
Figure 4—17: CTCF, SMC1 and SMC3 enrichment at the Kcnq1ot1 ICR and enhancer site in ES and TS cells

(A) Map of the primer test sites for Kcnq1ot1 ICR and enhancer region. (B). In ES cells CTCF, SMC1 and SMC3 enrichment was seen at the IC3 and IC4 regions. (C). No CTCF, SMC1 and SMC3 enrichment was seen at sites along the ICR and enhancer in TS cells. Error bars, s.e.m; *, significance p < 0.05 compared to IgG control.
Total CTCF enrichment levels

Allelic CTCF enrichment levels

Total SMC1 enrichment levels

Allelic SMC1 enrichment levels

Total SMC3 enrichment levels

Allelic SMC3 enrichment levels

ES cells

Maternal

Paternal

siNT
si107
si62
si153

siNT
si107
si62
si153

siNT
si107
si62
si153

siNT
si107
si62
si153

siNT
si107
si62
si153

siNT
si107
si62
si153
Figure 4—18: CTCF, SMC1 and SMC3 enrichment at the paternal *Kcnq1ot1* ICR is reduced upon nucleoporin depletion in ES cells.

(A) Quantitative ChIP analysis and allelic analysis for (A) CTCF, (B) SMC1 and (C) SMC3 at positive mAb414 and NUP153 enrichment sites in control and *Nup107, Nup62* and *Nup153*-depleted ES cells (n=3). Allelic proportions are represented as a percent of the total enrichment level. Error bars, s.e.m; *, significance p < 0.05 treatment compared to the siNT control.
Chapter 5

5 Discussion

The mechanisms by which the Kcnq1ot1 imprinted domain is regulated is poorly understood. It is not clear what regulates the paternal expression of the Kcnq1ot1 ncRNA. Furthermore, mechanisms regulating paternal allelic silencing of the neighbouring-protein coding genes are poorly understood. Currently, it is not known whether nucleoporins play a role in regulating Kcnq1ot1 domain imprinting. Furthermore, the mechanisms responsible for regulating the Kcnq1ot1 domain in three lineages of the preimplantation embryo are poorly understood. The purpose of this study was to determine the role of nucleoporin proteins in Kcnq1ot1 imprinted domain regulation in XEN cells and to determine whether this mechanism was conserved in ES and TS cells. Overall, I have identified a novel mechanism of imprinted domain regulation, namely nucleoporin-mediated imprinted domain regulation at the Kcnq1ot1 domain. I determined that nucleoporin proteins regulate Kcnq1ot1 ncRNA expression, Kcnq1ot1 domain localization to the nuclear rim and silencing of protein-coding genes on the paternal allele in a nucleoporin-specific manner in ES, TS and XEN cells. Very few epigenetic factors have been identified that regulate genomic imprinting. Perturbations in either imprint establishment or maintenance at the KCNQ1OT1 domain result imprinting defects that have severe consequences for growth and development including Beckwith-Wiedemann Syndrome. Therefore, my results establish an important role for nucleoporins NUP107, NUP62 and NUP153 in mediating imprinted domain regulation in all three cell lineages that represent the early embryo.

My data indicate that NUP107, NUP62 and NUP153 regulated paternal Kcnq1ot1 ncRNA expression, paternal Kcnq1ot1 domain positioning at the nuclear periphery, and paternal allelic silencing of specific imprinted genes in XEN stem cells (Figure 5-1). NUP107, NUP62 and NUP153 were bound at the Kcnq1ot1 ICR on the paternal allele in XEN cells. In addition, I found that NUP107, NUP62 and NUP153 maintained active chromatin at the Kcnq1ot1 ICR and a repressed conformation at the paternal alleles of imprinted genes that
had been reactivated upon \textit{Nup} depletion. The cohesin complex, but not CTCF, assembled at the same sites within the paternal \textit{Kcnq1ot1} ICR as NUP107, NUP62 and NUP153, and binding was reduced upon \textit{Nup} depletion. Similar to XEN cells, NUP107, NUP62 and NUP153 regulated paternal \textit{Kcnq1ot1} ncRNA expression and paternal \textit{Kcnq1ot1} domain positioning at the nuclear periphery in ES and TS cells (Figures 5-2, 5-3). While NUP107, NUP62 and NUP153 regulated paternal allelic silencing of specific imprinted genes in all three stem cell lines, the genes regulated differed. NUP107, NUP62 and NUP153 were bound at the \textit{Kcnq1ot1} ICR on the paternal allele in ES, TS and XEN cells, as well as at paternal alleles of imprinted genes that had been reactivated upon \textit{Nup} depletion. Compared to XEN cells, where the cohesin complex, but not CTCF, assembled at the paternal \textit{Kcnq1ot1} ICR with NUP107, NUP62 and NUP153, both CTCF and the cohesin complex bound to the paternal \textit{Kcnq1ot1} ICR in a NUP107, NUP62 and NUP153-dependent manner in ES cells, while in TS cells neither CTCF nor cohesin assembled at the paternal \textit{Kcnq1ot1} ICR. These results indicate that NUP107, NUP62 and NUP153 regulate imprinting at the \textit{Kcnq1ot1} domain by a nucleoporin-mediated mechanism in ES, TS and XEN cells, although in a cell lineage-specific manner. My work opens up a new dimension to understand imprinted domain regulation and role of nucleoporins.
Figure 5—1: Summary of nucleoporin-mediated regulation of the Kcnq1ot1 imprinted domain in XEN cells

