CHARACTERIZING THE ROLE OF NUCLEAR PORE COMPLEXES IN GENOME INSERTION OF THE YEAST TY1 RETROTRANSPOSON

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2018

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Characterizing the role of nuclear pore complexes in genome insertion of the yeast Ty1 retrotransposon

submitted by Savrina Manhas in partial fulfilment of the requirements for

the degree of Doctor of Philosophy

In Biochemistry and Molecular Biology

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Abstract

Nuclear pore complexes (NPCs) orchestrate cargo between the cytoplasm and nucleus and regulate chromatin organization. NPC proteins, or nucleoporins (Nups), are required for human immunodeficiency virus type 1 (HIV-1) gene expression and genomic integration of viral DNA. I utilize the Ty1 retrotransposon of Saccharomyces cerevisiae (S. cerevisiae) to study retroviral integration because retrotransposons are the progenitors of retroviruses and have conserved integrase (IN) enzymes. Ty1-IN targets Ty1 elements into the genome upstream of RNA polymerase (Pol) III-transcribed genes such as transfer RNA (tRNA) genes. Evidence that S. cerevisiae tRNA genes are recruited to NPCs prompted my investigation of a functional role for the NPC in Ty1 targeting into the genome. I find that Ty1 mobility is reduced in multiple Nup mutants that cannot be accounted for by defects in Ty1 gene expression, complementary DNA (cDNA) production or Ty1-IN nuclear entry. Instead, I find that Ty1 insertion upstream of tRNA genes is impaired. I also identify Nup mutants with wild type Ty1 mobility but impaired Ty1 targeting. The NPC nuclear basket, which interacts with chromatin, is required for both Ty1 expression and nucleosome targeting. Deletion of components of the NPC nuclear basket causes mis-targeting of Ty1 elements to the ends of chromosomes. The mis-targeting suggests that nuclear basket Nups are required directly or indirectly, perhaps as global architects or regulators of chromatin organization to orchestrate Ty1 targeting upstream of Pol III-transcribed genes.
Lay Summary

Globally ~37 million individuals are currently infected with human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS). Although new infections and AIDS-related deaths have been decreasing since the discovery of antiretroviral (ARV) drugs, in 2016, 2 million individuals were newly infected and 1 million individuals died of AIDS-related illnesses. HIV and other retroviruses share a similar life cycle to retrotransposons such as Ty1 in yeast; both HIV and Ty1 insert their DNA into the host genome and encode the enzyme integrase (IN) which is required for this process. Here, I study the role of nuclear pores, molecular tunnels that control movement of molecules in and out of the nucleus, for targeting Ty1-IN to the yeast genome. Identifying host proteins required for Ty1 survival can lead to a better understanding of how viral DNA is integrated into the genome which will aid in ARV drug design.
Preface

Chapter 1 is partially based on my co-first author publication (Manhas S, Cheung S, Measday V. 2018. Retrotransposon targeting to RNA Polymerase III-transcribed genes. Mobile DNA, Vol. 9 (14), p.1-15). This is a peer reviewed review article that I co-wrote with Stephanie Cheung, and Vivien Measday.

Chapters 2 and 3 are primarily based on my first author publication (Manhas S, Ma L, Measday V. 2018. The yeast Ty1 retrotransposon requires components of the nuclear pore complex for transcription and genomic integration. Nucleic Acids Research, Vol. 46 (7), p. 3552-3578). I performed the majority of the experiments and wrote the paper with Vivien Measday. Lina Ma generated the Ty1-IN expression plasmid that I used for the microscope work and she quantified tRNA and Ty1 mRNA levels in mutant yeast strains using qPCR (Figure 2.3A and 2.4).

Chapter 4 is partially based on a first author perspective paper that is being prepared for submission to Current Genetics.
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Δ Deletion
ΔΔCT method Comparative CT method
3C Chromosome conformation capture
aa Amino acids
AH Amphipathic helix
AI Artificial intron
AIDS Acquired immunodeficiency syndrome
bp Base pairs
C- Carboxy
CA Capsid
CCD Catalytic core domain
cDNA Complementary DNA
ChIP Chromatin immunoprecipitation
chr Chromosome
cNLS Classic nuclear localization signal
CTD C-terminal domain
Dex Dextrose
DNA Deoxyribonucleic acid
DSB Double strand break
ENV Envelope gene
FG Phenylalanine-glycine
FISH Fluorescence in situ hybridization
Gag3 Ty3 Gag protein
Gal Galactose
GAL Galactose inducible gene
GRS Gene recruitment sequence
GFP Green fluorescent protein
HBV Hepatitis B virus
HIS Histidine
HIV-1 Human Immunodeficiency Virus Type 1
HR Helical region
HSV-1 Herpes simplex virus type 1
HU Hydroxyurea
IN Integrase
INM Inner nuclear membrane
INO1 Inositol-1-phosphate synthase gene
Kap Karyopherin
kb Kilo base pairs
LEU Leucine
LTR Long terminal repeat
MMS Methyl methanesulfonate
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
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</tr>
<tr>
<td>N-</td>
<td>Amino</td>
</tr>
<tr>
<td>Nab2</td>
<td>Nuclear abundant poly(A) RNA-binding protein 2</td>
</tr>
<tr>
<td>NE</td>
<td>Nuclear envelope</td>
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<tr>
<td>NES</td>
<td>Nuclear export signal</td>
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<td>Nuclear localization signal</td>
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<td>NPC</td>
<td>Nuclear pore complex</td>
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<tr>
<td>Nup</td>
<td>Nucleoporin</td>
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<td>Outer nuclear membrane</td>
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<td>ORF</td>
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<td>Phosphoglycerate kinase</td>
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<td>Raf</td>
<td>Raffinose</td>
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<td>Ran GTPase-activating protein</td>
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<tr>
<td>RH</td>
<td>Ribonuclease H</td>
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<td>RIG</td>
<td>Recurrent integration gene</td>
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<td>RNA polymerase II</td>
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<td>RQ</td>
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<td>S. cerevisiae</td>
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<td>SAGA</td>
<td>Spt-Ada-Gcn5-acetyltransferase complex</td>
</tr>
<tr>
<td>S. pombe</td>
<td>Schizosaccharomyces pombe</td>
</tr>
<tr>
<td>SC</td>
<td>Synthetic complete</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SGD</td>
<td>Saccharomyces Genome Database</td>
</tr>
<tr>
<td>Sir</td>
<td>Silence information regulator protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>TD</td>
<td>Targeting domain</td>
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<tr>
<td>TE</td>
<td>Transposable element</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate EDTA</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA binding protein</td>
</tr>
<tr>
<td>TREX</td>
<td>Transcription-export complex</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>tRNA\textsuperscript{\text{I}Met}</td>
<td>Initiator methionine transfer RNA</td>
</tr>
<tr>
<td>ts</td>
<td>Temperature sensitive</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription start site</td>
</tr>
<tr>
<td>Ty</td>
<td>Transposon yeast</td>
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<tr>
<td>Ub</td>
<td>Ubiquitin</td>
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<tr>
<td>URA</td>
<td>Uracil</td>
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<tr>
<td>VLP</td>
<td>Virus like particle</td>
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<tr>
<td>Vpr</td>
<td>Viral protein R</td>
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<tr>
<td>WCE</td>
<td>Whole cell extract</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast peptone dextrose</td>
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<td>ZBD</td>
<td>Zinc binding domain</td>
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Acknowledgements

I thank my supervisor Vivien Measday for her guidance and support during my doctoral studies. I am grateful for the skills that I developed under Vivien’s supervision and I truly appreciate her mentorship and kindness. I thank my committee members Ivan Sadowski and Richard Harrigan for providing me with thoughtful and helpful feedback on my doctoral work. I thank the following researchers in the yeast community for their generosity in providing reagents for my thesis work: Jef Boeke, David Garfinkel, Anita Corbett, Alwin Köhler, Marco Foiani, Susan Wente, Catherine Dargemont, Katja Sträßer, and John Aitchison.

I thank all of the Measday Lab members, particularly Stephanie Cheung and Jay Martiniuk, your kindness and advice over the years has been very helpful and I am privileged to have worked with such talented people. Stephanie, thank you for being a great friend and an inspiring co-worker. I would also like to thank the members of the Sadowski Lab for being my second lab family at UBC. Pargol I thank you for your friendship-from help with experiments to talks about life and everything in-between.

I thank my parents for their support, generosity, and encouragement during my time as a graduate student. Thank you to my siblings and friends for being such wonderful supportive people.

Finally, I thank my husband Karthik for being brilliant and for making me laugh.
For my Parents and for Karthik
Chapter 1:

Introduction

1.1 Transposable Elements

Transposable elements (TEs) are genetic sequences that can move to new genomic locations and are thus colloquially referred to as “jumping genes” (1). In the late 1940’s Barbara McClintock discovered the first TEs, Ac and Ds, during her study of chromosome breakage in maize (2); this discovery challenged the field’s view of the genome as stable and static. In fact, it is now known that a significant portion of eukaryotic genomes are composed of TEs which have an impact on genome evolution as they can move and replicate thus contributing to genome plasticity (1). There are two classes of TEs: class I are retrotransposons and class II are DNA transposons (3). DNA transposons use a direct “cut and paste” mechanism to propagate whereas retrotransposons use a “copy and paste” mechanism to replicate through reverse transcription of their RNA and integration of the resulting complementary DNA (cDNA) into a new location (3). The movement of TEs from one location in the genome to another is often called transposition if TE movement occurs directly (cut and paste) or retrotransposition if TE movement occurs through an RNA intermediate (copy and paste) (3). Retrotransposons can be further classified into those containing flanking long terminal repeat sequences (LTR), termed LTR-retrotransposons and those without LTRs, called non-LTR retrotransposons (3). Typically retrotransposons encode for GAG and POL which produce the structural and enzymatic proteins, respectively, that are required for retrotransposition (Figure 1.1) (3). Phylogenetic analyses
indicate that LTR-retrotransposons are the progenitors of retroviruses, such as human immunodeficiency virus type 1 (HIV-1), because they encode functionally homologous structural and enzymatic proteins and share similar replication cycles (4). However, unlike retroviruses, retrotransposons do not have an infectious stage and solely replicate intracellularly (5). To enter and exit the cell, retroviruses, which have both GAG and POL genes, have acquired a third gene called envelope (ENV) that encodes for a transmembrane and surface glycoprotein and binds with receptors on the surface of cells thus enabling virion binding and entry (5).

Another feature common to both retrotransposons and retroviruses is that they require entry into the nucleus for integration of their genetic material into the host genome. Nuclear entry of retrotransposons and retroviruses requires movement through nuclear pore complexes (NPCs) embedded in the nuclear envelope (NE). NPC proteins have been previously identified in retrotransposon host factor screens in the yeast species Saccharomyces cerevisiae (S. cerevisiae), and Schizosaccharomyces pombe (S. pombe) which I discuss in section 1.10. In this thesis, I explore the functional role of NPC proteins on genomic targeting of a retrotransposon in the model organism S. cerevisiae.
Figure 1.1 Schematic of TEs with replication cycles affected by the NPC. \textit{S. cerevisiae} Ty1 ([5.9 kilobase pairs (kb)]) and Ty3 (5.4kb) are composed of two overlapping open reading frames (ORFs), \textit{GAG} (green) and \textit{POL} (blue) flanked by LTR sequences (black triangles) ranging from 334 to 358 base pairs (bp). The overlap between \textit{GAG} and \textit{POL} is 38bp. Tf1 (4.9kb) occurs in \textit{S. pombe} and is composed of \textit{GAG} and \textit{POL} on a single ORF. For all three TEs \textit{GAG} encodes for the structural capsid (CA) protein and \textit{POL} generates the enzymes protease (PR), integrase (IN) and reverse transcriptase with RNAse H activity (RT-RH).

1.2 Yeast Retrotransposons

\textit{S. cerevisiae}, also known as budding yeast, is a single-celled eukaryote with a 12.1 Mb haploid genome composed of 16 chromosomes that can divide as rapidly as once every 90 minutes (6). The common name “budding yeast” comes from the process of yeast cell division where daughter cells bud or pinch off from the mother cell; other common names of \textit{S. cerevisiae} are derived from its role in fermentation of alcoholic beverages (brewer’s yeast) and in baking to
raise bread dough (baker’s yeast) (6). The budding yeast is a model organism which has been used extensively in genetics and molecular biology among many other fields. In 1996, *S. cerevisiae* became the first complete eukaryotic genome sequenced (7) and in 2002 became the first organism with a complete deletion mutant strain collection (8). The *S. cerevisiae* genome is composed of ~6600 genes spread over 16 chromosomes and as of 2017 the Saccharomyces Genome Database (SGD) reports that 78% of these genes have been characterized, 10% are dubious ORFs that are unlikely to encode for proteins, and 11% remain uncharacterized (https://www.yeastgenome.org/genomesnapshot).

In total, retrotransposon sequences comprise 377 kb or 3.1% of the 12.1 Mb *S. cerevisiae* genome (9). The yeast genome contains five structurally and functionally similar LTR-retrotransposons known as Ty1 through Ty5 (10). All five elements transpose through an RNA intermediate and produce intracellular virus-like-particles (VLPs) (10). Ty1, Ty2, Ty4 and Ty5 elements belong to the *Pseudoviridiae* or Ty1/Copia group and Ty3 belongs to *Metaviridae* or gypsy-like element group; the two groups are distinguished by the order of their POL coding regions which proceeds as protease (PR)-integrase (IN)-reverse transcriptase (RT) for *Pseudoviridiae* and PR-RT-IN for *Metaviridae* (10). The reference yeast genome (S288C) carries circa 313 Ty1 insertions - of these 32 are full length Ty1 elements and the remainder are solo LTRs or LTR fragments (11). The 32 full-length Ty1 elements can be further categorized into three subfamilies, Ty1, Ty1/Ty2, and Ty1', of which Ty1 is presumed to be the ancestral subfamily and source of the majority of Ty1 insertions (11). Ty1' elements are characterized by a highly divergent *GAG* ORF nucleotide sequence and Ty1/Ty2 hybrid elements are characterized by a Ty1 coding region with Ty2-derived U3 LTR regions (11).
The S288C yeast genome also contains 13 full-length Ty2 elements along with two truncated Ty2 elements and 31 solo Ty2 LTRs (11). Ty2 elements have diverged less in comparison to other Ty families in *S. cerevisiae* which is consistent with the view that Ty2 elements have arrived more recently in the yeast genome as compared to other Ty elements (11). Two full-length Ty3 elements and 43 solo Ty3 LTRs of high sequence similarity are present in the S288c reference genome (11). There are also 15 solo LTRs belonging to the Ty3p sub-family which are thought to be ancient components of the yeast genome that lost the ability to replicate long ago (11). *S. cerevisiae* carries three full-length Ty4 elements and 45 solo Ty4 LTRs as well as 15 Ty5 insertions with only one full length but transpositionally inactive Ty5 element on chromosome (chr) III (11).

**1.3 Ty1 Replication**

Ty1 elements, the most abundant TEs in the yeast genome, consist of a 5.2 kb central coding region composed of two overlapping ORFs, called *TYA* and *TYB*, sandwiched between 334 bp LTR sequences (Figure 1.1) (12). *TYA*, analogous to retroviral *GAG*, encodes for structural proteins of the VLP, while *TYB*, similar to retroviral *POL*, produces a polyprotein of the enzymes: PR, IN, RT with a C-terminal ribonuclease H (RH) domain (Figure 1.2) (12). Ty1 replication begins with the transcription of a genomic Ty1 element by RNA polymerase II (RNA Pol II), the capping of Ty1 messenger RNA (mRNA) is followed by polyadenylation and exiting the nucleus (Figure 1.3) (12). In the cytoplasm, the Ty1 mRNA is translated into a 49 kDa TyA/Gag protein (p49) or a 199 kDa TyA-TyB/Gag-Pol fusion protein (p199) when a +1 ribosomal frameshift event, which occurs at an efficiency of 3 to 13% (13,14), puts Gag and Pol in frame (Figure 1.2 & 1.3) (12). Ty1 mRNA and proteins (Gag and Gag-Pol) colocalize to form
cytoplasmic foci called T bodies where they assemble to form immature VLPs (Figure 1.3) (15). Gag proteins form the structural outer shell of the immature VLP which contains 2 copies of the Ty1 mRNA transcript, the POL encoded enzymes, and an initiator methionine transfer RNA (tRNA\textsuperscript{Met}) (Figure 1.3) (12). The VLP then undergoes maturation whereby PR is autocatalytically cleaved from Gag-Pol and Gag p49 is processed by PR at the C-terminus to a 45 kDa Gag protein called capsid (CA) or p45 (Figure 1.2 & 1.3) (16). During VLP maturation, PR also cleaves the Gag-Pol precursor to yield CA and the enzymes: IN, RT and RH (Figure 1.2) (17).
**Figure 1.2** Synthesis and processing of Ty1 proteins. Genomic Ty1 elements are transcribed by RNA Pol II and the resulting Ty1 mRNA is translated primarily into Gag/p49 and upon a +1 frameshift event to Gag-Pol/p199. Both precursor proteins are proteolytically processed; Gag-p49 is processed to Gag-p45 and Gag-p4. Gag-Pol-p199 is processed to Gag-p45 and full-length Pol or p145 which is subsequently processed to protease (PR, Pol-p20), integrase (IN, Pol-p71), and reverse transcriptase (RT-RH, Pol-p63). The C-terminus of RT contains a ribonuclease H (RH) domain and is thus also referred to as RT-RH. Adapted from (18).

Following VLP maturation, Ty1 mRNA is reverse transcribed by RT within the VLP (19), utilizing tRNAiMet as a primer (Figure 1.3) (20). Synthesis of cDNA occurs upon initiation of reverse transcription involving the formation of complex whereby tRNAiMet binds to a 10 nucleotide minus-strand primer binding site (PBS) located near the C-terminus of Ty1 GAG on the Ty1 mRNA (21). Once the primer-template has been formed RT initiates synthesis of the cDNA. During minus-strand DNA synthesis, the template RNA strand of the newly formed RNA-DNA hybrid is degraded by the RT-associated RH activity (18). The newly synthesized Ty1 cDNA along with IN forms the pre-integration complex (PIC) (Figure 1.3) (12). The PIC localizes to the nucleus where Ty1 cDNA is inserted in a window ~1 kb upstream of genes actively transcribed by RNA polymerase III (RNA Pol III), such as transfer RNA (tRNA) genes in a process catalyzed by IN (22); Ty1 cDNA can also enter the genome via homologous recombination with pre-existing Ty1 elements or solo LTRs (23). While it is generally assumed that Ty1 cDNA and IN cross the NE as a PIC, it has not been excluded that they are transported independently to the nucleus.

In a haploid laboratory strain containing about 30 Ty1 elements, the rate of retrotransposition is between 3 x 10^-7 to 1 x 10^-5 per Ty1 element per generation under optimal conditions for Ty1 transposition (24). However, the rate of Ty1 replication is dramatically
increased when yeast strains are grown at temperatures lower (of 20°C to 15°C) than the optimal 30°C growth temperature of yeast (25).

**Figure 1.3** Ty1 Replication. Genomic Ty1 elements are transcribed by RNA Pol II to Ty1 mRNA which is then capped, polyadenylated and then exported to the cytoplasm. Translation of Ty1 mRNA produces Gag or Gag-Pol fusion proteins. These protein products along with two copies of Ty1 mRNA, an initiator methionine tRNA assemble into virus-like particles (VLPs). Proteolytic processing of the Ty1 proteins followed by reverse transcription of Ty1 mRNA to cDNA occurs in the VLP. Ty1 cDNA and integrase (IN) form a pre-integration complex (PIC) that traverses into the nucleus, presumably after uncoating of the VLP. Once in the nucleus, the Ty1 cDNA can insert into the genome either via homologous recombination with a pre-existing Ty1 element or by a transesterification reaction catalyzed by IN, upstream of genes transcribed by RNA Pol III.
1.3.1 Ty1 Integrase

Ty1 cDNA integration is performed by Ty1 IN, a 71 kDa protein composed of three domains that are similar to most retroviral and retrotransposon INs: N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD) (Figure 1.4) (18). Ty1 IN and other retrotransposon/retroviral INs also share two phylogenetically conserved regions: the N-terminal zinc-binding domain (ZBD) with conserved histidine and cysteine residues (HHCC) and the central catalytic triad motif (D, D$_{35}$, E) required to bind divalent metal ions (Mg$^{2+}$ or Mn$^{2+}$) and to perform the DNA breaking and joining reactions during Ty1 genomic integration (Figure 1.4) (18). The CTD of Ty1 IN is larger and less conserved in comparison to most retroviral and retrotransposon INs and contains a bipartite C-terminal nuclear localization signal (NLS) that is required for translocation of IN into the nucleus (26, 27) (Figure 1.4).