NUP107, NUP62 and NUP153 regulated paternal Kcnq1ot1 ncRNA expression, paternal Kcnq1ot1 domain positioning at the nuclear rim, and paternal allelic silencing of specific imprinted genes in XEN stem cells. NUP107, NUP62 and NUP153 were bound at the Kcnq1ot1 ICR on the paternal allele in XEN cells. In addition, we found that NUP107, NUP62 and NUP153 maintained active chromatin at the Kcnq1ot1 ICR. The cohesion complex, but not CTCF, assembled at the same sites within the paternal Kcnq1ot1 ICR as NUP107, NUP62 and NUP153. Upon Nup107 and Nup62 depletion, Kcnq1ot1 ncRNA expression was significantly reduced, which was correlated with a change in histone modifications at the Kcnq1ot1 ICR, and the paternal Kcnq1ot1 domain was shifted away from the nuclear rim. NUP107 and NUP62 had reduced binding at the the Kcnq1ot1 ICR and the enhancer element. NUP153 and cohesin binding were also reduced at the Kcnq1ot1 ICR. Furthermore, the paternal allele of the core group of genes, Slc22a18, Cdkn1c and Kcnq1 was reactivated, with a corresponding change in histone modifications. The silent paternal Osbpl5 and Phlda2 were also reactivated by little to no change in histone modifications. Similar alterations to Nup107- and Nup62-depleted XEN cells were observed upon Nup153 depletion. Here, the normally-silent, maternal Kcnq1ot1 ncRNA was reactivated. NUP153 had reduced binding at the Kcnq1ot1 ICR, the Kcnq1ot1 promoter and Cd8l promoter. NUP107, NUP62 and cohesin binding were also reduced at the Kcnq1ot1 ICR. Furthermore, the paternal allele of the core group of genes, Slc22a18, Cdkn1c and Kcnq1, as well as Cd8l were reactivated, with a corresponding change in histone modifications. For all Nup-depletion, DNA methylation state was maintained.
### ES cells

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Kcnq1ot1 Imprinted Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ospp65</td>
</tr>
</tbody>
</table>

#### Control ES cells

- **Nuclear Rim**
- **NUP107**
- **NUP62**
- **NUP153**

#### Nup107- and Nup62-depleted ES cells

- **5mC**
- **Active**
- **Repressive**
- **CTCF/COHESIN**

#### Nup153-depleted ES cells
Figure 5—2: Summary of nucleoporin-mediated regulation of the *Kcnq1ot1* imprinted domain in ES cells

NUP107, NUP62 and NUP153 regulated paternal *Kcnq1ot1* ncRNA expression, paternal *Kcnq1ot1* domain positioning at the nuclear rim, and paternal allelic silencing of specific imprinted genes in XEN stem cells. NUP107, NUP62 and NUP153 were bound at the *Kcnq1ot1* ICR on the paternal allele in XEN cells. In addition, we found that NUP107, NUP62 and NUP153 maintained active chromatin at the *Kcnq1ot1* ICR. The CTCF and the cohesion complex, assembled at the same sites within the paternal *Kcnq1ot1* ICR as NUP107, NUP62 and NUP153. Upon *Nup107* and *Nup62* depletion, *Kcnq1ot1* ncRNA expression was significantly reduced, which was correlated with a change in histone modifications at the *Kcnq1ot1* ICR, and the paternal *Kcnq1ot1* domain was shifted away from the nuclear rim. NUP107 and NUP62 had reduced binding at the *Kcnq1ot1* ICR and the enhancer element. NUP153 and CTCF/cohesin binding were also reduced at the *Kcnq1ot1* ICR. Furthermore, the paternal allele of *Cdkn1c* was reactivated, with a corresponding change in histone modifications. Similar alterations to *Nup107*- and *Nup62*-depleted XEN cells were observed upon *Nup153* depletion. Here, the normally-silent, maternal *Kcnq1ot1* ncRNA was reactivated. NUP153 had reduced binding at the *Kcnq1ot1* ICR, the *Kcnq1ot1* promoter and *Cd81* promoter. NUP107, NUP62 and cohesin binding were also reduced at the *Kcnq1ot1* ICR. Furthermore, *Cdkn1c* and *Cd81* were reactivated, with a corresponding change in histone modifications. For all *Nup*-depletion, DNA methylation state was maintained.
### TS cells

<table>
<thead>
<tr>
<th>Mouse Kcnq1ot1 Imprinted Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ospbl5</td>
</tr>
<tr>
<td>ICR</td>
</tr>
<tr>
<td>Kcnq1ot1</td>
</tr>
</tbody>
</table>

#### Control TS cells

- Nuclear Rim

#### Nup107- and Nup62-depleted TS cells

- NUP107
- NUP62
- NUP153

#### Nup153-depleted TS cells

- 5mC
- Active
- Repressive
Figure 5—3: Summary of nucleoporin-mediated regulation of the Kcnq1ot1 imprinted domain in TS cells

NUP107, NUP62 and NUP153 regulated paternal Kcnq1ot1 ncRNA expression, paternal Kcnq1ot1 domain positioning at the nuclear rim, and paternal allelic silencing of specific imprinted genes in TS cells. NUP107, NUP62 and NUP153 were bound at the Kcnq1ot1 ICR on the paternal allele in XEN cells. In addition, we found that NUP107, NUP62 and NUP153 maintained active chromatin at the Kcnq1ot1 ICR. Neither CTCF or cohesin complex binding was observed in TS cells. Upon Nup107 and Nup62 depletion, Kcnq1ot1 ncRNA expression was significantly reduced, which was correlated with a change in histone modifications at the Kcnq1ot1 ICR, and the paternal Kcnq1ot1 domain was shifted away from the nuclear rim. NUP107 and NUP62 had reduced binding at the the Kcnq1ot1 ICR and the enhancer element. NUP153 binding was also reduced at the Kcnq1ot1 ICR. Furthermore, the paternal allele of the core group of genes, Slc22a18, Cdkn1c and Kcnq1 was reactivated, with a corresponding change in histone modifications. Similar alterations to Nup107- and Nup62-depleted XEN cells were observed upon Nup153 depletion. Here, the normally-silent, maternal Kcnq1ot1 ncRNA was reactivated. NUP153 had reduced binding at the Kcnq1ot1 ICR, the Kcnq1ot promoter and Cd81 promoter. NUP107 and NUP62 binding were also reduced at the Kcnq1ot1 ICR. Furthermore, the paternal allele of the core group of genes, Slc22a18, Cdkn1c and Kcnq1 were reactivated, with a corresponding change in histone modifications. For all Nup-depletion, DNA methylation state was maintained.
5.1 Nucleoparin 107, 62 and 153 regulate Kcnq1ot1 domain both on- and off-pore

Previous studies have shown a coordinate role of NUP107 and NUP62 at spindles and kinetochores (Glavy et al., 2007), where they may play a role in chromatin reorganization upon nuclear membrane reformation (Kalverda et al., 2010). NUP107 and NUP62 may have a similar role at the Kcnq1ot1 domain by coordinating chromatin organization at the domain during each cell cycle. Alternatively, the regulatory role of NUP107 and NUP62 may be independent of mitosis and nuclear membrane reformation. Recently, NUP153 was reported to play a role in PRC1-mediated silencing of developmentally regulated genes in embryonic stem cells, along with tethering at the nuclear periphery (Jacinto et al., 2015). This is consistent with the reactivation of the normally silent maternal Kcnq1ot1 allele upon Nup153 depletion in XEN, ES and TS cells. The coordinate binding of NUP107 and NUP62 with NUP153 likely mediates its regulatory role in active Kcnq1ot1 ncRNA transcription, pointing to a dual role for NUP153. A recent study showed that human NUP98 binds to chromatin, and upon depletion decreased H3K4me2 and RNAPII at target promoters, suggesting a role in gene activation (Light et al., 2010; Light et al., 2013). Here, I observed increased Kcnq1ot1 ncRNA transcription upon Nup98/96 depletion in XEN cells (not in ES or TS cells), without a change in histone modification, leading us to theorize that NUP98 may mediate its function via enhancer interactions, although this requires further investigations.

Interactions between NUP107, NUP62 and NUP153 have previously been documented in HeLa cells. NUP107 depletion decreased NUP153 levels but not NUP62 levels, although both NUP153 and NUP62 localization at the nuclear periphery was reduced (Boehmer et al., 2003; Walther et al., 2003). NUP62 depletion produced a decrease in NUP153 levels (Hashizume et al., 2013), while NUP153 depletion led to patchy NUP107 localization at the nuclear periphery (Vollmer et al., 2015). A recent study investigating various nucleoporin interactions in U2OS 2-6-3 cells found that only four nucleoporins (NUP153, NUP50, NUP107, NUP133) of the eleven tested had the capacity to recruit multiple nucleoporins (Schwartz et al., 2015). NUP153 recruited multiple nucleoporins including
NUP62 and NUP107-160 complex members. This NUP153-initiated structure repositioned an integrated chromatin marker from the nuclear interior to the nuclear periphery in 76% of cells. NUP107 similarly recruited multiple nucleoporins and positioned the chromatin marker to the nuclear periphery, although with lower frequency (25% of cells). By comparison, NUP98 possessed very limited nucleoporin recruitment and lacked the capacity to target chromatin to the nuclear periphery. In keeping with this data, I found that Nup107, Nup62 and Nup153 depletion diminished Kcnq1ot1 domain positioning at the nuclear periphery.