**Figure 1.4** Ty1 and HIV-1 IN domain organization. Ty1 and HIV-1 IN are made up of a zinc-binding domain (ZBD), catalytic core domain (CCD) and a C-terminal domain (CTD). The ZBD contains a conserved zinc binding motif [H(X3-7)H(X23-32)C(X2)C], where X is any amino acid, and the CCD contains the IN hallmark catalytic triad motif [DD(X$_{35}$)E]. The CTD of Ty1 IN has a bipartite nuclear localization signal (NLS) [$\text{KKR}_{596-598}(X_{29})\text{KKR}_{628-630}$]. The HIV-1 IN NLS is not shown here due to conflicting reports on its location. Adapted from (18).
Integration of Ty1 cDNA into the genome occurs via a one-step transesterification reaction catalyzed by Ty1 IN (18). During this reaction the reactive hydroxyl groups on the blunt Ty1 cDNA 3’ ends act as nucleophiles to attack the phosphodiester bonds on each strand of the target DNA backbone at 5 bp staggered sites, breaking the target DNA and joining the retroviral DNA to the exposed 5’ phosphate groups of the target DNA in one step (18). This results in a newly exposed 3’ OH and five nucleotide gap on each strand of the target DNA at the cDNA:target DNA junctions (18). Repair of the gaps generates a 5 bp target site duplication (TSD) flanking the newly integrated Ty1 cDNA, which is a hallmark of Ty1 integration (18). Unlike retroviral DNA ends, Ty1 cDNA is blunt ended and therefore does not require an initial 3’ processing of the cDNA ends to expose the reactive hydroxyl groups (28).

1.4 Integration Site Specificity of Ty Elements

As early as 1979, it was observed that genomic copies of Ty1 are located near tRNA genes (29). After the sequencing of the S. cerevisiae genome was completed, a survey of the genomic sequence suggested that the majority of Ty1 elements are located within 750bp of RNA Pol III transcribed genes of which the 275 tRNA genes accounted for the majority of insertion sites (9). Induction of Ty1 element expression also resulted in targeting of Ty1 elements upstream of genes transcribed by RNA Pol III, (Figure 1.5) (9,22,30-32). Genome-wide mapping of Ty1 insertion sites on the S. cerevisiae genome showed that Ty1 specifically targets nucleosomal DNA which accounts for the ~80 bp periodicity in Ty1 insertion upstream of tRNA genes (31,33,34). Ty1 targeting also requires active RNA Pol III transcription as mutagenesis of the RNA Pol III promoter causes a reduction in Ty1 insertion (22). Many host factors, such as a subunit of the transcription factor (TF) IIIB complex called Bdp1 and the chromatin remodeling
complex Isw2 have been shown to contribute to Ty1 insertion (35,36). As chromatin structure plays a distinct role in Ty1 integration, chromatin maintenance factors like the histone deacetylase Hos3 and Trithorax group protein Set3 have been shown to enhance Ty1 integration at tRNA genes (37).

**Figure 1.5** Ty insertion sites. Ty1, Ty2 and Ty4 insert in a ~1 kb window upstream of genes transcribed by RNA Pol III, the majority of which are tRNA genes. Ty3 inserts ~2-3 bp upstream of RNA Pol III transcribed genes at transcription start sites. Ty5 inserts near silent loci such as HML, HMR and telomeres.

Along with Ty1, Ty2, Ty3 and Ty4 generally reside in a window upstream of RNA Pol III transcribed genes (Figure 1.5) (9,38,39). Ty3 insertion, mediated by Ty3 IN interacting with transcription factor (TF) IIIB components Brf1 and TATA binding protein (TBP, Spt15 in S. cerevisiae) occurs at transcription start sites (TSSs) 2-3 bp directly upstream of RNA Pol III transcribed genes (40-44). Ty5 preferentially integrates near regions of silent chromatin at the telomeres and the silent mating loci (HML and HMR) (Figure 1.5) (45-48). Chromatin at these sites is referred to as silent because transcription of genes located in these regions is repressed by
silent information regulator (Sir) proteins Sir2, Sir3 and Sir4 (49,50). A 9 amino acid motif (1092-LDSSPPNTS) at the C-terminus of Ty5 IN, termed the targeting domain (TD), is required for Ty5 insertion at telomeres and the silent mating loci (51). A single amino acid substitution in the TD at position 1094 (S1094L) drastically alters Ty5 insertion specificity (52). The TD of Ty5 IN interacts with the Sir4 protein which tethers the Ty5 integration complex to target sites for insertion and the TD/Sir4 interaction is necessary for Ty5 insertion specificity at telomeres and the silent mating loci (51).

1.5 Chromatin Organization

Eukaryotic DNA is assembled into chromatin which is comprised of ~70 000 nucleosomes in *S. cerevisiae* and millions of nucleosomes in humans, each consisting of roughly 147 bp of DNA wrapped around a histone octamer composed of two copies each of histones H2A, H2B, H3, and H4 (53). Nucleosomes are compacted into higher order organizational levels which control the structure and accessibility of nucleosomal DNA (54). Depending on the structural context, chromatin can both promote and impede most DNA processes including transcription, replication, recombination and DNA repair; thus, chromatin structure plays a central role in the control of these processes (54). DNA is packaged into heterochromatin and euchromatin with heterochromatin being tightly packed and largely inactive for transcription and is often referred to as “silent” chromatin whereas euchromatin is less condensed and accessible for transcription and is often referred to as “active” chromatin (Figure 1.6) (55). Components of the NPC interact with active chromatin whereas silent chromatin interacts with the telomere-binding complex Ku70/Ku80 and the SIR complex in between NPCs at the inner nuclear membrane (INM) of the NE (Figure 1.6) (56).
Figure 1.6 Heterochromatin and euchromatin in yeast. Chromatin is generally found in two forms: “active” euchromatin that is more loosely packed and ready for transcription and “inactive” heterochromatin that is tightly packed and mostly transcriptionally silent. Generally, active chromatin is associated with NPCs (grey) and inactive chromatin is located at the inner nuclear membrane (INM) in-between NPCs. Outer nuclear membrane (ONM). Adapted from (57).

Heterochromatin is established and maintained by regulatory DNA sequence elements called silencers which, in yeast, recruit the Sir proteins (58). Histones are also hypoacetylated in silenced chromatin which is required for Sir protein binding (58-60). In yeast, heterochromatin is present at HMR, HML, the ribosomal DNA (rDNA) locus that defines the nucleolus, subtelomeres and telomeres (55). Telomeres are composed of repeat sequences at the ends of chromosomes and are replicated by an enzyme called telomerase (61). Subtelomeric regions are located at chromosome ends proximal to telomeres in the yeast genome (62). Subtelomeres vary in length from ~2 to 33 kb, are generally low in gene density, and contain multiple similar sequence segments that are distinct from telomere repeat sequence (62,63). S. cerevisiae subtelomeres contain a conserved ~500 bp X element and 17 out of 32 subtelomeres contain Y'
elements which are highly variable in lengths (4-8 kb); the Y’ element includes the ORF of a helicase gene (62).

1.6 RNA Polymerase III Transcribed Genes

RNA Pol III is a 17 subunit complex that, along with transcription factors TFIIB, TFIIIC, and TFIIIB, transcribes all tRNAs, 5S ribosomal RNA (rRNA), and other essential small nonprotein-coding RNAs (64). Generally, RNA Pol III transcribed genes contain intragenic promoters and are classified into three types based on promoter structures and factor requirements. The promoter (type 1) of the yeast 5S rRNA gene RDN5 is composed of an internal A box and gene-specific C box (64). tRNA genes constitute type 2 genes and also contain an internal promoter consisting of two highly conserved sequence elements, a proximal A box and a more distal B box, within the transcribed region. Promoters for type 3 genes, such as the S. cerevisiae U6 snRNA genes, are composed of an upstream TATA box, an internal A box and a B box located downstream of the termination signal (64). tRNA gene activation first requires association of TFIIIC with DNA, followed by TFIIIB, which then recruits RNA Pol III (64,65). TFIIIC is a 6-subunit complex with a τA subcomplex that recognizes box A and a τB subcomplex that recognizes box B (66,67). TFIIIB is assembled from three proteins in yeast – Brf1, TBP and Bdp1 (68). Brf1 and TBP assemble first into the transcription complex followed by interaction with Bdp1 (69). Once TFIIIB is bound, the RNA Pol III transcription complex can assemble onto the promoter (70). The common features of all types of RNA Pol III promoters is that TFIIIC, TFIIIB and RNA Pol III are recruited to activate transcription. Mutation of the SUF16 glycine tRNA gene promoter, such as a point mutation in box B, that severely reduces
transcription, also dramatically reduces Ty1 element insertion suggesting that active Pol III transcription is required for Ty1 transposition (22).

1.6.1 Yeast tRNA Genes

Yeast tRNA genes have an open chromatin structure with strongly ordered upstream nucleosomes and nucleosome-depleted gene body regions (71,72). tRNAs are initially synthesized as precursors (pre-tRNAs) which undergo extensive post-transcriptional processing steps to become mature tRNAs. These steps include: trimming of nucleotides at both the 5’ and 3’ ends, nucleotide addition to 3’ ends and select 5’ ends, splicing of introns if present, and base modifications that include methylations, deaminations, isomerizations, and addition of small functional groups (73).

Given that tRNA genes are targets for many TEs, intranuclear positioning of tRNA genes can potentially affect dynamics of TE insertion in S. cerevisiae. The yeast nucleus has three major zones: the interior nucleoplasm, the nucleolus which is the site of ribosome synthesis, and the exterior nuclear periphery which is composed of the NE and NPCs which will be discussed in depth in subsequent paragraphs (74). Yeast tRNA genes, although dispersed on linear chromosome maps, have been shown to localize to the nucleolus (75,76). Thompson et al. first showed, by fluorescence in situ hybridization (FISH), that five different tRNA gene families are clustered at the nucleolus in fixed and permeabilized yeast cells (76). This result was confirmed using the same method by Haeusler et al. who also demonstrated that tRNA nucleolar clustering requires condensin, a protein complex involved in chromosome compaction (75). Subsequently, chromosome conformation capture (3C) studies of the yeast genome identified two clusters of tRNA genes: one cluster that cross-links with the nucleolar rDNA and another cluster that cross-
links with centromeric DNA (77-79). The 3C method is based on the principal that if different chromosomes are in close proximity in vivo, cross-linking will occur.

A comprehensive study of S. cerevisiae chromosome XII architecture, which carries the rDNA, used live-cell imaging to study the localization of three different tRNA genes (80). Each tRNA gene demonstrated unique localizations: tL(UAA)L was located at both the nucleolar and nuclear periphery, tP(UGG)L was associated with the nucleoplasm and tA(UGC)L was located at the nucleoplasm and nuclear periphery (80). Most recently, the same research group further explored the dynamic localization of seven additional tRNA genes (81). They visualized three tRNA genes located at the nuclear periphery, two at both the nucleolar and nuclear periphery, one (SUP4) in the nucleoplasm and nuclear periphery, and only one tRNA gene (SUF5) solely associated with the nucleolar periphery (81).

A combination of fluorescence microscopy and chromatin immunoprecipitation (ChIP) followed by quantitative polymerase chain reaction (ChIP-qPCR) was used by Chen et al. to demonstrate that tRNA genes are actively transcribed at the nuclear periphery (82). In M-phase arrested cells, coincident with the peak of tRNA synthesis, deletion of the Nup2 or Nup60 nuclear basket proteins reduced localization of the threonine tRNA gene tT(AGU)C to NPCs (82). ChIP-qPCR showed that all three tRNA genes tested (tT(AGU)C, tS(CGA)C, and tT(UGU)G1) cross-linked to Nup60 in M-phase arrested cells (82). Deletion of the tRNA exportin Los1 deterred the binding Nup60 with three tRNAs, including one with an intron (tS(CGA)C) and two without introns (tT(AGU)C and tT(UGU)G1) suggesting coordination between tRNA transcription at NPCs and export (82).

Based on these findings tRNA positioning can be thought of as dynamic and associated with the nucleolus and additional nuclear compartments including the nucleoplasm and nuclear
periphery and NPCs. The positioning of tRNAs likely has consequences on chromosome structure and Ty1 replication as Ty1 inserts upstream of tRNA genes.

1.6.2 RNA Pol III-transcribed Genes and Genome Evolution

The site upstream of Pol III transcribed genes seems to be prone to insertion and chromosome rearrangement. Regions upstream of tRNA genes are thought to be safe loci for TE insertion because they lack protein coding regions thus minimizing the damaging effect of TE insertion on the host and favoring the survival of the element (39,83). Interestingly, these same regions have been found to be sites of chromosome rearrangement which causes double strand DNA breaks (DSBs) labeling these sites as “fragile” (84-86).

Almost all chromosome aberrations arising from randomly induced DSBs from exposing *S. cerevisiae* cells to ionizing radiation were bordered by Ty elements (87). The majority of genome breakpoints in *S. cerevisiae* strains grown under glucose limitation were mapped to at least one Ty, solo LTR, or tRNA (88). The *S. cerevisiae* genome sequence was compared to the sequences of three other *Saccharomyces* species (*Saccharomyces paradoxus*, *Saccharomyces mikatae* and *Saccharomyces bayanus*) which are separated from *S. cerevisiae* by an estimated 5–20 million years of evolution (89). The comparison identified twenty sequence inversions among the three yeast species compared to *S. cerevisiae*, all of which were flanked by tRNAs, and seven of which were translocation events between transposable elements (89). Mapping of chromosomal rearrangement breakpoints caused by depletion of essential genes, including DNA replication genes, showed that chromosome break sites were proximal to Ty elements and LTRs making them fragile sites or sites of chromosome breakage (85,86,90). Furthermore, DNA DSBs induce homologous recombination of Ty1 elements distal to the break site (91). Taken together,
the region upstream of RNA pol III transcribed genes can be thought of as a dynamic site of genetic movement that can be a source of change in chromosome structure.

1.7 Nuclear Pore Complexes Assembly and Structure

Callan and Tomlin performed the first electron microscopy study of the NE, using amphibian oocytes in 1950 and discovered it to be punctured by pores (92). The NE is a double lipid bilayer membrane that compartmentalizes a cell’s genetic material within the nucleus separating it from the cytoplasm (93). The outer nuclear membrane (ONM) faces the cytoplasm and is continuous with the endoplasmic reticulum and the INM faces towards the inside of the nucleus (93). The nucleus is not entirely shut off from the cytoplasm as molecules such as mRNAs and tRNAs must exit the nucleus and proteins such as transcription factors and essential enzymes for DNA replication and transcription must enter the nucleus after synthesis in the cytoplasm. As such, the NE is studded with NPCs that mediate nucleo-cytoplasmic transport of molecules. Water, ions and small metabolites can diffuse through NPCs whereas larger molecules >40 kDa are actively transported (94).

At the beginning of mitosis in vertebrate cells, the NE breaks down and then reassembles around the newly segregated chromosomes; disruption of the NE is a hallmark of what is commonly referred to as “open” mitosis which occurs in most animals and plants (95). It is during NE reformation after mitosis and during interphase of the cell cycle that NPC assembly occurs in vertebrate cells (96,97). However, unlike vertebrates, yeast undergoes a “closed” mitosis where the NE remains intact and NPC assembly occurs continuously throughout the cell cycle (98). Turnover of NPC components and addition of new NPCs during cell growth occur in yeast to ensure optimal NPC function (98). 3D reconstructions of intact nuclei from electron
micrographs of serially sectioned cells showed that the number of NPCs increased steadily through the *S. cerevisiae* cell cycle (G₁, S, Early mitosis, late anaphase), from 65 during G₁ to 182 in late anaphase (99). NPC homeostasis is a process that is partially regulated by a newly discovered feedback loop that senses changes in NPC composition and responds to mitigate potential functional loss (100). The translational repression of a subset of mRNAs that encode for NPC subunits are maintained by binding of the RNA binding protein Hek2 (100). Hek2 binding of NPC mRNAs is prevented by Hek2 sumoylation which is controlled by the SUMO protease Ulp1 (100). NPC mutations that cause defects in nucleo-cytoplasmic transport were found to coincide with loss of Ulp1 activity and the downstream accumulation of sumoylated Hek2; sumoylated Hek2 no longer binds to NPC mRNAs, allowing for the translation of new NPC subunits (100).

Each NPC is composed of multiple copies (~16 each) of ~30 proteins termed nucleoporins or Nups resulting in over 450 polypeptides per NPC (101,102). NPCs have an overall cylindrical structure with eight-fold symmetry and are ~60-125 MDa and ~40-60 MDa in mammals and yeast respectively (102-104). Mammalian and yeast NPCs share an overall structural similarity and are both composed of ~30 Nups with the majority being homologs suggesting that NPCs are evolutionarily conserved across eukaryotes (105,106). The NPC also has structural plasticity allowing for conformational flexibility required for transport of macromolecules.

The ~30 Nups assemble to form the following 7 interacting substructures of the yeast NPC: outer ring, inner ring, cytoplasmic, transmembrane ring, central, linker, and nuclear basket (Figure 1.7). The central channel, where molecular exchange occurs, is composed of central phenylalanine-glycine (FG) repeat Nups (Nsp1, Nup49, Nup57, Nup100, Nup116, Nup145N)
(104,107,108) with FG repeat regions providing docking-sites for transport-factor cargo complexes (Figure 1.7) (107-109). The outer ring Nup84 subcomplex (Nup84, Nup85, Nup120, Nup133, Nup145C, Sec13, Seh1) and inner ring Nup170 subcomplex (Nup157, Nup170, Nup188, Nup53, Nup59, Nup192) form a core scaffold that the FG Nups attach to (Figure 1.7) (104,107,108,110-112). The NPC has structural asymmetry: on the cytoplasmic face of the NPC are the cytoplasmic Nups (Nup42, Nup159) whereas on the nuclear face Nup1, Nup2, Nup60, Mlp1 and Mlp2 form the nuclear basket (Figure 1.7) (104,107,108). The linker Nups (Nic96, Nup82), positioned towards the inside of the channel, connect the inner and outer rings (110) and serve as attachment sites for FG Nups whereas the transmembrane ring Nups (Ndc1, Pom34, Pom152) help anchor the NPC in the NE (Figure 1.7) (104,107,108). These basic structural components of the NPC are conserved across all eukaryotes (113).
**Figure 1.7** Schematic of the Yeast NPC. Major NPC structural subunits adapted from (113,114). The outer ring is composed of the Nup84 subcomplex (green) which sandwiches the inner ring Nup170 complex (purple). The cytoplasmic Nups and Nuclear basket Nups are shown in blue and red respectively. Transmembrane ring, also referred to as lumenal ring, Nups are shown embedded into the nuclear envelope (orange). Disordered, central channel phenylalanine-glycine (FG) repeat Nups (black) are attached to linker Nups (cyan). The underlined Nups were examined for a role in Ty1 replication in this thesis.
1.8 Nuclear Pore Complexes and Transport

NLS or nuclear export signals (NES) are amino acid sequences that target proteins into or out of the nucleus (113). The classic NLS (cNLS) is composed of a stretch of 5 basic amino acid residues (KKKRK); addition of this sequence is sufficient to mediate nuclear translocation of nonnuclear proteins (115). Many proteins, including Ty1 IN, carry non-classical bipartite NLSs composed of two clusters of basic amino acid residues separated by a spacer of about 10 amino acids (116). Soluble transport factors, mostly from the karyopherin (Kaps; also called importins, exportins, and transportins) protein family, recognize NLSs and bind cargo directly or indirectly via an adaptor protein to modulate NPC translocation (117). The association and dissociation of a karyopherin-cargo complex is regulated by direct binding of the small Ras-like GTPase Ran. The Ran concentration gradient, occurring based on the predominance of Ran-GTP in the nucleus and Ran-GDP in the cytoplasm, establishes the transport direction (113). Ran is maintained in its GTP-bound form in the nucleus by a GTP exchange factor (RanGEF) and the cytoplasmic Ran GTPase-activating protein (RanGAP) maintains Ran in the cytoplasm in the GDP form (113).