To determine the interdependency between NUP107/62 and NUP153 binding, I assessed NUP107/62 enrichment upon Nup153 depletion and NUP153 enrichment upon Nup107 and Nup62 depletion. In all three cell lineages, depletion of either Nup153, or Nup107 and Nup62 resulted in reduced enrichment of NUP107/62 and NUP153 at the Kcnq1ot1 ICR. This suggests that NUP107/62 and NUP153 act cooperatively at the Kcnq1ot1 ICR, thereby tethering the Kcnq1ot1 domain to nuclear periphery. Upon Nup107, Nup62 or Nup153 depletion, this nuclear peripheral positioning of the Kcnq1ot1 domain is lost. Having said this, other sites within the domain maintain their enrichment upon Nup153 depletion, or Nup107 and Nup62 depletion. For example, in XEN cells, NUP107 and NUP62 remained bound at the Osbpl5 promoter and the enhancer site upon Nup153 depletion. Similarly, NUP153 remained bound to Cd81 promoters in ES cells and XEN cells upon Nup107 and Nup62 depletion. However, even when these nucleoporin interactions are maintained, the Kcnq1ot1 domain shifted away from the nuclear periphery. The most likely explanation for this is that NUP107/62 and NUP153 bound to and tethered the Kcnq1ot1 ICR to the nuclear periphery through the nuclear pore complex, while NUP107/62 and NUP153 binding at other regions of the domain were through an off-pore function. Recent studies have identified multiple nucleoporins, including NUP62, NUP153, NUP98 and NUP50, that are present in the nucleoplasm as mobile soluble fractions and regulate genes away from the nuclear periphery by binding to their promoters (Buchwalter et al., 2014; Griffis et al., 2004; Liang et al., 2013; Jacinto 2015).
5.2 Requirement for the Kcnq1ot1 noncoding RNA

The Kcnq1ot1 noncoding RNA plays a role in paternal allelic silencing of both ubiquitous and placenta-specific imprinted genes in midgestation embryos. It was anticipated that upon reduced Kcnq1ot1 ncRNA expression, there would be similar domain-wide paternal allelic silencing, similar to what was observed for targeted deletion of the Kcnq1ot1 ICR and truncation of the Kcnq1ot1 ncRNA (Shin et al., 2008). While I observed a decrease in Kcnq1ot1 ncRNA abundance upon nucleoporin depletion in all three stem cell lines, paternal allelic silencing was not domain-wide; a different set of genes had paternal allelic reactivation across the three lineages. Furthermore, the Phlda2, Kcnq1, Tssc4, Cd81, Ascl2 and Th genes maintained paternal allelic silencing in at least one stem cell line upon nucleoporin depletion. This questions whether the Kcnq1ot1 ncRNA is required for paternal allelic silencing during early development stages, and if so, how the Kcnq1ot1 ncRNA mediates differential silencing within the Kcnq1ot1 domain. One possibility is that the Kcnq1ot1 ncRNA act in a gene-specific manner to silence the paternal Slec22a18, Cdkn1c and Kcnq1 alleles in XEN and TS cells, and Cdkn1c in ES cells. Genes more distal from the Kcnq1ot1 ICR would be regulated by a Kcnq1ot1 ncRNA-independent mechanism. Having said this, data from our lab showed that depletion of the Kcnq1ot1 ncRNA failed to reactivate paternal alleles of imprinted genes in the Kcnq1ot1 domain in XEN, ES and TS cells. Instead, it was concluded that it was likely the act of Kcnq1ot1 ncRNA transcription rather than the ncRNA itself which functioned to silence paternal alleles (Golding et al., 2011). Consistent with a transcription role, Nup107, Nup62 and Nup153 depletion reduced active chromatin modifications at the paternal Kcnq1ot1 ICR in ES and TS cells, and reduced active and increased repressive chromatin modifications at the paternal Kcnq1ot1 ICR in XEN cells. However, a Kcnq1ot1 transcription interference mechanism would not account for gene-specific paternal allelic reactivation, since only the paternal Kcnq1 and/or Cd81 alleles (not Tssc4 and Th) in XEN cells, the Cd81 allele (not Kcnq1 and Tssc4) in ES cells, and the Kcnq1 allele (not Ascl2) in TS cells were reactivated. Overall, these results suggest that it is an open chromatin conformation at the Kcnq1ot1 ICR, an active Kcnq1ot1 promoter and/or some yet to be determined function of the Kcnq1ot1 ncRNA (RNA adaptor; RNA scaffold) that mediates paternal allelic silencing.
Whichever the case, the mechanism by which gene-specific paternal allelic silencing is enacted requires further investigation.

Previous studies have shown that the Kcnq1ot1 domain is compartmentalized to the nuclear periphery or perinucleolar regions (Mohammad et al., 2008; Redrup et al., 2009). Here, Nup107, Nup62 and Nup153 depletion caused a loss of nuclear periphery localization for the paternal Kcnq1ot1 domain in G1-synchronized ES, TS and XEN cells. Despite this repositioning within the nucleus, the Kcnq1ot1 ncRNA remained associated with the Kcnq1ot1 domain in Nup107-, Nup62- and Nup153-depleted ES, TS and XEN cells, albeit with lower Kcnq1ot1 volumes. This indicates that the Kcnq1ot1 ncRNA per se does not regulate paternal Kcnq1ot1 domain positioning at the nuclear periphery.

5.3 Nucleoporin 107, 62 and 153 in higher order chromatin structure

One intriguing finding from my study was that imprinted genes within the Kcnq1ot1 domain were not co-ordinately regulated. To gain a better understanding of domain regulation, data from Pope et. al. was mined to map topological associated domains within the Kcnq1ot1 domain. We found that the Kcnq1ot1 domain mapped into three TADs in embryonic stem cells (Macdonald et al., 2015; Pope et al., 2014). The first TAD contains the Oshpl5 gene plus 3 non-imprinted genes, the second TAD extends from Phlda2 to the Kcnq1 promoter, while the third TAD covers Tssc4 to Th (Figure 5-4). Given the stable nature of TADs across cell types (Dixon et al., 2012; Korostowski et al., 2011), I propose a model for nucleoporin regulation at the Kcnq1ot1 domain. Within TAD 1, NUP107/NUP62 bound at the paternal Oshpl5 promoter region in XEN cells. This interaction may isolate Oshpl5 from non-imprinted genes in this TAD. Upon Nup107 and Nup62 depletion, paternal Oshpl5 may be relocated into the non-imprinted gene loop. Consistent with this, in ES and TS cells, the paternal Oshpl5 promoter region was not bound by NUP107/NUP62 and the paternal Oshpl5 allele was expressed, suggesting that it resides within the non-imprinted gene loop.
Within TAD 3, the paternal Tssc4, Cd81 and Th alleles were repressed in XEN cells, the paternal Tssc4 and Cd81 alleles were silenced in ES cells, while the paternal Ascl2 allele was repressed in TS cells. In XEN and ES cells, NUP153 bound to the paternal Cd81 promoter region. Upon Nup153 depletion, only paternal Cd81 silencing was disrupted in XEN and ES cells, suggesting that Cd81 may be incorporated into the neighbouring non-imprinted gene loop.

Within central TAD 2, NUP107/NUP62 and NUP153 were bound to the paternal Kcnq1ot1 ICR, and NUP107/NUP62 was bound to the paternal 8 kb-downstream enhancer element in all three cell lineages. In XEN cells, the cohesin complex also assembled at the paternal Kcnq1ot1 ICR. By comparison, in ES cells, both CTCF and the cohesin complex interacted with the paternal Kcnq1ot1 ICR. In ES cells, a second interaction with NUP107/NUP62 was observed at the paternal Cdkn1c promoter region, while in XEN cells, NUP107/NUP62 also interacted with the Kcnq1 promoter region. Thus, in XEN and ES cells, NUP107/NUP62 and NUP153 together with cohesin and/or CTCF may facilitate promoter-enhancer interactions and/or boundary function, enabling an active loop for Kcnq1ot1 ncRNA transcription, while excluding Cdkn1c and Kcnq1. Consistent with this, a previous study reported that a paternal 200 kb-intrachromosomal loop existed between the Kcnq1 promoter and enhancers in fibroblast cells, that maintained paternal Kcnq1 silencing (Korostowski et al., 2011; Zhang et al., 2014). Upon Nup107, Nup62 and Nup153 depletion, Kcnq1ot1 ICR-putative enhancer interaction or boundary function may be disrupted, repressing Kcnq1ot1 ncRNA transcription and allowing enhancer interactions or euchromatin spreading into other genes in this TAD. For XEN cells, this would include the paternal Slc22a18, Cdkn1c and Kcnq1 alleles while for ES cells, spreading would be limited to the paternal Cdkn1c allele. For TS cells, neither CTCF nor the cohesin complex assembled at the paternal Kcnq1ot1 ICR. This may suggest that other proteins may mediate chromatin interactions/chromatin looping within TAD2. Upon Nup107, Nup62 and Nup153 depletion, paternal Kcnq1ot1 ncRNA transcription would be repressed and the paternal Slc22a18, Cdkn1c and Kcnq1 reactivated.
Figure 5—4: Proposed model of topological organization of the paternal *Kcnq1ot1* imprinted domain orchestrated by nucleoporins in XEN, ES and TS cells.