To import cargo into the nucleus, a Kap binds its cargo in the cytoplasm where Ran-GTP levels are low (Figure 1.8) (113). The Kap-cargo complex translocates to the nucleus through a NPC, where Ran-GTP in the nucleus stimulates release and dissociation of the Kap-cargo complex (Figure 1.8) (113). Upon release, the Kap bound to Ran-GTP is recycled back to the cytoplasm (Figure 1.8) (113). Finally, GTP hydrolysis of Ran on the cytoplasmic side by a RanGAP, frees the Kap to interact with a new cargo for transport (Figure 1.8) (113). A similar process occurs for nuclear export, but in this case, Ran-GTP binding increases the affinity of the Kap for the export cargo unlike during import where Ran-GTP decreases the affinity of the Kap.
for its cargo (113). Generally, movement through the NPC does not require energy input; the affinity of Ran-GTP or Ran-GDP provide the energy for efficient cargo delivery and release (113).

**Figure 1.8** Schematic of nucleo-cytoplasmic transport. Nuclear import is depicted on the left side of figure and nuclear export is shown on the right. A cargo carrying a nuclear localization signal (NLS; purple) binds to a karyopherin (Kap; orange) in the cytoplasm and crosses the nuclear envelope (NE) through nuclear pore complexes (NPCs; grey). In the nucleus, RanGTP (yellow circled T) binds the cargo-Kap complex which causes dissociation. The export Kap (light orange) forms a trimeric complex with cargo carrying a nuclear export signal (NES; green) and RanGTP. Once in the cytoplasm, RanGAP then triggers hydrolysis of RanGTP into RanGDP (yellow circled D), leading to a conformational change that dissociates the export Kap-cargo complex. Adapted from (113).
1.9 Transport-independent Functions of Nuclear Pore Complexes

1.9.1 Nuclear Periphery and Silencing

The nuclear region directly adjacent to the INM, known as the nuclear periphery, has been commonly viewed as a site of transcriptional repression or silencing which refer to the inhibition of gene expression. In *S. cerevisiae* regions of the nuclear periphery contain transcriptionally repressed telomeres and silent mating-type loci (*HMR* and *HML*) located on chr III (118). As well, anchoring of a non-silenced locus to the nuclear periphery has been shown to cause silencing (119). NPC proteins have been shown to be involved in silencing of chromatin at the nuclear periphery. In wild type cells, the telomeric region localizes to the nuclear periphery. However, deletion of Nup84 subcomplex members (Nup120, Nup133, Nup84, Nup145C) resulted in localization of a labeled telomere into the interior of the nucleus (120). Deletion of the Nup84 subcomplex components also caused defects in telomere silencing as studied by measuring transcriptional silencing of *URA3* integrated into the subtelomeric region (120).

Nup2 and Nup60 have been shown to associate with a tRNA\(^{thr}\) gene adjacent to the silenced *HMR* locus (121). The transcriptionally active tRNA\(^{thr}\) is thought to act as a barrier insulator to restrict the repression of nearby active genes from the two silencers (*HMR-E* and *HMR-I*) flanking its neighboring *HMR* locus (121). The *HMR* locus was also observed to mislocalize from the nuclear periphery to the nuclear interior of *nup60Δ* yeast cells (121). This same study found that *HMR* silencing was restored by localization to the nuclear periphery upon expression of Gal-Nup fusion proteins (Nup133 and to a lesser extent Nup84 or Nup2) in a yeast strain lacking *HMR-I* and carrying Gal4 binding sites adjacent to a defective *HMR-E* silencer (121). Removal of Nup2 from the yeast nuclear basket has also been shown to disrupt telomere silencing in a single-cell telomeric silencing assay that monitors telomeric genes switching
between silent and active expression states (122). As well, deletion of Nup60 has been shown to release telomeres from the nuclear periphery (123). FISH experiments with a telomeric probe revealed a redistribution in telomere FISH signals in nup60Δ cells compared with the wild type (123). To measure targeted silencing Feuerbach et al. used an assay developed in 1998 by Andrulis et al. whereby the HMR-E silencer was replaced with Gal4-binding sites upstream of a TRP1 reporter gene located in a partially depressed HMR locus (119,123). This replacement causes silencing to be lost, however silencing can be restored by the introduction of a fusion protein of the Gal4 DNA-binding domain and the endoplasmic reticulum protein Yip1 which, upon overexpression, localizes to the INM essentially tethering the HMR locus to the nuclear periphery (119,123). Removing Nup60 caused the TRP1 reporter gene to not be silenced, counteracting the nuclear periphery-dependent silencing of the TRP1 reporter (123).

Early studies found that Mlp1 and Mlp2 nuclear basket proteins were required for telomere positioning. In 2000, Galy et al. performed FISH using a telomeric probe and found that deletion of both Mlp proteins disrupted telomere clustering at the nuclear periphery leading them to suggest that telomeres are tethered to the nuclear periphery by interaction with the Mlp NPC proteins (124). Similarly, in 2002 Feuerbach et al. also used FISH with a telomeric probe to show that a double mlp1Δmlp2Δ deletion strain had mislocalized telomeres (123). However, also in 2002, Hediger et al. found that both Mlp proteins are not required for nuclear positioning of telomeres or subtelomere regions (125). Instead, they proposed that Mlp proteins could play a role in regulating telomere length as cells carrying Mlp deletions have extended telomeres (125). Hediger et al. attribute the conflicting results due to nuclei instability in cells lacking both Mlp proteins that are exposed to spheroplasting and FISH protocols whereas in their study only cells with intact nuclei were analyzed (125). This was later confirmed by a separate group that
similarly found normal telomere position in a strain carrying a double deletion of *MLP1* and *MLP2* (126). Therefore, the Mlp proteins likely do not play a direct role in telomere positioning.

Nup170 is a subunit of the NPC inner ring located in the middle of the complex sandwiched between the outer rings and on the same plane as the transmembrane ring subunits which they interact with (127). In addition, Nup170 interacts with central FG Nup such as Nup145 (110). When Nup170 is deleted, transcription of normally silent genes in the subtelomeric region are up-regulated (128). Cells depleted for Nup170 had reduced occupancy of the +1 and to a lesser extent the -1 nucleosome which flank the nucleosome-free region adjacent to Pol II TSSs – this change in nucleosome positioning was most prominent within 25 kb of telomeres (128). Nup170 has been shown to bind Sir4 which is required for maintaining silenced chromatin and Sth1, the ATPase subunit of the RSC chromatin remodeling complex (128). Interestingly, it was recently found that Nup170, Sir4, the SUMO E3 ligase Siz2 and the NE associated protein Esc1 form a complex that is distinct from NPCs which could account for Nup170’s role in telomere silencing (129).

### 1.9.2 Gene Gating

Recent studies have shown that recruitment to the nuclear periphery is not always repressive. In fact, the gene gating hypothesis was first proposed in 1985 by Gunter Blobel who hypothesized that NPCs were gene gating organelles that interact with transcription competent regions of the genome (130). The positioning of active genes at the NPC could regulate transcription and allow rapid export of mRNA transcripts to the cytoplasm (130). One example of gene gating is the inducible inositol-1-phosphate synthase (*INO1*) gene (Figure 1.9). Inositol starvation triggers transcriptional activation and *INO1* localization to the nuclear periphery from
the nucleoplasm (131) (Figure 1.9). Interestingly, when inositol is added back INO1 transcription is repressed but INO1 remains at the nuclear periphery for up to four cellular divisions where it is primed for reactivation (132). The presence of H2A.Z, an H2A variant histone, at the INO1 promoter is associated with transcriptional memory of this and other genes which is a concept that will be discussed below. NPC tethering of INO1 is mediated by the binding of transcription factor Put3 to the INO1 gene recruitment sequences (GRSs; described in the next section) which does not directly regulate INO1 activity (133,134) (Figure 1.9). Many genes other than INO1 and tRNA genes (described earlier) have been shown to localize to NPCs upon transcriptional activation including: CTT1 (135), FIG2 (136), GAL (GAL1, GAL2, GAL7, GAL10) (132,137-141), HIS4 (142), HSP104 (137), HXX1 (139,143), PRM1 (142), STL1 (135,144), SUC2 (145), and TSA2 (146). Transcriptional activation of most of these genes does not require NPC localization but interacting with the NPC results in more efficient gene expression and transcriptional memory of some of these genes (147).

**Figure 1.9** NPC recruitment of INO1. The inactive INO1 gene (green) is positioned in the nucleoplasm and the active gene is positioned at the nuclear periphery through interaction with
the NPC (grey). Upon transcriptional activation through inositol starvation the Put3 transcription factor (orange) binds the genome recruitment sequence (GRS; dark green) upstream of *INO1* to recruit *INO1* to the NPC where is actively transcribed by RNA Pol II (blue). Adapted from (134).

Along with the NPC, other nuclear complexes are required to target transcribing genes to the nuclear periphery. Mex67 is an essential nuclear mRNA export factor that binds mRNA and localizes to NPCs *in vivo* (148). Mex67 forms a heterodimeric export complex with Mtr2 (149), which mediates translocation of messenger ribonucleoproteins (mRNP; mRNA in complex with proteins), through NPCs by interacting with FG Nups (150). Both the SAGA (Spt-Ada-Gcn5-acetyltransferase) complex which is involved in chromatin remodeling and transcription activation, and the transcription-mRNA export complex TREX-2 interact with mRNPs and the NPC basket by tethering actively transcribed genes to the NPC (151-155).

### 1.9.3 Transcriptional Memory

To adapt and survive, cells alter transcriptional activity based on changing cellular environments, collectively termed “stresses”, such as temperature shifts, nutrient availability, changes in osmolarity, and attacks by other microbes (156,157). To effectively and robustly respond to these stresses, several organisms including budding yeast possess “transcriptional memory” whereby previously expressed genes are primed for reactivation at the nuclear periphery, allowing for a more rapid transcriptional response to environmental changes previously experienced as these genes remain at the nuclear periphery for several generations, primed for reactivation (158). For a number of yeast genes including *INO1* and *GAL1* transcriptional memory is linked to NPCs by DNA “zip codes” known as GRSs in promoters that are responsible for localization to the periphery (132,159,160) (Figure 1.9). Insertion of these
GRSs at a locus such as *URA3* that normally resides in the nucleoplasm, can position *URA3* at the nuclear periphery (146). As mentioned above, continued association of *INO1* at NPCs even when inositol deprivation is reversed is an example of transcriptional memory (132). Another example are the *GAL* genes, which are also recruited to the nuclear periphery upon induction by galactose and remain at the NPC primed for reactivation in the absence of galactose (132). However, unlike *INO1* gene memory, *GAL* gene memory is achieved through the interaction of the gene promoter and the 3’ end of the gene in a loop that interacts with the Mlp1 nuclear basket protein (139).

### 1.9.4 mRNA Surveillance

Newly transcribed mRNAs undergo essential processing steps to produce export-competent mRNPs including the addition of a 7-methylguanosine (m$^\text{7}G$) cap structure at the mRNA 5’ end which is referred to as 5’ capping and is important for mRNA stability and translation, splicing, 3’ cleavage and polyadenylation (161). The recruitment of key proteins such as the Mex67-Mtr2 export factors and TREX to nascent mRNAs during mRNA nuclear export participates in the formation of exportable mRNPs, the quality of which is assessed before they can leave the nucleus (161). Dedicated mRNP surveillance/quality control pathways serve to prevent the synthesis of dysfunctional proteins by detecting and remove aberrant mRNPs that carry defects in nucleotide sequence and/or packaging (162,163). Unspliced mRNAs are actively retained at the nuclear periphery by the Mlp1 and Nup60 nuclear basket proteins and the NPC-associated Pml39 protein (164-166). Pml39, which is associated with Mlp proteins and Esc1 at the nuclear periphery, is involved in maintaining nuclear basket integrity (165). The retained
faulty mRNPs are degraded by the nuclear exosome or the Swt1 endonuclease that interacts with Mlp proteins and Nup60 (167).

1.9.5 Double Strand Break Repair

Homologous recombination is the repair of DNA lesions using homologous sequences and can be used to repair many types of lesions including DSBs, single-stranded gaps, interstrand cross-links, collapsed replication forks and eroded telomeres (168). Induction of a DSB at the MATα locus by galactose-induced expression of the HO endonuclease, resulted in MATα locus relocation to NPCs in a Nup84 subcomplex dependent manner (169). Homologous recombination is impaired in this strain because the usual donor for recombination, the sister chromatid, is also cut making this DSB “difficult to repair” (169). Conversely, long tracts of triplet CAG repeats are a natural barrier to DNA replication due to stalling of DNA replication forks and enhancement of fork reversal (170). It was observed that long tracts of CAG repeats (70-130 repeat units) relocalize to the NPC during DNA replication (S phase) and interact with the Nup84 subcomplex (171). CAG repeats have been associated with human neurodegenerative diseases including Huntington’s Disease (172). Along with DSBs and long tracts of triplet repeats it has been shown that eroded telomeres also move to NPCs for repair (173). Collapsed replication forks induced by prolonged incubation with the DNA replication inhibitor hydroxyurea (HU) also localize with NPCs (169, 171). It has been proposed that collapsed forks localize to NPCs for fork restart but this has yet to be tested. Mutations of the Nup84 complex renders cells hypersensitive to DNA damage inflicted by UV radiation and treatment with: the alkylating agent methyl methanesulfonate (MMS), the DNA DSB causing drug bleomycin and
1.10 Nuclear Pore Complexes and Transposable Elements

1.10.1 Nuclear Pore Complexes and Ty1

Nups have been identified as Ty1 host factors in three functional genomics screens. A screen of 4483 yeast deletion strains for Ty1 host factors identified 101 genes among which two were Nups (Table 1.1) (176). In this study, the two outer ring Nups, NUP84 and NUP133 were identified as deletion mutants with reduced Ty1 transposition but normal (nup84Δ) or increased (nup133Δ) levels of Ty1 cDNA indicating that Nup84 and Nup133 affect a late step in retrotransposition (176). NUP84 was identified in another genome-wide screen for deletion mutants with reduced Ty1 mobility along with the NUP170 inner ring nucleoporin (Table 1.1) (177). A genome-wide screen conducted in a mutant strain background with hyper transposition discovered reduced transposition upon deletion of NUP133, NUP120, NUP170 or NUP188 (Table 1.1) (178). Although members of both the Nup84 (Nup84, Nup120, Nup133) outer ring complex and Nup170 (Nup170, Nup188) inner ring complex have been identified as Ty1 host factors their specific role in Ty1 mobility remains to be characterized (176-178).

1.10.2 Nuclear Pore Complexes and Ty3

Nups have also been identified as host factors of the S. cerevisiae Ty3 LTR retrotransposon that is a member of the Ty3/Gypsy family and also integrates upstream of RNA Pol III transcribed genes. Unlike Ty1 which inserts in a 1-2 kb window upstream of RNA Pol III transcribed genes, Ty3 inserts 1 to 4 nucleotides upstream of the RNA Pol III TSS (41). A screen that monitored Ty3 insertion in a collection of mutants identified 53 genes, of which three were
Nups (NUP116, NUP159, NUP157), as potential Ty3 host factors (Table 1.1) (179).

Additionally, a screen of 4457 S. cerevisiae nonessential knockout strains identified 130 mutants with increased or decreased Ty3 transposition measured using a high-copy inducible pGAL-Ty3-HIS3 plasmid. Of the 130 mutants, 8 genes that function in nuclear transport had increased or decreased Ty3 transposition (NUP100, NUP84, NUP120, NUP133, NUP59, KAP120, SAC3, NPL3) (Table 1.1) (180).

Table 1.1 NPC proteins identified in TE host factor screens

<table>
<thead>
<tr>
<th>TE (species)</th>
<th>NPC proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ty1 (S. cerevisiae)</td>
<td>Nup133 (176,178), Nup84 (176,177), Nup170 (177,178), Nup120 (178), Nup188 (178)</td>
</tr>
<tr>
<td>Ty3 (S. cerevisiae)</td>
<td>Nup116 (179), Nup159 (179), Nup157 (179), Nu100 (180), Nup84 (180), Nup120 (180), Nup133 (180), Nup59 (180)</td>
</tr>
<tr>
<td>Tf1 (S. pombe)</td>
<td>Nup124 (181), Nup61 (182)</td>
</tr>
</tbody>
</table>

Yeast cells carrying a mutation in either NUP120 or NUP133, display clustering of pores on the NE (183,184). nup120Δ and nup133Δ strains, which also display NPC clustering, were used to determine if Ty3 VLPs associate with the NPC versus the NE using immunofluorescence microscopy (185). In the nup133Δ and nup120Δ mutant backgrounds, Ty3 VLPs localize to clustered NPCs within 6 hours of Ty3 induction (185). The association between Ty3 VLPs and NPC clusters argues that Ty3 VLPs interact physically with the NPC at the time when complete Ty3 cDNA products are first detected. Ty3 Gag (Gag3) binds directly to Nup100 and Nup116 GLFG repeat domains in vitro (185). Therefore, Ty3 VLP docking on the NPC is likely mediated by interactions between Ty3 Gag3 and GLFG Nups.
1.10.3 Nuclear Pore Complexes and Tf1

*S. pombe*, commonly referred to as “fission yeast”, is an ascomycete yeast species that diverged from *S. cerevisiae* between 330 to 420 million years ago (186). Tf1 is an active LTR retrotransposon in *S. pombe* containing a single ORF that encodes Gag, PR, RT, and IN proteins that assemble along with copies of RNA to form VLPs (187-189). Tf1 inserts in promoter regions of RNA Pol II transcribed genes in a window 100-400 bp upstream of ORFs throughout the genome (190,191). Phylogenetic analyses have confirmed that Tf1 belongs to the Ty3/Gypsy family of LTR-retrotransposons (192).

A genetic screen for Tf1 host factors identified the FG NPC protein Nup124 (Table 1.1) (181). A *nup124-1* mutant strain had a 12-fold decrease in Tf1 transposition compared to a wild type strain and caused a significant defect in nuclear localization of Tf1 Gag (181). Tf1 Gag carries three NLSs that are required for Tf1 transposition and the nuclear localization activity (193). Interestingly, when the N-terminal NLS of Tf1 Gag was fused to green fluorescent protein (GFP)-LacZ it was discovered that nuclear localization depended on Nup124 (193). The human NPC protein Nup153 (huNup153) was identified as a homolog for *S. pombe* Nup124 with similarity between the Nup124 N-terminal amino acid sequence (AA 272–454) and the N-terminal region (AA 448–634) of huNup153 (194). Nup124 is also required for nuclear entry of the HIV-1 viral protein R (Vpr), a viral protein involved in replication efficiency and PIC nuclear import (195-197), when Vpr is expressed in *S. pombe* (198). Given that Nup124 residues 272-454 are a part of the Tf1-Gag binding domain (181), it is not a surprise that removing this domain dramatically reduces Tf1 activity in *S. pombe*; however, replacing these residues with amino acids 448-634 from human Nup153 rescues Tf1 activity (198).
1.11 Nuclear Pore Complexes and Viruses

Many viruses must cross the NE barrier to enter the nucleus in order to undergo transcription and replication. In order to do so, viruses have evolved different strategies to access the nucleus which include: capsid docking at NPCs which allows the viral genome to traverse the nuclear pore, direct movement of the viral genome containing capsid through NPCs, disruption of the NE and entry through the breaks, or by waiting for mitotic NE breakdown (199).