The paternal *Kcnq1ot1* imprinted domain separated in three distinct topologically associated domains (TADs, dashed lines) in XEN, ES and TS cells. Within TAD2, the *Kcnq1ot1* ICR (orange) and putative enhancer (green) interact with NUP107, NUP62 and NUP153, and the cohesin complex in XEN cells and CTCF/cohesin complex in ES cells, facilitating promoter-enhancer interactions or chromatin boundary function. This results in active chromatin loop formation, which allows for *Kcnq1ot1* noncoding RNA transcription. The remaining genes in TAD2 reside outside the active chromatin loop and are repressed. In TS cells, this interaction is independent of CTCF and cohesin complex.

In XEN cells, within TAD1, an additional site of NUP107 and NUP62 binding is present at the paternal *Osbpl5* promoter. This may generate a chromatin boundary between repressive and active chromatin, placing *Osbpl5* in repressive chromatin while other expressed, non-imprinted genes reside in an active chromatin loop. In ES and TS cells, *Osbpl5* is expressed and possibly located within the active chromatin loop. TAD3 consists of paternally silenced genes as well non-imprinted genes that are expressed. NUP153 binds at the *Cd81* promoter in XEN and ES cells, placing *Cd81* in repressive chromatin and the neighboring expressed, non-imprinted genes in an active chromatin loop. In TS cells, the paternal *Cd81* is expressed. For non-imprinted genes in the domain, please note their imprint and expression status has not been determined in XEN cells. Also note that I have drawn the domain linearly and that there may be subTAD interactions generating different conformation or loops than what is displayed here. Model is not to scale.
5.4 Future Directions

Based on the results of the present study, I propose a model where NUP107/NUP62 and NUP153 facilitates chromatin-chromatin interactions and chromatin loop formation on the paternal allele in partnership with the cohesin complex and/or CTCF, or other epigenetic factors in XEN, ES and TS cell respectively. Future studies need to be directed toward understanding the role of nucleoporins in chromatin structure at the Kcnq1ot1 domain in ES, TS and XEN cells. These studies would include performing Hi-C, which is a chromatin conformation capture method followed by high-throughput sequencing. Hi-C analysis in control stem cells will determine chromatin-chromatin interactions and potential chromatin boundaries, as well as allow the comparison of TAD profiles between the three stem cell lineages. Furthermore, Hi-C analysis in nucleoporin-depleted stem cells will determine which chromatin interactions and chromatin boundaries are dependent on nucleoporins. Once TAD boundaries important to imprinted domain regulation are identified, CRISPR/Cas9 technology can be used to generate mutations that alter these boundaries to determine their functional role during development.

Future studies are also needed to understanding the role of nucleoporins in the nuclear architecture at the Kcnq1ot1 domain. Lamin-associated domains (LADs) are regions of chromatin that are bound to Lamin B1 (Guelen et al. 2008). Unlike TADs that remain largely stable across cell and tissue types, LADs can be constitutive or dynamic. LaminB1-ChIP sequencing could be performed to identify LADs in XEN, ES and TS cells. Mapping of LADs within the Kcnq1ot1 domain may account for genes that maintain paternal-allelic silencing as well as those that differentially lose paternal-allelic silencing in stem cells upon Nup107, Nup62 and Nup153 depletion. A previous study also reported an interaction between HDAC4 and NPC proteins at boundary locations between active and repressed regions (Kehat et al., 2011). Chromatin immunoprecipitation of various HDAC proteins such as HDAC9 (identified in our shRNA screen) and HDAC4 at regions between or coinciding with NUP107, NUP62 and NUP153 and LADs might therefore be informative.
In my thesis, the *Kcnq1ot1* domain was used as a model domain. Currently, there are two models of imprinted domain regulation, the ICR-mediated enhancer-blocking model and the ncRNA-mediated model. The *H19* and the *Gtl2* domains are examples of the former, while the *Airn* and *Snrpn* domain are examples of the latter. Future studies are required to investigate the role of NUP107, NUP62 and NUP153 in regulating other imprinted domains in XEN, ES and TS cells. Investigation of the *H19*, *Gtl2*, *Airn* and *Snrpn* domain will determine whether NUP107, NUP62 and NUP153 play a role in the regulation of these domains, and whether they are regulated in manner similar to the *Kcnq1ot1* domain.

Once the mechanisms are clearly determined in ES, TS and XEN cells, future studies will be required to investigate the role of nucleoporins during development. To determine whether nucleoporins are involved in *Kcnq1ot1* imprinted domain regulation during preimplantation development, genetic mutations could be generated using CRISPR technology. To determine whether nucleoporin mutations lead to aberrant *Kcnq1ot1* ncRNA and paternal allelic reactivation, imprinted expression analyses can be conducted for the *Kcnq1ot1* ncRNA and protein-coding genes in blastocyst stage embryos. Imprinted methylation analysis can also be performed to assess whether DNA is maintained as it was in stem cells. DNA/RNA FISH using *Kcnq1ot1* probes can be performed to visualize the effects of nucleoporin mutations in individual cells of the blastocyst embryo, allowing us to examine cells with different fates, i.e. epiblast precursors, primitive endoderm and trophectoderm. These investigations will delineate the mechanism of nucleoporin-mediate regulation of the *Kcnq1ot1* domain during preimplantation development. Further investigation of postimplantation stages of development will also be important. Midgestation embryos carrying a paternally-inherited *Kcnq1ot1* ICR deletion or *Kcnq1ot1* ncRNA truncation exhibit domain-wide loss of paternal allelic silencing. To determine whether nucleoporins act domain-wide in control and mutant postimplantation conceptuses, embryonic, placental and yolk sac tissues can be examined using the same assays that I used for XEN, ES and TS cells. These experiments will address the developmental regulation of the *Kcnq1ot1* imprinted domain by nucleoporins.
Overall, the novel mechanistic action of nucleoporin proteins at the *Kcnq1ot1* imprinted domains opens a new dimension to understand imprinted domain regulation and the role of nucleoporins as epigenetic regulators.
References


Engel, N., Thorvaldsen, J. L. and Bartolomei, M. S. (2006). CTCF binding sites promote transcription initiation and prevent DNA methylation on the maternal allele


Weksberg, R., Nishikawa, J., Caluseriu, O., Fei, Y. L., Shuman, C., Wei, C., Steele, L., Cameron, J., Smith, A., Ambus, I., et al. (2001). Tumor development in the


Appendix 1: Copyright Release


NRC RESEARCH PRESS LICENSE
TERMS AND CONDITIONS

Jun 24, 2016

This Agreement between Saqib S Sachani ("You") and NRC Research Press ("NRC Research Press") consists of your license details and the terms and conditions provided by NRC Research Press and Copyright Clearance Center.

License Number 3895560301751
License date Jun 24, 2016
Licensed Content Publisher NRC Research Press
Licensed Content Publication Biochemistry and Cell Biology
Licensed Content Title A role for chromatin topology in imprinted domain regulation
Licensed Content Author William A. MacDonald, Saqib S. Sachani, Carlee R. White, et al
License Content Date Feb 1, 2016
Licensed Content Volume Number 94
Licensed Content Issue Number 1
Type of Use Thesis/Dissertation
Requestor type Author (original work)
Format Electronic
Portion Figure/table
Number of figures/tables 1
Order reference number
Title of your thesis / dissertation NUCLEOCOPIN-MEDIATED REGULATION OF THE KCNQ1OT1 IMPRINTED DOMAIN
Expected completion date Aug 2016
Estimated size (pages) 166
Requestor Location Saqib S Sachani
A4-146 Victoria Research Labs
800 Commissioners Road East
London, ON N6C 2V5
Canada
Attn: Saqib S Sachani

Billing Type Invoice
Billing Address Saqib S Sachani
A4-146 Victoria Research Labs
800 Commissioners Road East
London, ON N6C 2V5
Canada
Attn: Saqib S Sachani

Total 0.00 CAD

Terms and Conditions

From: Canadian Science Publishing
To: Saqib Sachani
RE: Permission to use copyrighted material in a doctoral thesis
Date: Monday June 27, 8:51 AM

Dear Dr. Sachani,

Please review:  http://www.nrcresearchpress.com/page/authors/information/rights

As one of the authors of these paper, you may reuse your published material. Permission is granted.

Thank you for checking.

Regards,
Thera Pritchard
Customer Service and Sales Coordinator
Canadian Science Publishing
Curriculum Vitae

SAQIB S. SACHANI

EDUCATION

**Doctor of Philosophy – Biochemistry and Developmental Biology**  
2011 – Present  
Department of Biochemistry, Collaborative Program in Developmental Biology  
Children’s Health Research Institute  
University of Western Ontario, London, Ontario, Canada  
Thesis: Nucleoporin-mediated regulation of the *Kcnq1ot1* imprinted domain

**Masters of Science – Biological Sciences**  
2009 - 2011  
Department of Biological Sciences  
University of Windsor, Windsor, Ontario, Canada  
Thesis: Role of Six3 and Pax6 in regulating the gene networks involved in vertebrate eye development.