Herpes viruses are a large group of enveloped DNA viruses of which the role of nuclear transport has been well studied in herpes simplex virus type 1 (HSV-1) (200). HSV-1 virions are composed at their core of a 152 kb ds DNA genome packaged within a 120 nm icosahedral capsid (200). The DNA containing HSV-1 capsid is surrounded by a proteinacious layer called the tegument which is the next structural layer of the virion envelope (200). HSV-1 enters cells by fusion of viral and host cell membranes or endocytosis (201). Similar to Ty3 and other viruses, replication of HSV-1 has a NPC nucleocapsid docking step whereby the nucleocapsid binds to cytoplasmic Nups which triggers release of the HSV-1 genome across the NPC (202-205) (Figure 1.10). Hepatitis B virus (HBV) is a small enveloped DNA virus with a diameter of 42-47 nm (200). HBV enters a cell by endocytosis and during nuclear entry the intact HBV capsid, which is ~32-36 nm in diameter, crosses the NPC (200) (Figure 1.10). Parvoviruses are small (~25 nm), non-enveloped DNA viruses that enter cells via endocytosis (199). Parvoviruses are small enough to directly enter the nucleus through NPCs (206), but they also disrupt the NE using caspase enzymes to cause partial dissolution to allow nuclear access (207) (Figure 1.10).
Figure 1.10 Viral nuclear entry. The HSV-1 capsid (orange) docks on the cytoplasmic face of a NPC (grey) through an interaction with importin β (green) which triggers release of the viral genome. The intact HBV capsid (blue), via an interaction with importin α (light green) and importin β, traverses through the NPC. The HBV capsid is then disassembled at the nuclear face of the NPC and the contents of the HBV capsid (circular viral genome and viral proteins) enters the nucleus. The capsids of paroviruses (parvo; pink) can enter the nucleus directly or physically disrupt the NE to gain nuclear entry. Adapted from (199,200)

1.11.1 Nuclear Pore Complexes and HIV-1

HIV-1, a retrovirus that primarily infects mammalian CD4+ T cells, was discovered to be the causative agent of acquired immunodeficiency syndrome (AIDS) in the early 1980’s (208,209). HIV infection begins with virion binding to CCR5 or CD4 receptors host cell receptors, virion entry, followed by reverse transcription of the viral RNA genome to double-stranded DNA, nuclear import and integration of the viral DNA into the genome (210). Three independent genome-wide small interfering RNA (siRNA) screens for host factors required for HIV-1 replication identified Nups involved in PIC trafficking and integration, thus lending
support for the evolutionarily conserved role of Nups in retrotransposon and retroviral life cycles (211-213).

Functional conservation of Ty1-IN with HIV-1 IN suggests that studies of Ty1-IN protein interactions and targeting in yeast will be applicable to the mechanism of HIV-1 propagation. Nups, such as the human Nup153 nuclear basket protein, have been a recent topic of interest in viral replication (214). Chromatin nuclear architecture dictates HIV-1 integration, which predominantly occurs in chromatin located at the outer edge of the nucleus, in close proximity with nuclear pores (215,216). HIV-1 recurrent integration genes (RIGs), which are genes that have been targeted for HIV-1 integration in more than one integration site mapping study, are clustered into specific regions on human chromosome maps and are positioned near the NE (215). Integrated and transcriptionally active HIV-1 DNA was visualized in the same area and was shown to interact with Nups, suggesting that integration occurs at the NE region in the presence of NPC proteins (215). Using 3D confocal microscopy Albanese et al. found that fluorescently labeled PICs preferentially distribute in decondensed areas of the chromatin at the nuclear periphery, while heterochromatin regions are largely disfavored providing the first indication of nuclear architecture influencing retroviral integration sites (217). In addition, visualization of DNA repair foci from viral DNA carrying DSBs demonstrated that integrated HIV-1 localizes to the nuclear periphery (218). Burdick et al. engineered YFP tagged APOBEC proteins, host factors that incorporate into virions and restrict virus replication, to track HIV-1 PIC movement and found that HIV-1 associates with the NE and preferentially integrates at chromatin adjacent to the NE at the nuclear periphery (219). Taken together, these studies find that HIV-1 integration likely occurs at the nuclear periphery placing HIV-1 IN in proximity to NPCs (218,219).
HIV-1 replication is significantly decreased in the absence of the nuclear basket Nup153 protein (216). Nup153 binds directly with HIV-1 IN, CA, and Vpr in an importin-independent manner for nuclear import (198,220,221). There is no consensus as to which yeast nuclear basket proteins are the homologues of mammalian Nup153. Earlier reports state that Nup153 has no identifiable yeast homolog (222,223). However, others have identified Nup1 of *S. cerevisiae* as the Nup153 homolog (106,224). Nup60 has also been described as a potential homologue of Nup153 given that both are located on the nuclear basket and interact with homologous NPC proteins: Tpr proteins in mammalian cells and Mlp proteins in *S. cerevisiae* (225). Based on both sequence comparison and functional analysis, *S. pombe* Nup124 may also be a possible homologue of human Nup153 as Nup124 is required for both Tf1-Gag and HIV-1 Vpr transport from the cytoplasm to the nucleus (194).

### 1.12 Research Objectives

NPCs are dual gateways for nucleo-cytoplasmic transport of macromolecules. Alongside their transport roles, NPCs are involved in numerous genomic processes such as gene silencing, gene gating, transcriptional memory, mRNA surveillance and DSB repair. Yeast tRNA genes are one of the many genes that are actively transcribed at NPCs and they are the genomic targets of the Ty1 retrotransposon. Previous Ty1 host factor screens have identified Nups as both Ty1 restriction and host factors. In addition, Nups play a central role in the propagation of other yeast TEs and viruses. My hypothesis was that NPC proteins play a role in Ty1 replication and influence integration site selection.

The yeast Ty1 retrotransposon is an excellent model system to study retroviral integration because Ty1 and retroviruses share similar replication cycles. Although Ty1 elements have no
infectious stage, Ty1 replicates through reverse transcription of an RNA intermediate, as do retroviruses such as HIV-1. Both Ty1 and HIV-1 also require the structural Gag proteins for generating the outer shell of VLPs and virions respectively, RT for synthesis of cDNA and IN for insertion of their cDNA into the host genome. Mounting evidence also suggests that NPC proteins directs the integration of HIV-1 viral DNA into chromatin via an “import-coupled integration” mechanism.

1.12.1 Specific Aims:

Aim 1. Identify NPC proteins that play a role in Ty1 replication

I screened NPC non-essential deletion and essential temperature-sensitive (ts) mutants for Ty1 transposition defects. To identify Nups required for Ty1 transposition I transformed wild type, NPC deletion and ts yeast strains with a Ty1 plasmid carrying a uracil (URA3) selectable marker and HIS3 reporter gene. Next, I induced transposition by growing the strains at 20°C for 5 days, plated cells on synthetic complement (SC)-URA and SC-URA-histidine (HIS) for viability and transposition frequency respectively; I counted colonies on these plates and calculate the transposition frequency. I also isolated genomic DNA from NPC mutant yeast strains, induced endogenous transposition by growing the strains for 3 days at 20°C in yeast peptone dextrose (YPD) media, to analyze Ty1 integration levels upstream of tRNA genes using a polymerase chain reaction (PCR) assay.
Aim 2. Determine at which step(s) (ie. transcription or integration) that Nups identified in Aim 1 affect Ty1 replication

I was interested in Nups that direct IN-mediated Ty1 cDNA insertion into the genome. Since the NPC functions as a two-way transporter, I needed to eliminate Nups from my study that solely function in the steps of the Ty1 replication cycle preceding the formation of a PIC, such as Ty1 mRNA export. To determine if changes in transposition are due to differences in the availability of transposition intermediates, I quantified Ty1 mRNA levels in yeast strains by quantitative PCR and I measured Ty1 cDNA levels by southern blotting. To test if observed transposition levels were due to changes in Ty1 mRNA translation or export, I quantified Ty1 Gag levels by western blotting. I also examined Ty1-IN nuclear localization by microscopy with a reporter construct carrying Ty1 expressed from a phosphoglycerate kinase (PGK) promoter and a mCherry tagged IN.

Aim 3. Explore the influence of nuclear basket proteins on the regulation of Ty1 integration

I studied Ty1 replication using methods in Aims 1 and 2 in nuclear basket (Nup1-Nup2-Nup60-Mlp1-Mlp2) mutant strains with normal Ty1 mRNA and protein levels but impaired Ty1 insertion. I also characterized the role of complexes known to interact with NPCs, such as TREX and TREX-2, on Ty1 replication.
Chapter 2:
The Nuclear Pore Complex Plays a Role in Ty1 Replication

2.1 Introduction

An obligate and irreversible step in retroviral replication is insertion of retroviral DNA into the host cell’s genome. Host cell machinery, although commonly hijacked by retroviruses for replication, can pose challenges to genomic insertion of retroviral genetic material. One such cellular structure, the NE, surrounds the nucleus creating a physical barrier between retroviruses and their target genomic DNA. In mammalian cells, the NE breaks down during mitosis allowing retroviruses, such as Moloney Murine Leukemia Virus, access to genomic DNA for integration (226,227). However, HIV-1 can productively infect non-dividing, terminally differentiated cells, and therefore, has a mechanism to overcome the NE barrier to gain access to genomic integration sites (228,229). Because the yeast S. cerevisiae NE does not break down during mitosis, retrotransposons in yeast and HIV-1 may possess similar mechanisms to access chromatin.

The S. cerevisiae Ty1 retrotransposon, which is a member of the Ty1/copia family, resembles a retrovirus in structure and life cycle except that Ty1 does not have an extracellular phase (12). The Ty1 element consists of GAG and POL ORFs flanked on either side by LTR sequences (12). GAG encodes a structural protein of the VLP, while POL produces the enzymes PR, IN, RT-RH (12). Early stages in Ty1 replication include Ty1 transcription, translation, VLP assembly, Ty1 Gag and Pol processing followed by reverse transcription of the Ty1 mRNA...
intermediate to cDNA. The newly synthesized Ty1 cDNA forms a complex with IN called a PIC (12). The PIC localizes to the nucleus where Ty1 cDNA is inserted upstream of genes transcribed by RNA Pol III, such as tRNA genes by a mechanism that depends on Ty1-IN (22). Recently my lab, and Bridier-Nahmias et al., discovered that Ty1-IN interacts with RNA Pol III subunits and that this interaction is necessary for targeting Ty1 element insertion upstream RNA Pol III transcribed genes (230,231).

To translocate across the NPC, soluble transport factors (karyopherins/importins/exportins) recognize protein cargoes carrying a NLS for import substrates or a nuclear export signal for export substrates. Ty1-IN has a bipartite NLS that is required for entry into the nucleus and binds the importin-alpha transporter (Kap60), suggesting that Ty1-IN uses the classical nuclear import pathway for nuclear entry (26,27,232). It is not known if the Ty1 VLP or Ty1 PIC is delivered to the NPC or how the Ty1 PIC travels through the NPC. As well, the role of NPC proteins, termed Nups, in Ty1 integration remains unknown but potentially significant given the emerging role for Nups in gene regulation and chromatin organization as well as retroviral integration (233,234).

Here I use a Ty1 reporter plasmid to monitor Ty1 mobility, resulting from both IN-mediated retrotransposition of Ty1 to a new genomic location and Ty1 cDNA recombination with a pre-existing genomic Ty1 element, in a panel of Nup mutant yeast strains. I also measure endogenous Ty1 element insertion in these strains using a PCR assay. My findings indicate that deletion or mutation of multiple Nups alters Ty1 mobility and Ty1 element insertion upstream of tRNA genes without affecting the levels of Ty1 replication intermediates (Ty1 mRNA, Gag protein, Ty1 cDNA) and Ty1-IN nuclear localization. As such, Nups appear to play a part in the Ty1 replication cycle during Ty1 element transcription and integration. Taken together with the
well-defined role of actively transcribed genes relocating to NPCs, my results further support the role of the NPC as a dynamic functional and regulatory hub for coupling cellular processes such as a PIC import and integration.

2.2 Materials and Methods

2.2.1 Yeast Strains and Media

Both non-essential deletion (Δ) and essential ts mutant yeast strains were previously engineered (8,235). Yeast strains used in Chapter 2 are listed in Table 2.1 and deletion mutants were verified by colony PCR. *S. cerevisiae* strains were grown in YPD, SC media or SC media lacking URA, Leucine (LEU) or HIS and supplemented with combinations of dextrose (Dex), and/or hygromycin B (Invitrogen) as required.

**Table 2.1 Yeast strains used in Chapter 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
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<td>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
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<td>MATa his3Δ1 leu2Δ0 lys2Δ0 met15Δ ura3Δ0 nup188ΔkanMX6</td>
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</tbody>
</table>
2.2.2 Ty1 Mobility Quantification Assay

This assay was performed as described in (237). Briefly, strains were transformed with a CEN based URA3 marked Ty1-his3-AI reporter plasmid (pBDG922, pBDG924 or pBDG633, all kind gifts from Dr. David Garfinkel). Single colonies were isolated and patched. 2000 cells were inoculated into SC-URA media and grown for 5 days at 20°C. $10^7$ cells were plated on SC-URA-HIS plates and 200 cells on SC-URA plates to test for transposition frequency and viability, respectively. After incubation for 2 days at 25°C, the transposition frequency of the marked Ty1 element was calculated by the following formula: # colonies on SC-URA-HIS/$10^7$ divided by # colonies on SC-URA/200. At least 2 single colonies were tested in triplicate for each strain. My Ty1 mobility data does not account for possible changes in plasmid copy numbers due to NPC disruption. Statistical analyses were performed using GraphPad Prism 6.

2.2.3 Quantitative PCR

Quantitative PCR (qPCR) was performed and analyzed as described (231). Yeast strains were grown in YPD media at 20°C to log phase; $10^7$ cells were pelleted and total RNA was isolated

<table>
<thead>
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<th>Yeast</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
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<tr>
<td>YSM98</td>
<td>MATa leu2Δ ura3Δ his3Δ NAB2::HIS3 (pAC636)</td>
<td>(236)</td>
</tr>
</tbody>
</table>
with a Qiagen RNeasy kit. The Superscript VILLO cDNA synthesis kit (Invitrogen) was used for cDNA synthesis from 1 µg of RNA. Real-time PCR was set-up in triplicate with 2 µl of a 1:50 cDNA dilution and Power SYBR Green Master Mix (Invitrogen). A standard two-hour comparative PCR analysis was performed using a 7500 Real Time PCR system (Applied Biosystems). The primers for Ty1 mRNA expression were: OVM760: 5’-TCGCATGGTCAGAAGATCGA-3’ and OVM761: 5’-ACCCACAGCAGTGCATGATG-3’.

The primers for amplifying mature tLEU (CAA) (10 genes) are OVM959 5’-GGTTGTTTGCCGAGCGGTCTAAG-3’ and OVM960 5’-TGTTTGCTAAGGATCGAATTCTCT-3’ (230). The primers for amplifying unspliced tLEU (CAA) (tLEU de novo, 7 genes) are OVM959 5’-GGTTGTTTGCCGAGCGGTCTAAG-3’ and OVM961 5’-TATTCCCACAGTAACTGCGGTC-3’ (230). The primers for amplifying mature tGLY (GCC) expression (16 genes) are OVM962 5’-GCGCAAGTGGTTTAGTGG-3’ and OVM963 5’-AAGCCCGGATCGAACC-3’ (230). qPCR analysis was performed using the comparative C_T method (ΔΔC_T method, Applied Biosystems), with TAF10 used as an internal control. The TAF10 primers were: OVM695 5’-GGCGTGCAGCAGATTTCAC-3’ and OVM696 5’-TGAGCCCGTATTCAAGCAACA-3’ as previously described (231).

2.2.4 Ty1 Gag Westerns

Yeast strains were grown on selection plates (YPD supplemented with G418 at 1 mg/ml) for 3 days at 20°C. For each strain, colonies were transferred to 10mL of YPD and grown overnight at 20°C to log phase (OD_600 0.5-1.0) in duplicate. Cells were pelleted, washed with ice-cold water and then re-suspended in ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 0.5 mM EDTA pH 8.0, 250 mM NaCl, 0.1% NP40 and protease inhibitors). After glass bead lysis, samples were
centrifuged at 14 000 rpm for 10 min and the supernatant [whole cell extract (WCE)] was transferred to a new eppendorf tube. The WCE protein concentration was determined by Bio-Rad Protein Assay. Sodium dodecyl sulfate (SDS) loading dye (2X) was added to 60 µg of each WCE sample, which were then boiled for 3-5 min and loaded on a 12% SDS-PAGE gel and transferred to a methanol activated polyvinylidene difluoride (PVDF) or nitrocellulose membrane using the Bio-Rad Transblot Turbo system (25 V, 2.5 A, 15 min). Membranes were probed with anti-Gag sera (1:15 000, provided by Dr. Jef Boeke) and anti-GAPDH (1:5000, UBC Antibody Lab) as a loading control. Membranes were incubated with the following infrared secondary antibodies from Mandel Scientific: IRDye® 680RD Goat anti-Rabbit IgG (H+L), IRDye® 800CW Goat anti-Mouse IgG (H+L), both at 1:10 000, and imaged on an Odyssey CLx. Ty1 Gag protein levels (p49 and p45) were quantified relative to GAPDH levels using Image Studio Ver 2.1 software.

2.2.5 pPGK1-Ty1-IN-mCherry Construction

The PGK1-mCherry expression vector (pCW1; BVM460) was a gift from Dr. Chris Walkey. pCW1 carries both hygromycin and ampicillin resistance genes and has a single Sal I restriction site between the PGK1 and mCherry ORFs (238). pCW1 was digested with Sal I and then dephosphorylated with calf intestinal phosphatase. Ty1-IN was amplified from a plasmid carrying a full length Ty1 element called pGAL1-Ty1-H3 (pJEF724, generous gift from Dr. Jef Boeke) with IN-XhoI-F (5’-GGTGGTGTCTCGAGATGAATGTCCATACAAGTGAAAGTACACGCAAA-3’) and IN-XhoI-R (5’-GGTGGTGTCTCGAGTGAATCCAGGTGAATTCGTTCTT CGATCT-3’).
primers which have 8 bases in front of Xho I site (underlined). The resulting PCR product was purified, digested with Xho I then ligated with Sal I digested pCW1.

2.2.6 Microscopy

Yeast strains were transformed with pPGK1-Ty1-IN-mCherry or empty vector (pCW1; BVM460) and grown at 25°C on YPD plates supplemented with hygromycin B for 2-3 days. Two isolates per mutant strain were grown to log phase overnight at 25°C in 5 ml of SC media supplemented with hygromycin B and 2% Dex. 30 min prior to imaging, 1 ml of each culture was stained with 0.5 µg Hoechst 33342 (Life Technologies). Live cells were imaged with a Zeiss Axio Observer inverted microscope equipped with a Zeiss Colibri LED illuminator and a Zeiss Axiocam ultrahigh-resolution monochrome digital camera Rev 3.0; ten image stacks were acquired with a 40X objective, at a step of 0.3 µm, and analysed with Zeiss Axiovision software as in (239).

2.2.7 Ty1 cDNA

Ty1 cDNA analysis was generally performed as described (240-242). Briefly, strains were grown in YPD for 2 days at 20°C, followed by genomic DNA extraction using phenol-chloroform. Ten micrograms of genomic DNA was digested with the enzyme PvuII (New England Biolabs) to release a ~2.4 kb fragment of Ty1 cDNA. Digestion reactions were run on a 0.8% Tris-Borate EDTA (TBE) agarose gel and then transferred to a Hybond-XL charged nylon membrane (GE Healthcare). Membranes were subsequently probed with a 32P-α-dATP (Perkin Elmer) radiolabelled 1517 bp PvuII/SnaBI C-terminal fragment of the Ty1 element using a
DecaLabel DNA labeling Kit (ThermoScientific). A Typhoon Trio Variable Mode Imager was used for imaging and ImageQuant software was used for quantitation.

2.2.8 **SUF16, SUP61 and tGLY Ty1 Integration Assay**

This assay was performed as described (237) with the following modifications. Yeast strains were grown on plates for 3 days at 20°C then colonies were transferred to liquid media and grown for 3 days at 20°C to induce endogenous Ty1 transposition. Glass beads and lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10mM Tris-HCl (pH 8.0), 1mM EDTA] were used to lyse cells, and genomic DNA was extracted using phenol-chloroform. Ty1 insertions upstream of the **SUF16** and **SUP61** loci on chromosome III were amplified using **SNR33** out (OVM890 5’-GTGACACCATCGTACAAGAGGGC-3’) and **BUD31** out (OSM27 5’-GGCTTGGATCTTCTGGTCTTTATGC-3’) primers, respectively, along with the TyB out: (OVM889 5’-GCTGATGTGATGACAAAACCTCTTCCG-3’) primer (237). The primers were used to amplify Ty1 insertions from 1 µg of genomic DNA using the following touchdown PCR cycling parameters: 96°C, 2 min; 5 cycles at 96°C, 30 sec; 70°C, 30 sec; 68°C, 1 min followed by 5 cycles at 96°C, 30 sec; 67°C, 30 sec; 68°C, 1 min followed by 5 cycles at 96°C, 30 sec; 65°C, 30 sec; 68°C, 1 min followed by 5 cycles at 96°C, 30 sec; 63°C, 30 sec; 68°C, 1 min followed by 10 cycles at 96°C, 30 sec; 60°C, 30 sec; 68°C, 1 min. The presence of quality genomic DNA was verified by PCR amplification of the **CPR7** locus with 5 ng of genomic DNA (CPR7-forward: 5’-GT TTGATTTATCTCTGGACTGCT-3’ and CPR7-reverse: 5’-AGTTCGTCTCTCCTCATATTCTCA-3’) by executing the following PCR parameters: 94°C, 2 min; 30 cycles at 94°C, 30 sec; 60°C, 30 sec; 72°C, 2 min followed by 72°C, 10 min. Each yeast strain was assayed in triplicate.
2.2.9  *NAB2* plasmid shuffle assay

I used a previously characterized set of Nab2 mutant plasmids to characterize a role for Nab2 in Ty1 replication using a plasmid shuffle assay (243). To study the essential protein Nab2, a *nab2Δ* yeast strain carrying a plasmid expressing wild type *NAB2* marked with *URA3* and also containing *NAB2*, *nab2ΔN* (lacking residues 4–97), *nab2F72D* or *nab2F73D* marked with *LEU2* were plated onto SC-URA-LEU plates (where the *NAB2 URA3* maintenance plasmid is retained) and SC-LEU plates containing 5-Fluoroorotic acid (5-FOA) at 2 µg/ml to remove the *NAB2 URA3* maintenance plasmid and leave the *NAB2 LEU2* test plasmids as the only copy of *NAB2* in each strain (243).

2.3  Results

2.3.1  Subunits of the yeast nuclear pore complex are involved in Ty1 mobility.

I tested 15 non-essential deletion and 4 essential ts Nup mutant yeast strains for significant changes in Ty1 mobility. Ty1 mobility was quantified by monitoring the formation of HIS+ colonies in NPC mutant yeast strains carrying a plasmid (pBDG922) with a *HIS3* marked Ty1 element (24). The *HIS3* gene is interrupted by an artificial intron (AI) that is removed from the *Ty1-H3mHIS3AI* mRNA intermediate by splicing, followed by reverse transcription and Ty1-IN mediated integration of *Ty1HIS3 de novo* into the genome or by homologous recombination with a pre-existing chromosomal Ty1 element or solo LTR (24). The 19 strains tested here carry ts mutations or deletions of Nups that are representative of NPC substructures (Figure 1.7). Of the 19 strains tested, 9 were significantly decreased and 2 were significantly increased for Ty1
mobility (Figure 2.1 and Table 2.2). Four of the 7 members of the Nup84 outer ring subcomplex were tested (\textit{nup84\textDelta}, \textit{nup120\textDelta}, \textit{nup133\textDelta}, \textit{sec13-1}) and of these, \textit{nup120\textDelta} and \textit{sec13-1} strains had a \(-3.5\)-fold increase and \(-8.2\)-fold decrease in Ty1 mobility respectively (Figure 2.1 and Table 2.2). I tested 5 of the 6 members of the Nup170 inner ring subcomplex of which 4 (\textit{nup170\textDelta}, \textit{nup188\textDelta}, \textit{nup53\textDelta}, \textit{nup192-15}) had significantly decreased Ty1 mobility (Figure 2.1 and Table 2.2). The transmembrane ring subunit, Pom34, and the Nup159 cytoplasmic filamentous Nup are both required for wild type levels of Ty1 mobility with a 4-fold reduction in the \textit{pom34\textDelta} mutant and a 50-fold reduction in the \textit{nup159-1} mutant (Figure 2.1 and Table 2.2). Three of the nuclear basket deletion strains tested had a significant difference in Ty1 mobility compared to wild type with the \textit{mlp1\textDelta} and \textit{nup60\textDelta} strain each reduced by \(-3\)-fold and the \textit{mlp2\textDelta} strain increased by \(-2.3\)-fold (Figure 2.1 and Table 2.2). Although Nup2 is not a core member of the NPC I included it in my study as a nuclear basket Nup (Figure 1.7) because Nup2 is a mobile Nup that binds both the cytoplasmic and nuclear faces of the pore, interacts with Nup60, and has a role in chromatin organization and tRNA transcription (82,122,160,244,245). However, I did not detect a significant change in Ty1 mobility in the \textit{nup2\textDelta} strain compared to the wild type strain (Figure 2.1 and Table 2.2).
Figure 2.1 The NPC has a role in Ty1 mobility. (A) Diagram of the Ty1 mobility assay plasmid pBDG922 - a CEN plasmid, carrying a URA3 marker and a HIS3AI tagged Ty1 element expressed from the Ty1 endogenous promoter. (B) Ty1 transposition frequency of wild type (WT) and 19 Nup mutant strains [outer ring (nup84Δ, nup120Δ, nup133Δ, sec13-1), inner ring (nup170Δ, nup188Δ, nup192-15, nup59Δ, nup53Δ) cytoplasmic (nup159-1, nup42Δ), transmembrane ring (ndc1-4, pom152Δ, pom34Δ), central (nup100Δ), nuclear basket (nup60Δ, mlp1Δ, mlp2Δ and nup2Δ)] each carrying pBDG922. Cells from two colonies, grown in triplicate, were induced to transpose for 5 days at 20°C. A two-tailed t test was used for analysis and mutants with a statistically significant difference in transposition frequency from wild type (p<0.05) are marked with an asterisk (*).
Table 2.2 Summary of Ty1 replication data for NPC mutant yeast strains

<table>
<thead>
<tr>
<th>NPC substructure</th>
<th>Yeast strain</th>
<th>Ty1 mobility frequency (SD)</th>
<th>Ty1 mobility ratio$^a$</th>
<th>SUF16$^b$</th>
<th>SUP61$^b$</th>
<th>Ty1 mRNA$^c$</th>
<th>Ty1 Gag protein levels$^d$</th>
<th>Ty1 cDNA$^c$</th>
<th>tLEU$^f$</th>
<th>tLEU (denovo)$^f$</th>
<th>tGLY$^f$</th>
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<td><strong>Outer ring</strong></td>
<td>wild type</td>
<td>6.80E-06 (3E-06)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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</tr>
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<td>nup84A</td>
<td></td>
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<td>0.99</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
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<td>3.53</td>
<td>9.8</td>
<td>2.4</td>
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<td>-</td>
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<td>0.05</td>
<td>0.07</td>
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<td>0.84</td>
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<td><strong>Inner ring</strong></td>
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<td>0.19</td>
<td>0.0</td>
<td>1.1</td>
<td>0.53</td>
<td>0.6</td>
<td>0.5</td>
<td>0.6</td>
<td>0.6</td>
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<tr>
<td>nup188A</td>
<td></td>
<td>6.22E-07 (9.9E-07)</td>
<td>0.09</td>
<td>0.07</td>
<td>0.02</td>
<td>2.2</td>
<td>0.4</td>
<td>2.1</td>
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<td>0.6</td>
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<tr>
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<td>0.08</td>
<td>0.09</td>
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<td>2.9</td>
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<td>0.21</td>
<td>0.16</td>
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<td>1.12E-07 (5E-08)</td>
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<td>0.05</td>
<td>0.003</td>
<td>0.52</td>
<td>0.37</td>
<td>0.5</td>
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<td>0.02</td>
<td>0.04</td>
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<td>0.04</td>
<td>0.3</td>
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<td>0.27</td>
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<td><strong>Central FG</strong></td>
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<td>0.07</td>
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<td>0.21</td>
<td>0.25</td>
<td>1.6</td>
<td>-</td>
<td>-</td>
<td>0.51</td>
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<td>0.32</td>
<td>0.0</td>
<td>0.03</td>
<td>0.19</td>
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<td>0.6</td>
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<td>-</td>
<td>0.4</td>
<td>0.78</td>
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</table>

$^a$ compared to wild type Ty1 mobility
$^b$ compared to wild type Ty1 insertion upstream of the S UF16 or SUP61 loci
$^c$ compared to wild type Ty1 mRNA levels with TAF10 as an internal control
$^d$ compared to wild type Ty1 Gag protein levels normalized to a GAPDH loading control
$^e$ compared to ratio of Ty1 cDNA to an endogenous Ty1 element
$^f$ compared to wild type tRNA levels with TAF10 as an internal control

Standard deviation (SD), experiments not done indicated with a dash
The Ty1 mobility assay monitors Ty1 insertion into the genome mediated by Ty1-IN but also by homologous recombination with a pre-existing genomic Ty1 element or LTRs (23). To assess the frequency of Ty1 insertion mediated via IN versus homologous recombination, I transformed a wild type strain with a plasmid expressing wild type Ty1-IN \([Ty1-H3mHIS3AI\text{ plasmid (pBDG633)}]\) or a \(Ty1-H3mHIS3AI\text{ plasmid coding for a catalytically inactive Ty1-IN enzyme (pBDG924)}\). I carried out the quantification assay with both plasmids and found that circa 35% of Ty1 mobility in the wild type strain can be attributed to homologous recombination (Figure 2.2). Indeed, I demonstrate below that mutants with significant reductions in Ty1 mobility display severe reductions in Ty1 targeting upstream of tRNA genes which is Ty1-IN mediated (Fig. 4, 5).

**Figure 2.2** Ty1 mobility in the absence of functional Ty1-IN. Transposition frequency of a wild type (WT) strain carrying either a plasmid expressing \(Ty1-H3mHIS3AI\) (pBDG633) or \(Ty1-H3mHIS3AI\) with an catalytically inactive IN enzyme (pBDG924). Cells from 2 colonies were grown in triplicate for 5 days at 20°C then plated on selection plates to test for viability and transposition.
2.3.2 **Nup60 is required for optimal Ty1 mRNA, Ty1 Gag and tRNA levels.**

NPCs are bidirectional transporters that interact with cellular cargo entering and exiting the nucleus. Therefore, NPC mutants may restrict Ty1 transposition at various stages of the Ty1 life cycle such as transcription of the Ty1 element, nuclear export of processed Ty1 mRNA transcripts, reverse transcription of the Ty1 mRNA to cDNA, import of Ty1-IN into the nucleus and/or Ty1 cDNA integration into the genome. To determine if the significant differences in quantitative Ty1 mobility in NPC mutant strains were due to defects in Ty1 mRNA transcription and/or export I measured endogenous levels of Ty1 mRNA by quantitative real-time PCR (qPCR) and Ty1 Gag protein [unprocessed (p49) and processed (p45)] by quantitative western blot analysis in all NPC mutants with significant changes in Ty1 mobility.

To assess Ty1 mRNA expression in Nup mutant strains, cells were grown at 20°C to logarithmic phase, mRNA extracted, converted to cDNA and qPCR analysis performed. The *spt3Δ* mutant was used as a negative control as Spt3 is required for transcription of Ty1 elements (246). Of the 11 mutants with altered Ty1 mobility, the *nup60Δ* strain had the most dramatic reduction in Ty1 mRNA, generating only 19% of wild type Ty1 mRNA levels, suggesting that Nup60, which is located on the nuclear face of the yeast NPC, is required for expression of Ty1 elements (Figure 2.3A and Table 2.2). Interestingly, Nup60 interacts with tRNA genes but NPC contact is not specifically required for the binding of Pol III to tRNA genes or tRNA gene expression (82). Two other nuclear basket mutants, *mlp1Δ* and *mlp2Δ* expressed Ty1 mRNA at ~79% and 133% of wild type levels, respectively and Ty1 Gag at 77% and 75% of wild type levels, respectively (Figure 2.3 and Table 2.2). The only other Nup mutant with reduced Ty1 mRNA levels was *nup159-1*, which is located on the cytoplasmic side of the NPC, and expressed
Ty1 mRNA at 52% of wild type levels (Figure 1.7 and 2.3A). All other NPC mutants had Ty1 mRNA levels similar to or higher than the wild type strain (Figure 2.3A and Table 2.2).

Figure 2.3 Nup60 is required for Ty1 mRNA expression and Ty1 Gag production. (A) qPCR analysis of Ty1 mRNA was performed on the following mutant yeast strains: wild type (WT), nup60Δ, pom34Δ, mlp1Δ, mlp2Δ, nup170Δ, nup188Δ, nup170Δ, nup120Δ, sec13-1, nup192-15, nup159-1 and nup53Δ. spt3Δ serves as a negative control. Strains were grown at 20°C to log phase for RNA extractions. The bar graph shows relative quantification (RQ) of Ty1 transcript compared to a control (TAF10) and relative to wild type Ty1 mRNA set to 1. (B) Ty1 Gag protein levels were analyzed in the same strains as (A), grown at 20°C to log phase. Anti-Gag sera was used to detect unprocessed (p49) and processed (p45) Gag levels by quantitative western blot with fluorescently labeled secondary antibodies. Ty1 Gag fluorescence signals (p49 and p45) were normalized to GAPDH, averaged, quantified using Image Studio software and are reported relative to wild type.
Because all Ty1 polypeptides are generated from the same Ty1 mRNA, monitoring Ty1 Gag protein levels is a proxy for nuclear export of Ty1 element mRNA and also convenient as Ty1 Gag is the only Ty1 protein that can be detected at endogenous levels. I generated cell lysates from wild type and Nup mutant logarithmically growing cells and performed quantitative immunoblot analysis with anti-Ty1 Gag antibodies to assess Ty1-Gag protein levels and anti-GAPDH antibodies as a loading control (Figure 2.3B). The spt3Δ strain was used as a negative control as described above. As expected from the reduction in Ty1 mRNA expression, the nup159-1 and nup60Δ mutants displayed reduced Ty1 Gag protein at 37% and 23% of wild type levels, respectively (Figure 2.3B and Table 2.2). Not predicted by the Ty1 mRNA expression data, however, was the reduction in Ty1 Gag levels detected in a subset of mutants from the Nup170 inner ring subcomplex. The nup170Δ, nup188Δ and nup192-15 mutants had 53%, 40% and 73% of wild type Ty1 Gag levels, respectively (Figure 2.3B, Table 2.2). Therefore, the Nup170 inner ring subcomplex may have a role in export of Ty1 mRNA into the cytoplasm. The remainder of the Nup mutants displayed 75% or higher levels of Ty1 Gag when compared to wild type suggesting that mRNA export and translation of Ty1 transcripts is not dramatically altered (Figure 2.3B and Table 2.2).

Active Pol III transcription is thought to be required for Ty1 insertion. A point mutation in B box of the SUFI6 tRNA promoter that dramatically reduces transcription also reduces Ty1 element insertion (22). Also, Ty1 does not target to the tRNA relic gene ZOD1 which has very low transcription levels (31,33). As well, since tRNA genes are recruited to the nuclear pore in M phase it was important to determine if the NPC mutants with Ty1 mobility defects had reduced tRNA gene expression (82). To determine if Pol III transcription was affected in my panel of NPC mutants, Dr. Lina Ma and I measured the level of leucine (tLEU and unprocessed
tLEU called tLEU denovo) and glycine tRNA transcripts (tGLY) by qPCR in cells grown to log phase at 20°C. We found that tLEU and tLEU denovo transcript levels were reduced by ~50% in nup60Δ, mlp1Δ, mlp2Δ, nup170Δ and nup188Δ strains (Figure 2.4A, 2.4B and Table 2.2).

Notably, the nup60Δ mutant also had a ~50% reduction in tGLY levels whereas all other NPC mutants expressed tGLY at 60% of wild type levels or higher (Figure 2.4C). Some yeast strains (pom34Δ, nup192-15, nup159-1) expressed higher levels of one or two of the tRNA genes, with the highest value for pom34Δ tLEU de novo (1.7-fold above wild type), but no strain generated higher levels of all three tRNA gene families tested here (Figure 2.4). Our tRNA expression data suggests that a 2-fold reduction in Ty1 genomic insertion in NPC mutants may be attributed to a reduction in Pol III transcription. However, any further reduction in Ty1 targeting in NPC mutants may be due to a defect in the integration process.
Ty1 cDNA levels are affected in only a subset of Nup mutant strains

One of the limiting steps in the Ty1 life-cycle is the reverse-transcription of the Ty1 mRNA to cDNA (12). It has previously been shown that deletion of *NUP120* or *NUP133* results in increased Ty1 cDNA and deletion of *NUP84, NUP120* or *NUP133* also causes an increase in Ty3 cDNA levels (176,178,180). One interpretation of these data is that the Ty1 cDNA increase is due to accumulation of the cDNA in the cytoplasm in the absence of efficient nuclear transport (176). Another interpretation is that PIC nuclear entry is enhanced upon disruption of the Nup84 outer ring subcomplex (180). I analyzed endogenous Ty1 cDNA levels in wild type and Nup

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**Figure 2.4** tRNA levels are reduced in a subset of NPC mutant strains. qPCR analysis was used to measure (A) *tLEU*, (B) *tLEU* denovo and (C) *tGLY* levels in wild type (WT), *nup60Δ, pom34Δ, mlp1Δ, mlp2Δ, nup170Δ, nup188Δ, nup120Δ, sec3-1, nup192-15, nup159-1* and *nup53Δ* strains. RNA was isolated from strains grown to log phase at 20°C and qPCR was performed in triplicate with *TAF10* expression used as an internal control. Mutant gene expression values are derived by comparing levels to wild type, which is set to 1.
mutants with altered Ty1 mobility by southern blot analysis of yeast genomic DNA digested with PvuII, which liberates a ~2kb fragment of Ty1 cDNA, and hybridization of a probe containing a 1517 bp C-terminal fragment of the endogenous Ty1 element. I used software to quantify the ratio of Ty1 cDNA compared to a Ty1 element fragment (Ty1 control). Of the 11 Nup mutant strains I tested, 3 (mlp2Δ, nup192-15, pom34Δ) had a wild type ratio of Ty1 cDNA to Ty1 control and 3 (nup53Δ, nup120Δ, nup188Δ) had increased ratios of Ty1 cDNA to Ty1 control compared to a wild type strain (Figure 2.5 and Table 2.2). These data suggests Ty1 mobility defects in these mutants are not due to low Ty1 cDNA production. The nup120Δ strain, which has increased Ty1 mobility, had an ~8-fold higher ratio of Ty1 cDNA to Ty1 control than the wild type strain (Figure 2.5 and Table 2.2); which has been previously observed (178). The remainder of the Nup mutants had a 1.5 to 3-fold reduction in Ty1 cDNA ratios (mlp1Δ, nup159-1, nup170Δ, sec13-1) whereas the nup60Δ mutant had a 6-fold reduction in Ty1 cDNA levels compared to wild type, which is not surprising considering the defects in Ty1 mRNA synthesis and Ty1 protein levels in this mutant (Figure 2.5 and Table 2.2). The reduced Ty1 cDNA levels in the mlp1Δ, nup159-1, nup170Δ, sec13-1 mutants could be in part causing Ty1 mobility defects although the nup170Δ mutant has ~60% of wild type Ty1 cDNA levels but a 17-fold reduction in transposition mobility (Figure 2.1B and 2.5, Table 2.2).
Figure 2.5 Analysis of endogenous Ty1 cDNA in NPC mutant strains. (A) (B) Southern blots of yeast genomic DNA extracted from the indicated mutants and wild type (WT) after growth for 2 days at 20°C then digested with the PvuII restriction enzyme. Endogenous Ty1 elements and cDNA were detected by a 1517 bp radiolabelled C-terminal fragment of the Ty1 element (PvuII/SnaBI). Image Quant software was used to measure the ratio of Ty1 cDNA to an endogenous Ty1 element (Ty1 control). The spt3Δ strain was used as a negative control for cDNA production.