**Bachelor of Science (Honours) – Biology and Biotechnology**  
2005 – 2009  
Minor in Applied Information Technology  
Department of Biological Sciences  
School of Computer Science, Faculty of Science  
University of Windsor, Windsor, Ontario, Canada

ACADEMIC AWARDS AND SCHOLARSHIP

Lawson Health Research Institute Student Fellowship  
2014 – 2015  
Children’s Health Research Institute Travel Award  
2015  
Curtis Cadman Studentship, Children’s Health Research Institute  
2012 – 2013  
Obstetrics and Gynaecology Graduate Scholarship  
2011 – 2012  
Graduate Student Society Scholarship  
2010 – 2011  
Travel Award, 13th International *Xenopus* Conference  
2010  
International Student Graduate Scholarship for Academic Excellence  
2009-2011
REFEREED PUBLICATIONS
1. Sachani SS, White CR and Mann MRW (2016). Conserved nucleoporin-mediated regulation of the Kcnq1ot1 imprinted domain in embryonic and trophoblast stem cells, manuscript under preparation

NON-REFEREED PRESENTATIONS (ORAL AND POSTER)
2. Fakhereddin, M, Sachani SS; Crawford MJ. Exploring the Role of Rax1 in Eye Development. Canadian Developmental Biology Conference, Banff, Alberta Canada, March 17-20, 2016. Poster Presentation


13. Sachani SS, Landschoot LS, Zhang L, Mann MRW. Epigenetic regulation of the Kcnq1ot1 imprinted domain at the nuclear membrane. M3: Mostly Mammals in Montreal Meeting, Montreal, Quebec, Canada. March 21-23, 2013. (Selected oral presentation)


15. Sachani SS, Landschoot LS, Zhang L, Mann MRW. Role of nuclear pore complex proteins and laminas in regulating imprinting at the Kcnq1ot1 domain. Harold B. Stewart Memorial Lecture and Research Showcase, Department of Biochemistry, University of Western Ontario. November 2012 (Poster Presentation)

16. Baert T, Sachani SS and Crawford MJ. Factors affecting crystallin expression and lens differentiation in Xenopus laevis (Seminar Talk) – Undergraduate Biology Colloquium, University of Windsor, April 21, 2011


PROFESSIONAL MEMBERSHIPS
2009 – Present, Member Society of Developmental Biologists
2009 – Present, Canadian Society of Biochemistry, Molecular and Cellular Biology
WORK EXPERIENCE

Globalink Graduate Advisor 2012 – 2015
Mitacs Globalink Foundation, Canada
• Advisor and support for students attending Western University in the STEM (Science, Technology, Engineering and Mathematics) fields during Summer from around the world.

Graduate Teaching Assistant 2009 – 2011
University of Windsor, Windsor, ON. Canada.
• Introductory Microbiology and Techniques, Laboratory Instructor (Fall 2009)
• Medical Microbiology and Techniques, Laboratory Instructor (Winter 2010)
• Embryology & Developmental Biology – Teaching Assistant (Fall 2010)
• Guest Lecturer, Embryology & Developmental Biology, (Fall 2010, 4 lectures)
• DNA Biotechnology Molecular Techniques, Laboratory Instructor (Winter 2011)

Assistant Instructor/Course Coordinator 2008 - 2011
Canterbury College, University of Windsor, Windsor, ON. Canada
• International Engineering/Medical Graduate Program (June 2010 – April 2011)
• International Medical Graduate Program (January 2008 – April 2010)

SUPERVISING EXPERIENCE

University of Western Ontario
2014 Summer - Taylor Smith, Biotechnology Internship candidate
2013 – 2014 – Alisha Bester, 4th Year undergraduate project student)
2013 Summer – Josef Ianni, Volunteer summer student
University of Windsor
2011 Summer – Touba Warsi (NSERC Summer Student)
2010-2011 – Candace Rapchak (NSERC Summer/4th year undergraduate project student)
2010 – 2011 – Todd Baert (4th Year undergraduate project student)
2010 – 2011 – Ahsan Farooqi (Volunteer)

UNIVERSITY INVOLVEMENT

University of Windsor
2010 – 2011, University Senate, Student Standing Committee
2010 – 2011, International Student Retention Working Committee
2010 – 2011, President of International Student Society
2009 – 2010, Vice President of Accessibility, International Student Society
2009 – 2010, I.T. Steering Committee, Canterbury College
2008 – 2010, Education Program Development Committee, Canterbury College
2010 – 2011, International Student Health Insurance Working Committee
2010 – 2011, Student Diversity Action Committee, Human Rights
2009 – 2011, International Student Scholarship Committee, ISS.
2008 – 2009, Awards and Scholarship Selection Committee, Canterbury College