2.3.4 Ty1 IN can enter the nucleus of NPC mutant strains

To determine if the transposition defect observed in the NPC mutant strains was due to a Ty1-IN nuclear localization defect I analyzed Ty1-IN localization in the mutant strains by fluorescence microscopy. Dr. Lina Ma constructed a Ty1-IN expression plasmid with a PGK1 promoter and the fluorescent mCherry tag appended to the C-terminus of Ty1-IN. The constitutively expressed PGK1 promoter is not among the list of genes with inducible promoters, such as the GAL genes, that have been shown to interact directly with NPCs (234,245), therefore Ty1-IN-mCherry expression should not be affected by the NPC mutation. NPC mutant strains
carrying the Ty1-IN-mCherry plasmid were grown to log phase followed by nuclear staining and live cell fluorescence imaging. All of the eleven NPC mutants with altered Ty1 mobility demonstrated localization of Ty1-IN into the nucleus suggesting that none of these NPC components are critical for Ty1-IN nuclear entry (Figure 2.6).
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Figure 2.6 Ty1-IN-mCherry localizes to the nucleus in NPC mutant strains. Live cell imaging of the indicated yeast strains carrying a plasmid expressing mCherry fluorescently labeled Ty1-IN (red) was performed on a Zeiss Axio Observer inverted microscope. Yeast strains were grown to log phase overnight at 25°C and were stained with Hoechst 33342 DNA dye for nucleus visualization (blue). Strains transformed with the empty vector were used as a negative control. Images were taken in the differential interference contrast (DIC), Hoechst (blue), and mCherry (red) channels with a 40X objective. Images from the Hoechst and mCherry channels are superimposed (overlay) in the far right column.

2.3.5 Ty1 insertion requires NPC subunits.

Since all but one (nup60Δ) of the Nup mutants with low Ty1 mobility have relatively normal (pom34Δ) or higher (nup53Δ, nup192-25) Ty1 mRNA, Gag or cDNA levels or levels higher than predicted from the reduction in Ty1 mobility (sec13-1, mlp1Δ, nup159-1, nup170Δ, nup188Δ) in addition to proper Ty1-IN nuclear import, I hypothesized that the reduction in Ty1 mobility is due to an inability of the Ty1 element to insert into the genome. To test my hypothesis, I induced endogenous transposition of pre-existing chromosomal Ty1 elements by growing wild type and Nup mutant strains at 20°C for 3 days, isolating genomic DNA and analyzing Ty1 insertion events upstream of the SUFI6 glycine tRNA gene [tG(GCC)C] on chromosome III, which has previously been shown to be a transposition hotspot (30). The SUFI6 locus is located within the pericentromere of chromosome III which is a 20-50kb region to the left and right side of the ~125 bp centromere that is enriched for the cohesion complex which holds sister chromatids together during mitosis (247). tRNA genes have a role in the enrichment of condensin at pericentromeres, therefore I postulated that Ty1 element insertion upstream of tRNA genes in the pericentromere may have different requirements from Ty1 element insertion upstream of tRNA genes located on the chromosome arm (248). The serine tRNA gene SUP61 [tS(CGA)C] is located outside the pericentromere on the arm of chromosome III. Interestingly,
SUP61 is recruited to and actively transcribed at NPCs during M phase, evoking a potential role for the Nups in targeting Ty1 element insertion upstream of SUP61 (82).

I designed a primer complementary to either the SNR33 or BUD31 locus on chromosome III adjacent to SUF16 and SUP61, respectively (Figure 2.7). A second primer that hybridizes within the Ty1 element was used for both PCR assays (Figure 2.7). Each NPC mutant strain was assayed in triplicate with a spt3Δ strain used as a negative control for Ty1 replication. Quantification of Ty1 integration was achieved by software that calculated the total band intensity for each strain as described (240). PCR amplification of CPR7 located on chromosome X was used as a control to ensure that PCR competent genomic DNA was present. Due to the position of the primers, periodic insertion of Ty1, is detected in a window of ~750-1500 bp upstream of SUF16 and SUP61 in a ladder due to nucleosome positioning. Although Ty1 elements can insert in either orientation (31), this PCR assay only detects Ty1 elements inserted in the forward direction.

**Figure 2.7** Schematic of Ty1 insertion Assay. Genomic DNA was extracted from yeast strains and subjected to PCR to amplify Ty1 insertion upstream of either SUF16 or SUP61 (pink) with a primer that binds to Ty1 (TyB-out) and either a primer that binds to SNR33 (orange; SNR33-out) or BUD31 (orange; BUD31-out). Both tRNA genes are located on chromosome III (Chr III). SUF16 is 155 bp from SNR33 and SUP61 is 276 bp from BUD31. Ty1 inserts in a ~1 kb window upstream of these tRNA genes.
When compared to the wild type strain, Ty1 insertion upstream of both the **SUF16** and **SUP61** tRNA genes was almost negligible in the 8 of the 9 Nup mutant strains with reduced Ty1 mobility: **nup53Δ, nup60Δ, nup159-1, nup170Δ, nup188Δ, nup192-15, pom34Δ, and sec13-1** (Figure 2.8 and 2.9, Table 2.2). Three NPC mutants had different Ty1 insertion frequencies depending on the locus tested. The **mlp1Δ** mutant, which had ~3-fold reduced Ty1 mobility compared to wild type (Figure 2.1B and Table 2.2) had 0.83 Ty1 insertion frequency upstream of **SUF16** but only 0.2 Ty1 insertion frequency upstream of **SUP61** when compared to wild type (Figure 2.8A and 2.9A, Table 2.2). The **mlp2Δ** strain which had significantly increased Ty1 mobility (~2.3-fold compared to wild type) had a 2.1-fold increased Ty1 insertion upstream of **SUF16** but 63% of wild type Ty1 insertion upstream of **SUP61** (Figure 2.8A and 2.9A, Table 2.2). In corroboration with the ~3.5-fold increase in Ty1 mobility, the **nup120Δ strain** had increased Ty1 insertion upstream of both tRNA genes, however the increase was more pronounced upstream of **SUF16** (9.8-fold) compared to **SUP61** (2.4-fold) (Figure 2.1B, 2.8B, and 2.9B, Table 1). As well, in 3 independent cultures, the majority of the **nup120Δ** Ty1 insertions occurred in one nucleosome position upstream of the **SUF16** gene but did not favour a particular nucleosome upstream of the **SUP61** gene (Figure 2.8B asterisk and Figure 2.9B).

The Ty1 insertion data suggests that a subset of Nups are absolutely required for Ty1 element insertion upstream of pericentromeric and chromosome arm tRNA genes, whereas the Mlp1 nuclear basket protein may be more important for insertion at tRNA genes located on chromosome arms. As well, the Mlp2 nuclear basket protein and the Nup120 outer ring protein are more inhibitory to Ty1 element insertion upstream of pericentromeric tRNA genes than chromosome arm tRNA genes.
Figure 2.8 Ty1 insertion upstream of the SUF16 tRNA hotspot is severely impaired in multiple NPC mutant strains. The indicated strains (A) wild type (WT), spt3Δ, nup159-1 mlp1Δ, sec13-1, mlp2Δ (B) WT, spt3Δ, nup192-15, pom34Δ, nup170Δ, nup120Δ and (C) WT, spt3Δ, nup60Δ, nup188Δ, nup53Δ were each grown in triplicate for 3 days at 20°C. Genomic DNA was extracted, then used for PCR analysis with one primer located in the Ty1 element and the other located adjacent to the SUF16 locus. Ty1 insertion events are represented by a ladder of bands coincident with nucleosome positioning. The lower panel is a control PCR of the CPR7 locus to demonstrate the presence of PCR-competent genomic DNA in each sample. Image Lab software was used to quantify the intensity of Ty1 insertion events in each lane, averaged for each yeast strain and compared to wild type which was set to a level of 1.0. An asterisk (*) denotes a preferred Ty1 element insertion site in the nup120Δ strain.
Figure 2.9 Ty1 insertion upstream of the SUP61 tRNA arm locus is dependent on the NPC. The indicated strains (A) wild type (WT), spt3Δ, nup159-1 mlp1Δ, sec13-1, mlp2Δ (B) WT, spt3Δ, nup192-15, pom34Δ, nup170Δ, nup120Δ and (C) WT, spt3Δ, nup60Δ, nup188Δ, nup53Δ were each grown in triplicate for 3 days at 20°C. Genomic DNA was extracted, then used for PCR analysis with one primer located in the Ty1 element and the other located adjacent to the SUP61 locus. The range of Ty1 insertion events is marked to the right of each gel. The lower panel is a control PCR for the CPR7 locus to demonstrate the presence of genomic DNA in each sample. Image Lab software was used to quantitate the intensity of Ty1 insertion events in each lane, averaged for each yeast strain and compared to WT set to a level of 1.0.
Mlp1 interacts with the Nuclear abundant poly(A) RNA-binding protein 2 (Nab2) which is an essential protein involved in mRNA poly(A) tail length control, mRNP formation, and nuclear mRNP export (243,249-252). Interestingly, Nab2 also occupies all RNA Pol III transcribed genes and therefore may have a role in Ty1 transposition (253). The N-terminus of Nab2 contains a hydrophobic patch centred on 2 phenylalanine residues (Phe72 and Phe73) (252). Phe73 is absolutely required for the interaction between Nab2 and Mlp1 whereas mutating Phe72 still allows Nab2 to bind Mlp1, albeit with lower affinity (252). I tested a set of NAB2 mutants (nab2ΔN, nab2F73D, nab2F73D) that eliminate the Mlp1-Nab2 interaction (243) for defects in Ty1 insertion at tGLY but found that all of the NAB2 mutants had Ty1 insertion levels that were comparable to wild type (Figure 2.10). The NAB2 mutants and wild type strain did not exhibit the periodic integration pattern, as Ty1 insertion depends on nucleosome positioning, commonly observed when monitoring Ty1 insertions. This could be caused by growing the yeast strains in minimal media to maintain the plasmids. Alternatively, the expression of NAB2 from a plasmid may affect nucleosome positioning at tRNA genes since Nab2 occupies Pol III genes. I also analyzed Ty1 tGLY insertion levels in a nab2-34 essential ts mutant (253). Although the conditions I used allow for Ty1 transposition in the S288C strain background, in the nab2-34 isogenic wild type strain (W303) very little Ty1 insertion was detected (Figure 2.10). However, I detected a 6-fold increase in Ty1 insertion in the nab2-34 mutant suggesting that Nab2 may be inhibitory to Ty1 transposition in the W303 background (Figure 2.10).
Figure 2.10 Mutating the RNA binding protein Nab2 does not affect Ty1 insertion upstream of tGLY genes. (A) Schematic representation of the Nab2 protein with the N-terminal domain (residues 4-97) and F72 and F73 residues identified. (B) Ty1 insertion levels monitored in Nab2 N-terminal (residues 4-97; nab2ΔN) and phenylalanine to aspartic acid mutants (nab2F72D and nab2F73D) (243). CPR7 control PCR is shown in the lower panel. (C) PCR amplified Ty1 insertions upstream of 16 tGLY genes monitored in a nab2-34 mutant yeast strain. spt3Δ is a negative control for Ty1 replication. For (B) and (C) Genomic DNA was extracted from strains grown for 3 days at 20°C to induced endogenous Ty1 transposition and quantified using Image lab software and normalized relative to wild type.
2.3.6 Ty1 is mistargeted in the absence of Nup proteins

The HIS3 marked Ty1 element mobility assay (Figure 2.1) cannot distinguish between targeting of Ty1 elements upstream of Pol III-transcribed genes and mis-targeting of Ty1 elements into the genome and only depends on insertion into a locus that does not impede HIS3 gene expression. Therefore, I assessed Ty1 insertion in Nup deletion strains that had wild type levels of Ty1 mobility (*nup84Δ, nup133Δ, nup59Δ, nup42Δ, ndc1-4, pom152Δ, nup100Δ, and nup2Δ, Figure 2.1B). I first analyzed insertion upstream of *SUF16* and, surprisingly, noticed that all Nup mutants with wild type Ty1 mobility were impaired for Ty1 insertion upstream of the *SUF16* locus (Figure 2.11). Deletion of *NUP84* and *NUP133*, both members of the Nup84 outer ring subcomplex, caused Ty1 insertion at *SUF16* to diminish by 50% and 30%, respectively (Figure 2.11A and Table 2.2). In addition, Ty1 insertion in the *nup133Δ* mutant appeared to favour the first insertion site (asterisk, Figure 2.11A) and one of the *nup84Δ* cultures had an extra insertion site not detected in the wild type strain (arrow, Figure 2.11A). Deletion of the Nup100 central FG-repeat Nup and Nup59 inner ring Nup, caused a ~6-fold and ~5-fold reduction, respectively, in Ty1 insertion upstream of *SUF16* compared to wild type (Figure 2.11B and Table 2.2). The *ndc1-4* and *pom152Δ* transmembrane Nup mutants had a ~25-fold and ~5-fold reduction, in Ty1 insertion upstream of *SUF16* compared to wild type (Figure 2.11A and 2.11B, Table 1). Deletion of the cytoplasmic FG Nup, Nup42, had the most severe effect on Ty1 insertion with a ~50-fold reduction (Figure 2.11A and Table 2.2). I observed similar results upon testing Ty1 targeting upstream of the *SUP61* serine tRNA gene (Figure 2.12). There were two notable exceptions - *nup133Δ* had a 1.6-fold increase (compared to a 30% reduction for *SUF16*) and *nup84Δ* had an 11-fold decrease in *SUP61* targeting (compared to a 2-fold decrease for *SUF16*) when compared to wild type (Figure 2.12A and Table 2.2).
NPC mutants with wild type Ty1 mobility have defects in Ty1 insertion upstream of the SUF16 hotpot locus. The following strains with wild type Ty1 mobility: (A) wild type (WT), nup84Δ, nup133Δ, nup42Δ, ndc1-4. (B) WT, nup100Δ, nup59Δ, pom152Δ and (C) WT, nup2Δ were grown for 3 days at 20°C in triplicate. spt3Δ serves as a negative control. Genomic DNA was extracted then used for PCR analysis with a Ty1 primer and a primer adjacent to SUF16. The CPR7 panel is a control PCR to demonstrate the presence of genomic DNA in each sample. Image Lab software was used to quantitate the intensity of Ty1 insertion events in each lane, averaged for each yeast strain and compared to WT set to a level of 1.0. An arrow (→) denotes a novel insertion site in the nup84Δ strain and an asterisk (*) denotes a preferred insertion site in the nup133Δ strain.

Figure 2.11 NPC mutants with wild type Ty1 mobility have defects in Ty1 insertion upstream of the SUF16 hotpot locus. The following strains with wild type Ty1 mobility: (A) wild type (WT), nup84Δ, nup133Δ, nup42Δ, ndc1-4. (B) WT, nup100Δ, nup59Δ, pom152Δ and (C) WT, nup2Δ were grown for 3 days at 20°C in triplicate. spt3Δ serves as a negative control. Genomic DNA was extracted then used for PCR analysis with a Ty1 primer and a primer adjacent to SUF16. The CPR7 panel is a control PCR to demonstrate the presence of genomic DNA in each sample. Image Lab software was used to quantitate the intensity of Ty1 insertion events in each lane, averaged for each yeast strain and compared to WT set to a level of 1.0. An arrow (→) denotes a novel insertion site in the nup84Δ strain and an asterisk (*) denotes a preferred insertion site in the nup133Δ strain.
Figure 2.12 NPC mutants with wild type Ty1 mobility have defects targeting Ty1 elements upstream of the SUP61 tRNA arm locus. The following strains with wild type Ty1 mobility: (A) wild type (WT), nup84Δ, nup133Δ, nup42Δ, ndc1-4, (B) WT, nup100Δ, nup59Δ, pom152Δ and (C) WT, nup2Δ were grown for 3 days at 20°C in triplicate. spt3Δ as a negative control. Genomic DNA was extracted, then used for PCR analysis with a Ty1 primer and a primer adjacent to SUP61. The lower panel is a control PCR of the CPR7 locus to demonstrate that genomic DNA is present in each sample. Image Lab software was used to quantitate the intensity of Ty1 insertion events in each lane, averaged for each yeast strain and compared to WT set to a level of 1.0.
Removing two of the nuclear basket Nups did not affect Ty1 mobility but did impact the efficiency of Ty1 targeting upstream of tRNA genes. Deletion of Nup2, a mobile Nup that is known to interact with chromatin and tRNA genes (82,122,160,244), resulted in wild type Ty1 mobility but reduced Ty1 targeting upstream of \textit{SUF16} to 21% of wild type levels (Figure 2.11C and Table 2.2). Deletion of Nup2 also reduced Ty1 targeting upstream of the \textit{SUP61} gene ~4-fold compared to wild type Ty1 levels (Figure 2.12C and Table 2.2).

### 2.4 Discussion

In the mid 1980’s Blobel postulated the “gene gating” hypothesis, asserting that actively transcribed genes are tethered at NPCs to allow transcription and mRNA export to be coupled events (130). This hypothesis sparked exploration of transport-independent functions of NPCs resulting in mounting evidence supporting the influence of NPCs on chromatin structure and organization which in turn can affect gene regulation in yeast and higher order eukaryotes (234). Active transcription of at least a subset of genes has been demonstrated to occur at NPCs in a variety of eukaryotic organisms (131,137,143,151,155,159,160,254-257). Of particular importance to this study is that tRNA genes, the preferred genomic target of Ty1, are actively transcribed at NPCs during the peak of tRNA synthesis (M phase of the cell cycle) (82).

Moreover, three studies have reported that mutations of a sub-set of Nups cause nuclear retention of intron-containing tRNAs (258-260). Given this functional link between Nups, chromatin, and transcription, I explored the role of Nups in Ty1 element integration into the genome.

In this study, I surveyed a panel of 19 NPC mutants for quantitative defects in Ty1 mobility and identified 11 mutants (\textit{mlp1A, mlp2A, nup53A nup60A nup120A, nup159-1, nup170A, nup188A, nup192-15, pom34A, sec13-1}) with a statistically significant difference in
Ty1 mobility (Figure 2.1B). The majority of these Nups were subunits of the Nup170 inner ring complex and the nuclear basket, however Nup mutants from all NPC substructures impacted Ty1 transposition (Figure 2.1, Table 2.2). Most Nup mutants with reduced Ty1 mobility also displayed decreased insertion of Ty1 elements upstream of the SUF16 and SUP61 tRNA genes (Figures 2.1B, 2.8, and 2.9, Table 1). I was surprised to discover, however, that Nup mutants with wild type levels of Ty1 mobility also had defects in Ty1 targeting upstream of the SUF16 and SUP61 genes (Figures 2.1B, 2.11, 2.12, Table 2.2). Included in this group is the nuclear basket nup2Δ mutant and due to the proximity of the nuclear basket Nups to chromatin I will focus my study on the nuclear basket proteins in Chapter 3. I have demonstrated that deletion of individual nuclear basket genes affects Ty1 mRNA expression (nup60Δ), Ty1 mobility (nup60Δ, mlp1Δ, mlp2Δ) and Ty1 targeting (nup2Δ, nup60Δ, mlp1Δ, mlp2Δ) (Figures 2.1B, 2.8, and 2.9).

This is the first study to demonstrate that the Ty1 promoter may be regulated by the NPC. Ty1 mRNA expression was reduced 5-fold in the nup60Δ strain (Figure 2.3A). Multiple studies have demonstrated that Nup60 can interact with chromatin. For example, ChIP experiments have demonstrated that Nup60 can interact with chromatin (122,261) including tRNA genes (82). Genome-wide ChIP studies demonstrated that Nic96, Nup116, Nup2, and Nup60 associate with highly transcribed genes (245). If Nup60 indeed tethers the Ty1 element to the NPC, it would be interesting to determine if Nup60 interacts with any of the nine transcription factors known to bind to the Ty1 promoter. Of these nine transcription factors, Rap1 and Gcr1 interact with the Nup84 subcomplex (257). Rap1 also interacts with Nup170 (128). However a Nup84 complex mutant (sec13-l) and the nup170Δ mutant both had wild type levels of Ty1 mRNA suggesting that these Nup interactions are not required for Ty1 transcription (Figure 2.3A). Deletion of Nup60 can result in mislocalization of telomeres perhaps through the loss of interaction with the
silencing protein Sir3 which is also mislocalized in nup60Δ cells (123). Cells lacking Nup60 have also been shown to be defective in their ability to silence a URA3 reporter within a partially depressed HML locus presumably as a result of a loss of interaction with Nup2 (122).

I observed defects in Ty1 mobility in a subset of mutants from every NPC complex, except for the one central FG Nup I tested (nup100Δ) (Figure 2.1B, Table 1). Every mutant I tested in the Nup170 inner ring complex, except for nup59Δ, had greatly reduced Ty1 mobility and negligible insertion of Ty1 elements upstream of the SUF16 or SUP61 tRNA genes (Figures 2.1B, 2.8, and 2.9, Table 2.2). Nup170 interacts with genomic regions containing ribosomal protein and subtelomeric genes to mediate nucleosome positioning and transcription repression (128). Ty1 insertion in an RNA pol III mutant has been shown to be redirected to subtelomeric loci (230) which could involve Nup170, given its role in chromatin organization of this region.

Mutation of the Nup84 outer ring complex resulted in variable Ty1 mobility defects with an 8-fold reduction in the sec13-1 mutant, a 3.5-fold increase in the nup120Δ mutant and wild type Ty1 mobility in the nup84Δ and nup133Δ strains (Figure 2.1B, Table 2.2). All Nup84 outer ring mutants, however, displayed defects in Ty1 targeting upstream of the SUF16 and SUP61 genes (Figures 2.8A-B, 2.9A-B, 2.11A and 2.12A). As predicted from the increase in Ty1 cDNA and Ty1 mobility, the nup120Δ mutant had a ~10-fold increase in Ty1 targeting mostly in the second nucleosome upstream of the SUF16 gene (asterisk, Figure 2.8B). I also detected ~2.4-fold increased Ty1 insertion upstream of the SUP61 gene in the nup120Δ mutant but no specific nucleosome position was targeted (Figure 2.9B). This may reflect a difference in NPC interactions with the pericentromere (where SUF16 is located on Chr. III) versus the chromosome arm (where SUP61 is located on chr III).
Interestingly, Ty1 mRNA in the *nup120Δ* mutant was increased by 60% above wild type levels but Ty1 Gag protein levels in the *nup120Δ* mutant were similar to wild type levels (Figure 2.3 and Table 2.2). Both Nup170 inner ring mutants *nup188Δ* and *nup192-15* generated twice as much Ty1 mRNA as the wild type strain but Ty1 mobility and Ty1 insertion upstream of *SUF16* and *SUP61* was severely impaired in these strains (Figure 2.3A, 2.1B, 2.8B-C, 2.9B-C). This suggests that the increase in Ty1 mRNA in the *nup120Δ* might not account for the observed significant increase in Ty1 mobility and insertion. Instead, Nup120 could regulate Ty1 cDNA production as the *nup120Δ* mutant strain had a 7-fold increase in Ty1 cDNA levels compared to wild type (Figure 2.5A and Table 2.2). Perhaps clustering of NPCs, which occurs as a result of *NUP120* mutation (183), causes clustering of transposition-competent Ty1 VLPs or PICs at the NE that enhances Ty1 mobility and insertion. Conversely, deletion of *NUP120* could result in the mislocalization of an unknown Ty1 restriction factor. Ty1 replication studies with yeast strains carrying *NUP120* mutations that do not cause NPC clustering will likely shed light on the role of Nup120 in the Ty1 life cycle.

Two members of the Nup84 subcomplex, Nup84 and Nup133, have been previously identified as Ty1 host factors in large-scale genetic screens (176-178), and have been shown to associate with chromatin (120,123,124,169,171). The Nup84 subcomplex physically interacts with long tracts of CAG repeats that are known to interfere with DNA replication (171). The interaction of Nup84 and CAG repeats represses the fragility and instability of CAG repeats (171). Also, recruitment of DNA DSBs to the NPC require the presence of the Nup84 subcomplex (169). As well, mutations in members of the Nup84 complex have been shown to alter telomere organization (120,123,124). Taken together, the Nup84 subcomplex could
influence Ty1 integration as members of this complex are associated with essential genomic processes.

Another interesting observation was that in the \textit{nup84}\textsuperscript{Δ} mutant, Ty1 elements were targeted into a non-canonical position in the second nucleosome (arrow, Figure 2.11A) and in the \textit{nup133}\textsuperscript{Δ} mutant Ty1 elements were primarily targeted to the first nucleosome (asterisk, Figure 2.11A). It is important to mention that deletion mutations of \textit{NUP84}, \textit{NUP133} or \textit{NUP120} cause NPC clustering (183,184,262-264) while the \textit{NUP188} deletion mutant has defects in NE morphology (265). Global structural changes in NPCs could in turn affect chromatin organization and the positioning of actively transcribed genes which may explain the alterations of Ty1 insertion in these Nup mutant strains.

In the next Chapter I will focus on the nuclear basket Nups which, given their proximity to chromatin and previously defined roles in chromatin organization and transcription, are likely to direct Ty1 site selection.
Chapter 3:

The Nuclear Basket Dictates Ty1 Integration

3.1 Introduction

The yeast NPC is composed of multiple subcomplexes including the nuclear basket. The proteins of the nuclear basket converge to form a ring like structure that projects from the NPC into the nucleus (113). The S. cerevisiae nuclear basket consists of five proteins: Nup60, Nup1, Nup2, and the two myosin-like proteins Mlp1 and Mlp2 (113). Nup60, Nup1 and Nup2 are also classified as FG (phenylalanine-glycine) Nups which are characterized by FG repeat sequences required for cargo transport (109). In vertebrates, the bulk of the nuclear basket is composed of the translocated promoter region (Tpr) protein (266) which is a homolog of the yeast Mlp1 and Mlp2 proteins (267). Vertebrate nuclear baskets are composed of two additional proteins: Nup153 which may be a homolog for both Nup1 and Nup60 in yeast, and Nup50 which is a Nup2 homolog (113).

Based on my findings in Chapter 2 and given that the NPC’s nuclear basket substructure (Nup1-Nup2-Nup60-Mlp1-Mlp2) has been strongly linked to genomic processes such as chromatin organization, transcription and mRNA export (147,268,269) I chose to focus on these Nups, and protein complexes that interact with the nuclear basket, for further study.

Nup2 is a mobile nuclear basket protein that binds Nup60 and soluble transport factors such as Kap60 and Kap95 (270-273). Deletion of Nup60 causes mislocalization of Nup2 and inhibits Nup2’s role in transport (270). Although primarily located on the nuclear basket through a physical interaction with Nup60, Nup2 is also present in the nucleoplasm (270,271,274,275).
The Nup2 protein is composed of three domains: the N-terminal domain (residues 1-172), the central domain (residues 182-546) and the C-terminal domain (residues 556-720) (274). The Nup2 N-terminal domain interacts with Kap60 and Nup60 (273,275). The central domain contains 15 FxFG repeat domains necessary for transport (109) and the C-terminal domain contains a Ran binding domain (RBD) required for nucleo-cytoplasmic transport and NPC localization of Nup2 (270,276). Nup2 interacts with chromatin boundary elements which are DNA sequences that prevent enhancer–promoter interactions and prevent active genes from repression by nearby silent loci (122). Interestingly, genome-wide studies have shown that Nup2 is associated with transcriptionally active genes, including tRNA genes (82,122,133,160,245). Recently, a role for Nup2 in meiotic progression has also been discovered (277).

Nup60 is a nonessential NPC protein that is exclusively located at the nuclear basket (113). Nup60 can serve as a docking site for Kaps as well as Nup2 and the Mlp nuclear basket proteins (271,278). Structurally, Nup60 contains FG repeats and an RNA recognition motif (RRM) fold composed of a two-layer α/β sandwich secondary structure (279). Nup60 also contains an N-terminal amphipathic helix (AH) and an adjacent α-helical region (HR), which are required to maintain NPC integrity and promote membrane curvature via insertion into the lipid bilayer (280). Along with the AH and HR regions, Nup60 contains target lysine residues for SUMOylation and ubiquitylation (281). Deletion of Nup60 causes nuclear retention of poly(A)+ RNA thus Nup60 has been proposed to function in mRNA surveillance and processing (164,282). Although the major role of Nup60 seems to be recruitment and tethering of the Mlp proteins and Nup2, Nup60 has been shown to interact with active genes (245), including tRNA genes (82), and telomeres (123). Also, Nup60 physically associates with chromatin associate proteins such as the Asf1 histone chaperone (261).
The Mlp proteins are tethered to the NPC by a coiled-coil N-terminal domain and a globular C-terminal domain that binds to mRNA export factors (251,267,283). The majority of Mlp proteins colocalize with NPCs, but are excluded from NPCs near the nucleolus (164). Mlp1 and Mlp2 are associated with the NPC through an interaction with Nup60 as deletion of NUP60 leads to mislocalization of both Mlp proteins and release of telomeres from the nuclear periphery to the nuclear interior (123,164). Furthermore, a double deletion of Mlp1 and Mlp2 can lead to loss of telomere anchoring at the nuclear periphery and loss of telomere repression (124). Also, an mlp1Δmlp2Δ yeast strain has been shown to have extended telomeres suggesting that both proteins could play a regulatory role in telomere length control (125). Intron-containing mRNAs are retained in the nucleus by Mlp1 as deleting Mlp1 impairs mRNA retention but not splicing (164). Along with the telomere interaction, both Mlp proteins interact with active genes (245) and Mlp1 is required for NPC tethering of GAL10 and HSP104 (137). Intron-containing mRNAs are retained in the nucleus by Mlp1 as deleting Mlp1 impairs mRNA retention but not splicing (164). Mlp1 also physically interacts with the chromatin associated complexes SAGA (284), and the mRNA exporter Nab2 (251). On the other hand, Mlp2 directly binds the spindle pole body (SPB) and is required for localization of SPB components with loss of Mlp2 leading to smaller SPBs and mitotic disruption (285).

Of the five nuclear basket proteins only Nup1 was not previously studied in Chapter 2. Nup1 is located on the nuclear side of the yeast NPC at the nuclear basket substructure and is required for mRNA export (101,151). Removing Nup1 causes temperature-dependent structural changes in the nucleus and defects in nucleo-cytoplasmic transport (286,287). Nup1, similar to Nup60, contains AH and HR domains that are required for NPC membrane curvature (280). Nup1 physically interacts with the Sac3 component of TREX-2 and is required for NPC tethering
of the TREX-2 complex (151). *GAL* genes are also tethered at NPCs via an interaction with Nup1 (152). Interestingly, Nup1 Cdk phosphorylation is likely required for NPC tethering of the active *GALI* and *INO1* genes as mutation of Nup1 phosphorylation sites disrupts targeting of *INO1* and *GALI* to the nuclear periphery (140).

The nuclear basket performs numerous cellular functions along with its role in transport. Here I studied the influence of nuclear basket proteins on Ty1 replication and insertion site selection. In addition to analyzing Ty1 element mobility and genome insertion in complete gene deletions of nuclear basket proteins I also acquired Nup1, Nup2 and Nup60 mutants that retained partial activity. I found that removal of the Nup60 AH and HR regions, which have been recently shown to be required for NE membrane curvature (280), allows for Ty1 expression and targeting upstream of tRNA genes but instead alters the nucleosome pattern of Ty1 element integration. I show that a defect in the typical nucleosome pattern of Ty1 integration also occurs when the AH and HR regions of Nup1, also required for NE membrane curvature, are deleted (280). I find that removal of the RBD from Nup2 eliminates Ty1 targeting upstream of *tGLY* genes. Finally, I show that when nuclear basket proteins are deleted, Ty1 elements are re-targeted to subtelomeric regions, similar to an Rpc40 mutant that no longer interacts with Ty1-IN (7). Taken together with the well-defined role of actively transcribed tRNA genes relocating to NPCs, my results suggest that the nuclear basket couples cellular processes such as tRNA expression, Ty1 expression and Ty1 integration.
3.2 Materials and Methods

3.2.1 Yeast Strains, Plasmids and Media

Both non-essential deletion (Δ) and essential ts mutant yeast strains were previously engineered (8,235). Yeast strains used in this study are listed in Table 3.1. *S. cerevisiae* strains were grown in YPD, SC media or SC media lacking URA, LEU or HIS and supplemented with combinations of Dex, raffinose (Raf), and/or galactose (Gal) as required. The NUP1 (pRS313-NUP1-mCherry, pRS313-nup1Δ1-32-mCherry, pRS313-nup1Δ85-123-mCherry) and NUP60 (pRS315-NUP60-mCherry, pRS315-nup60Δ1-47-mCherry, pRS315-nup60Δ48-162-mCherry, pRS315-nup60Δ1-162-mCherry) AH and HR mutant plasmids were a gift from Dr. Köhler (280). The Nup1 (pRS413-Nup1, pRS413-Nup1^{S11A/T159A/S161A}) and Nup2 (pRS413-Nup2, pRS413-Nup2^{T361A}) phosphorylation site mutant plasmids were kindly gifted by Francesc Posas (144). The Ty1 expression plasmid (pBDG922) that was used in this study for Ty1 mobility assays was a gift from Dr. Garfinkel (24,27). Ty1 insertion PCR with the ΔFG nuclear basket mutants was performed with pJBe376 (a kind gift from Dr. Boeke) which is a 2 micron plasmid that expresses a Ty1 element from a GAL1 promoter.

Table 3.1 Yeast strains used in Chapter 3

<table>
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<th>Strain</th>
<th>Description</th>
<th>Source</th>
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3.2.2 Ty1 Gag Westerns

Yeast strains were grown on selection plates for 3 days at 20°C. For each strain, colonies were transferred to 10mL of YPD and grown overnight at 20°C to log phase (OD$_{600}$ 0.5-1.0) in duplicate. Preparation of WCEs and western blots were performed as described in section 2.2.4. Briefly, samples were loaded on a SDS-PAGE gel then transferred to a PVDF or nitrocellulose membrane. Membranes were probed with anti-Gag sera and anti-GAPDH then incubated with IRDye® secondary antibodies. Ty1 Gag protein levels (p49 and p45) were quantified relative to GAPDH levels.

3.2.3 Ty1 Mobility Quantification Assay

This assay was performed as described in (237) and section 2.2.2. Briefly, strains were transformed with a Ty1-his3-AI reporter plasmid (pBDG922 from Dr. David Garfinkel). Cells were grown for 5 days at 20°C in SC-URA media then plated on SC-URA-HIS plates and SC-URA plates. Ty1 transposition frequency was calculated as # colonies on SC-URA-HIS/10$^7$ divided by # colonies on SC-URA/200.

3.2.4 Quantitative PCR

qPCR was performed and analyzed as described in section 2.2.3 (231). Briefly, cDNA was synthesized from RNA isolated from yeast strains grown to log phase. A standard two-hour comparative PCR analysis was performed using a 7500 Real Time PCR system to measure Ty1 mRNA and tRNA (tLEU, tLEUdenovo, and tGLY) levels. qPCR analysis was performed using the ΔΔC$_T$ method, with TAF10 used as an internal control.
3.2.5 **SUF16, SUP61 and tGLY Ty1 Integration Assay**

This assay was performed as described in section 2.2.8 (237). Yeast strains were grown on plates for 3 days at 20°C then colonies were transferred to liquid media and grown for 3 days at 20°C to induce endogenous Ty1 transposition. For the ΔFG Nup mutants (*nup1ΔFxFG, nup2ΔFxFG, nup60ΔFxF, nup1ΔFxFG nup2ΔFxFG, nup1ΔFxFG nup2ΔFxFG nup60ΔFxF*), each strain was transformed with a pGAL-TyH3mHIS3AI-URA3 plasmid (pJBe376) and induced in Gal media for 24h as described (240). Cells were lysed in lysis buffer with glass beads and genomic DNA was extracted using phenol-chloroform. Ty1 insertions upstream of the *SUF16* chromosome III was amplified using the *SNR33* out and the TyB out primers (237). Ty insertion upstream of 16 copies of the glycine tRNA gene (GCC anticodon), collectively called *tGLY*, were amplified with OVM807 (5’-GGATTTTACCACTAAACCACTT-3’) and TyB out (289). The presence of quality genomic DNA was verified by PCR amplification of the *CPR7* locus. Each yeast strain was assayed in triplicate.

3.2.6 **PCR Assay for Ty1 Insertion Near Chromosome Ends**

This assay was performed as previously described (231) with 1.5 µg of genomic DNA, the TyB out primer (OVM889), and either one of the following primers: OSC66, 5’-

CCAAGGATCTAGGTAGGCTTTGAGAA-3’ (Chr XIV left end 13,634–13,669 bp (*SNZ2*) and Chr VI left end 11,739–11,765 bp (*SNZ3*)); OSC68, 5’-

GACATGGGCCCCTGTTGTTATATTGT-3’ (Chr IV left end 12,021–12,047 bp (*HXT15*) and Chr X right end 733,753–733,779 bp (*HXT16*)). The PCR cycling parameters were identical to the Ty1 integration assay in section 2.2.8 apart from the final step which was 15 cycles at 96°C, 30 sec; 60°C, 30 sec; 63°C, 1 min.
3.3 Results

3.3.1 Deletion of nuclear basket Nups impairs Ty1 insertion upstream of 16 glycine tRNA genes

Given that the NPC’s nuclear basket substructure (Nup1-Nup2-Nup60-Mlp1-Mlp2) is located on the nuclear side of the NPC and has been strongly linked to genomic processes such as chromatin organization, transcription, mRNA export and tRNA gene expression (147,268,269) I chose to focus on these Nups and protein complexes that interact with the nuclear basket, for further study.

I analyzed Ty1 insertion using the TyB-out primer that hybridizes to Ty1 and a tGLY primer that hybridizes to all 16 glycine tRNA genes in total (collectively termed tGLY; GCC) in four of the nuclear basket deletion mutant strains (mlp1Δ, mlp2Δ, nup2Δ, and nup60Δ). The nup60Δ, mlp1Δ and nup2Δ yeast strains had a 100-fold, ~3-fold, and ~5-fold decrease in Ty1 insertion upstream of tGLY respectively (Figure 3.1A). Ty1 insertion upstream of tGLY was at 70% of wild type levels in the mlp2Δ strain and extra Ty1 insertion sites (indicated by arrows) were detected that were not observed in the wild type or other nuclear basket mutant strains (Figure 3.1A). The elimination of Ty1 insertion upstream of all tGLY genes in the nup60Δ is consistent with the lack of Ty1 insertion upstream of SUF16 (tGLY) and SUP61 (tSER) in the nup60Δ mutant and likely because of the reduction of Ty1 gene expression (Figure 3.1A, 2.8C, 2.9C and 2.3A). However, the mlp1Δ and mlp2Δ strains vary in Ty1 insertion levels at different tRNA genes. In the mlp1Δ strain that had a 3-fold reduction in Ty1 mobility (~3-fold, Figure 2.1B), Ty1 insertion decreased by 17%, 80%, and 34% upstream of SUF16 (tGLY), SUP61 (tSER) and tGLY respectively when compared to a wild type strain (Figure 2.8A, 2.9A and 3.1A).
Ty1 insertion levels also varied in the \textit{mlp2Δ} yeast strain which had significantly increased Ty1 mobility (~2.3-fold) when compared to wild type (Figure 2.1B). Ty1 insertion upstream of \textit{SUF16} (tGLY) was increased by 2-fold in the \textit{mlp2Δ} strain (Figure 2.8A) but decreased by 0.63-fold and 0.7-fold upstream of \textit{SUP61} (tSER) and all tGLY genes respectively when compared to wild type (Figure 2.9A and 3.1A). Although deletion of Nup2 did not impair Ty1 mobility, Ty1 insertion upstream of \textit{SUF16} and \textit{SUP61} was decreased by 79% and 75% respectively when compared to wild type (Figure 2.11C and 2.12C). A similar result was found when Ty1 targeting upstream of 16 glycine tRNA genes was analyzed in a \textit{nup2Δ} mutant where Ty1 insertion was decreased by 78% compared to wild type (Figure 3.1A) (289). The levels of tRNAs (\textit{tLEU}, \textit{tLEUdenovo} and \textit{tGLY}) are at approximately half of wild type tRNA levels when Nup2 is deleted (Figure 3.1B). Removing Nup2 causes impairment to tRNA levels similar to the removal of Nup60 which also results in a reduction of the three tRNAs to approximately half of wild type levels (Figure 2.4). However, the deletion of Nup2, unlike the deletion of Nup60, does not affect Ty1 mobility as the \textit{nup2Δ} strain has normal Ty1 mobility (Figure 2.1B). The impaired tRNA levels are likely caused by changes in tRNAs tethering the NPC via Nup2 which could explain the impaired Ty1 insertion in the \textit{nup2Δ} strain (2.11C, 2.12C and 3.1A.).
Figure 3.1 The nup2Δ strain has wild type Ty1 mobility but impaired Ty1 insertion upstream of tGLY genes. (A) Genomic DNA was extracted from wild type (WT), spt3Δ, mlp2Δ, nup60Δ, mlp1Δ and nup2Δ yeast strains grown for 3 days at 20°C in YPD media in triplicate. Ty1 insertion events were detected using PCR amplification with a primer that hybridizes to Ty1 and a primer that hybridizes to 16 different glycine tRNA genes (collectively termed tGLY; GCC). spt3Δ serves as a negative control. Image lab software was used to quantify total Ty1 insertion for each strain (number and intensity of bands) relative to wild type (WT) which was set to 1. A control CPR7 is shown to confirm the presence of genomic DNA. (B) nup2Δ Ty1 mRNA and (C) tRNA (tLEU, tLEU de novo, tGLY) expression measured by qPCR relative to TAF10. Relative quantification (RQ) values for nup2Δ are compared to wild type Ty1 mRNA or tLEU, tLEU de novo or tGLY which were set to 1.0. Arrows (→) denote novel insertion sites in the second and third lanes of the mlp2Δ strain.
Removal of the Nup1 nuclear basket protein, which is required for mRNA export (101,151), did not alter Ty1 mobility (Figure 3.2A) but resulted in an 8.3-fold decrease in Ty1 insertion upstream of tGLY genes (Figure 3.2B). The nup1Δ mutant produced 1.65-fold more Ty1 mRNA than wild type and produced Ty1 Gag at 81% of wild type which is consistent with the fact that Ty1 mobility in the nup1Δ strain is similar to wild type (Figure 3.2C, 3.2D). The nup1Δ mutant had a 60% reduction in tLEU levels compared to wild type but expressed tLEU_{denovo} and tGLY at levels similar to wild type (Figure 3.2E). It has been previously determined that nup1Δ strains do not accumulate unspliced tRNAs suggesting that tLEU_{denovo} should be exported into the cytoplasm for splicing at the mitochondrial outer surface (259,290). Therefore, the low tLEU levels in the nup1Δ strain may reflect defects in another aspect of tRNA maturation or stability. The fact that Ty1 mobility is similar to wild type levels in both the nup1Δ and nup2Δ nuclear basket mutants, yet Ty1 element targeting is impaired, suggests that Ty1 elements may be mistargeted to alternative loci.
Figure 3.2 Nup1 is required for Ty1 insertion upstream of tGLY genes. (A) Ty1 mobility measured in a nup1Δ yeast strain compared to wild type (WT). Yeast strains carrying a Ty1-HIS3AI plasmid were grown for 5 days at 20°C in SC-URA media after which cells were plated on SC-URA and SC-HIS plates and grown for 2 days at 25°C. Colonies were counted to calculate transposition frequency of each strain. (B) Genomic DNA was extracted from WT, nup1Δ, and spt3Δ yeast strains grown in YPD media for 3 days at 20°C in triplicate. Ty1
insertions upstream of \textit{tGLY} tRNA genes (GCC; 16 copies) were amplified by PCR and quantified relative to WT. (C) Relative quantification (RQ) of Ty1 mRNA levels in WT, \textit{spt3A} and \textit{nup1A} strains normalized to an internal \textit{TAF10} control and compared to wild type which was set to 1. (D) Bar graph generated from quantitative immune blots monitoring Ty1 Gag (p49 and p45) protein levels relative to GAPDH used as a loading control in WT, \textit{spt3A} and \textit{nup1A} strains (E) \textit{tLEU}, \textit{tLEU de novo} and \textit{tGLY} tRNA levels in logarithmically growing WT and \textit{nup1A} strains. tRNA levels were normalized to \textit{TAF10} and compared to wild type tRNA levels which were set to 1.0.

### 3.3.2 NPC membrane curvature by Nup1 and Nup60 plays a role in Ty1 insertion

Thus far, my studies indicate that the nuclear basket has a role in Ty1 element insertion into the genome, however we employed complete gene deletions which remove all protein function and therefore have pleiotropic phenotypes. To assess nuclear basket mutants that retain partial activity I acquired Nup mutants that retained some activity but removed specific protein domains, to test the impact of the mutation on Ty1 element insertion. It has recently been discovered that Nup1 contains an AH and an adjacent α-HR that are required for NE membrane curvature at the NPC insertion site and anchoring the nuclear basket to the NPC, respectively (280).

I tested for Ty1 insertion defects in yeast strains carrying Nup1 lacking the AH (nup1Δ1-32) or HR (nup1Δ85-123) domains which results in partial mislocalization of Nup1 into the nucleoplasm (280). Both mutants had higher Ty1 insertion levels than a wild type strain - nup1Δ1-32 had \approx11-fold higher and nup1Δ85-123 had \approx1.8-fold higher when compared to a wild type strain (Figure 3.3A, 3.3B). Not only is Ty1 inserted more frequently but there is a strong bias in the Ty1 element insertion position with the second nucleosome primarily targeted in the nup1Δ1-32 mutant and a single site targeted in the nup1Δ85-123 mutant \approxfive nucleosomes upstream of the tRNA gene (Figure 3.3B). Both mutants had no impairment in Ty1 mRNA or Gag levels and the nup1Δ1-32 mutant had wild type tRNA levels whereas the nup1Δ85-123
mutant expressed tRNA genes at ~61-69% of wild type levels (Figure 3.3C, 3.3D and 3.3E). The reduction in tRNA gene expression does not appear to affect overall Ty1 insertion frequency.

**Figure 3.3** The Nup1 N-terminal AH and HR domains affect Ty1 element positioning upstream of tGLY loci. (A) Nup1 protein schematic with the AH and HR highlighted as in (280). (B) Ty1 insertion upstream of tGLY (GCC) loci for a nup1Δ yeast strain carrying a plasmid expressing either full-length nup1 (NUP1), nup1Δ1-32 or nup1Δ85-123 fused to mCherry. Ty1 insertion levels were quantified for each strain and normalized to wild type levels. The spt3Δ yeast strain is a negative control for Ty1 replication (C, E) Ty1 mRNA and tRNA (tLEU, tLEU de novo,
tGLY) levels were measured by qPCR and normalized relative to TAF10. Relative quantification (RQ) values of the mutant strains were compared to wild type which was set to 1. (D) Ty1 Gag (p49 and p45) protein levels measured with quantitative western blots and presented in a bar graph. Gag levels were quantified relative to GAPDH which was used as a loading control and normalized to wild type Gag/GAPDH.

Nup60, similar to Nup1, has AH and HR regions implicated in membrane curvature and nuclear basket tethering, respectively (280). Removal of the Nup60 AH (nup60Δ1-47) or HR (nup60Δ48-162) domains results in partial detachment of Nup60 from the nuclear basket (280). Removal of both AH and HR domains (nup60Δ1-162) causes the majority of Nup60 to mislocalize to the nucleoplasm and abnormal clustering of Mlp1 and Mlp2 (280). Since the nup60Δ mutant is required for Ty1 mRNA expression (Figure 2.3A), I first tested Ty1 mRNA levels in the Nup60 AH and HR mutants. All of the Nup60 AH and HR mutants expressed Ty1 mRNA at wild type levels suggesting either that sufficient Nup60 remains attached to the nuclear basket to allow Ty1 mRNA expression or that the role of Nup60 in Ty1 expression does not require tethering to the NPC (Figure 3.4C). Likewise, Ty1 Gag was expressed at 64% of wild type, or above wild type levels and tRNA gene expression was not affected in the Nup60 AH and HR mutants (Figure 3.4D and 3.4E). I tested Ty1 insertion upstream of tGLY genes in the Nup60 AH and HR mutants and noticed a change of Ty1 insertion pattern in each mutant suggesting that either nucleosome positioning or Ty1 targeting is affected in these mutants (Figure 3.4B). The insertion frequency was reduced to 61% and 47% of wild type in the nup60Δ1-47 and nup60Δ48-162 mutant, respectively, whereas the nup60Δ1-162 mutant had wild type Ty1 insertion levels (Figure 3.4B). Therefore, the restoration of Ty1 mRNA and Ty1 Gag levels, compared to the nup60Δ mutant, allows Ty1 element insertion upstream of tGLY genes but the
nucleosome targeting is altered when the interaction between Nup60, the inner nuclear membrane and the nuclear basket is deficient.

Figure 3.4 The Nup60 N-terminal AH and HR domains determine the pattern of Ty1 insertion upstream of tGLY genes. (A) Nup60 schematic with the AH and HR regions highlighted as in (280). (B) PCR amplification of endogenous Ty1 insertion levels in a nup60Δ yeast strain carrying a plasmid expressing either full-length nup60 (NUP60), nup60Δ1-47, nup60Δ48-162, or nup60Δ1-162 fused to mCherry. Genomic DNA was extracted from yeast strains grown for 3 days at 20°C. Amplification of a 2000 bp product containing the CPR7 locus (lower panel) was performed as a control. Ty1 insertion levels, based on total number of bands and band intensities,
were quantified using Image lab software. (C, E) Ty1 mRNA levels and tRNA (\textit{tLEU}, \textit{tLEU de novo}, \textit{tGLY}) levels were normalized to a \textit{TAF10} internal control and relative quantification (RQ) values were calculated based on wild type Ty1 mRNA or tRNA levels which were set to 1. (D) Ty1 Gag (p49 and p45) protein levels measured with quantitative western blots relative to GAPDH used as a loading control, compared to wild type Gag/GAPDH, and presented in a bar graph.

Monoubiquitylation of Nup60, at one of 8 lysines between Lys105 and Lys175 (Figure 3.5A), controls the dynamics of Nup60 association with the NPC and contributes to the DNA-damage response and telomere repair (281,291). Some of the Nup60 ubiquitylated lysine residues are in the Nup60 HR region that impact Ty1 targeting (Figure 3.4A, 3.5A). To characterize the role of Nup60 ubiquitylation on Ty1 targeting I utilized a Nup60 ubiquitin (Ub) deficient mutant [nup60-K(105-175)R] that can no longer be conjugated to Ub (281). In the nup60-K(105-175)R mutant, the efficiency of Ty1 insertion upstream of \textit{tGLY} genes was ~58% that of a wild type strain, however, unlike the Nup60 HR mutant, the targeting pattern was similar to wild type (Figure 3.5B). The nup60-K(105-175)R Ub mutant has a 35% increase in Ty1 mRNA compared to wild type but a 26% reduction in Ty1 Gag protein levels compared to wild type (Figure 3.5C and 3.5D). Genotoxic stress, which prompts Nup60 ubiquitylation, may change the role of Nup60 in Ty1 replication, especially since the nup60-K(105-175)R mutant has an increase of unrepaired DNA lesions (281). However, since ubiquitylated Nup60 preferentially interacts with Nup84-Nup133, the 42% reduction of Ty1 insertion in a Nup60 Ub mutant could be due to an impaired interaction with the Nup84 outer ring subcomplex or indirect effects due to changes to the NPC structure (281).
Figure 3.5 Nup60 SUMOylation and the SUMOylation pathway have a role in Ty1 insertion. (A) Schematic of Nup60 protein with Ub (K105-107) and SUMO (K440-442, K505) sites labeled as in (281). (B) PCR amplification of Ty1 insertion events upstream tGLY genes (GCC, 16 copies) in yeast strains carrying mutated Nup60 Ub (nup60-K105-175R) or SUMO (nup60-K105-175R, K440, 442, 505R) sites assayed in triplicate. spt3Δ serves as a negative control. Image lab software was used to quantify total Ty1 insertion for each strain relative to wild type. A control
CPR7 is shown to confirm the presence of genomic DNA. (C) Ty1 mRNA in the indicated strains was assessed by qPCR relative to a TAF10 control. The bar graph shows relative quantification (RQ) values of Nup60 mutants compared to wild type Ty1 mRNA which was set to 1. (D) Ty1 Gag (unprocessed or p49 and processed or p45) protein levels detected by western blot and quantified relative to GAPDH loading control in duplicate. The bar graph shows quantification of Ty1 Gag in mutant strains relative to wild type Ty1 Gag and GAPDH levels which was set to 1. (E) qPCR of tLEU, tLEU de novo and tGLY, relative to a TAF10 control. RQ values are relative to wild type tLEU, tLEU de novo, or tGLY which were set to 1. (F) PCR amplification of Ty1 insertion upstream of tGLY in wild type (WT), spt3Δ, ulp1-333 and mms21-1 yeast strains. (G) PCR amplification of Ty1 insertion upstream of tGLY in WT, spt3Δ, siz1Δ and siz2Δ yeast strains. Ty1 insertion PCR gels in (F) and (G) were analysed as in (B).

Nup60 contains 2 SUMOylation sites: Lys440,442 and Lys505, however the function of Nup60 SUMOylation is not yet known (281). I studied the role of Nup60 SUMOylation with a Nup60 yeast strain containing a triple SUMO site mutation [Nup60-K(440-42, 505)R] integrated into the genome. The Nup60-K(440-42, 505)R mutant, which cannot be SUMOylated, had a ~3-fold reduction in Ty1 insertion compared to wild type (Figure 3.5B). Importantly, the Nup60-K(440-42, 505)R SUMO mutant expressed Ty1 mRNA (92%), Ty1 Gag (83%) and tRNA genes at levels similar to wild type (Figure 3.5C, 3.5D and 3.5E). The Siz1 and Siz2 SUMO ligases are responsible for SUMOylation of Nup60 and the Ulp1 SUMO protease is responsible for removing the Nup60 SUMOylation (281). To determine the importance of SUMOylation on Ty1 targeting I analyzed Ty1 insertion upstream of tGLY genes in siz1Δ, siz2Δ, and ulp1-333 mutant strains. I also included an MMS21 mutant (mms21-1) because Mms21 is a SUMO E3 ligase that mediates the relocation of DNA double strand breaks to the nuclear periphery (292). Deleting Siz1 did not reduce Ty1 insertion upstream of tGLY while removing Siz2 caused a modest 32% decrease in Ty1 insertion upstream of tGLY as compared to wild type (Figure 3.5G). As well, the ulp1-333 mutant had 82% of wild type Ty1 insertion upstream of tGLY. However, the mms21-1
mutant had a more dramatic phenotype with a reduction in Ty1 insertion of ~3.7-fold compared to wild type (Figure 3.5E). It has not yet been tested if Nup60 SUMOylation depends on Mms21.

### 3.3.3 Mutating Nup1 phosphorylation sites reduces Ty1 insertion at tGLY genes

The Hog1 stress-activated protein kinases (SAPK), among other roles, phosphorylates the Nup1, Nup2 and Nup60 nuclear basket proteins (144). In response to osmotic stress and resultant Hog1 activation, Nup1, Nup2 and Nup60 interact with the *CTT1* and *STL1* stress-response promoters and are essential for expression of these genes (144). It has also been shown that Cdk1 phosphorylation of Nup1 is necessary for localization of active *GAL1* and *INO1* to the nuclear periphery (140).

I looked at the role of Nup1 phosphorylation by Hog1 on Ty1 integration using a Nup1 triple mutant (nup1 S11A/T159A/S161A) that has strongly reduced Hog1 mediated phosphorylation *in vitro* (144). The nup1 S11A/T159A/S161A mutant had an 8-fold reduction in Ty1 insertion compared to wild type insertion levels (Figure 3.6A). Ty1 mRNA expression was reduced by 34% and Ty1 Gag protein levels were elevated by 30% relative to wild type in this Nup1 phospho mutant (Figure 3.6C and 3.6D). The levels of tRNA expression was also assessed in the nup1 S11A/T159A/S161A mutant with *tLEU* at 88%, *tLEU*denovo at 81%, *tLGY* at 89% of wild type (Figure 3.6B). Given the normal Ty1 Gag protein levels and modest reductions in Ty1 mRNA and tRNA levels in the nup1 S11A/T159A/S161A mutant, the 8-fold reduction in Ty1 insertion observed with this Nup1 phospho mutant is likely due to disruption of the Ty1 integration process and not defects in Ty1 transcription, translation or tRNA expression.

Similar to Nup1, Nup2 is also phosphorylated by Hog1 with mutation of the threonine residue at position 361 (T361A) resulting in a circa 70% decrease in Hog1 mediated
phosphorylation of Nup2 (144). Ty1 insertion upstream of *tGLY* was minimally impaired in the Nup2 phospho mutant (nup2 T361A) which was at 62% of wild type (Figure 3.6E). Given the modest decrease in Ty1 insertion along with the normal Ty1 insertion pattern I did not assess the nup2 T361A mutant for defects in Ty1 mRNA, Ty1 Gag or tRNA levels.
Figure 3.6 Mutating Nup1 but not Nup2 Hog1 phosphorylation sites reduces Ty1 insertion. (A) Genomic DNA was extracted from a spt3Δ strain and a nup1Δ strain carrying a plasmid expressing either a wild type copy of Nup1 (NUP1) or a nup1 phosphorylation site mutant (nup1 S11A/T159A/S161A). Ty1 insertion products were PCR amplified and quantified based on the number and intensity of bands for each strain and normalized to wild type Ty1 insertion levels using Image Lab software. Amplification of the CPR7 locus (lower panel) was performed as a control. (B, C) RNA was extracted from yeast strains grown at 20°C to log phase. tRNA (tLEU, tLEU de novo, tGLY) and Ty1 mRNA levels were quantified relative to TAF10 (D) Ty1 Gag
(p49 and p45) protein levels measured with quantitative western blots relative to GAPDH used as a loading control. (E) PCR amplified Ty1 insertion levels in a spt3Δ strain and a nup2Δ strain carrying a plasmid expressing either wild type Nup2 (NUP2) or nup2 carrying a T361A mutation (nup2 T361A).

### 3.3.4 Nuclear basket FG repeats are not required for Ty1 insertion

*S. cerevisiae* NPCs have eleven Nups, including three nuclear basket Nups (Nup1, Nup2 and Nup60), that contain unstructured FG repeat domains required for nucleo-cytoplasmic transport and maintaining the selective permeability barrier of NPCs (109,293-296). FG repeat motifs are defined as: FG, FxFG, GLFG; where x denotes any amino acid (297,298). Nup2 and Nup1 contain 16 and 23 FxFG peptide repeats, respectively, which serve as karyopherin docking sites (272,273,275,299-301). Nup60 contains 4 FxF repeats at its C-terminus (109). I acquired nup1, nup2 and nup60 strains carrying combinations of deletions in the FG repeats from Dr. Wente’s lab (109). The Wente lab strain background is W303 which I found has undetectable levels of Ty1 transposition using the same conditions that enable transposition in the S288C background (growth at 20°C for 3 days). To increase Ty1 transposition levels in W303, I transformed the nuclear basket FG mutant yeast strains with a pGAL-TyH3mHIS3AI-URA3 plasmid (pJBe376) and induced Ty1 element expression for 24 hours with 2% Gal media. I found that removal of the nup1, nup2, nup60 nuclear basket FG repeats had no effect on the Ty1 targeting pattern and also did not significantly affect Ty1 insertion efficiency (Figure 3.7). A triple nup1ΔFxFG nup2ΔFxFG nup60ΔFxF strain deleted for all nuclear basket FG repeats demonstrated Ty1 insertion upstream of tGLY genes at 89% of wild type levels (Figure 3.7D). My data implies that Nup1, Nup2 and Nup60 FG repeat domains, and therefore karyopherin docking at the nuclear basket, do not play a role in Ty1 targeting.
Figure 3.7 The nuclear basket FG repeats are not required for Ty1 insertion (A-D) *nup1ΔFxFG, nup2ΔFxFG, nup60ΔFxF, nup1ΔFxFG nup2ΔFxFG* and *nup1ΔFxFG nup2ΔFxFG nup60ΔFxF* yeast strains transformed with a GAL-TY1 plasmid and grown in triplicate in galactose media (GAL) to induce Ty1 expression or dextrose media (D) as a negative control for 24 hours at 25°C. Ty1 insertion was amplified at 16 *tGLY* (GCC) loci by PCR and Ty1 insertion levels were quantified by the number and intensity of insertions for each strain as compared to wild type (WT) which was set to 1. *CPR7* control PCR is shown in the lower panel for each gel.

3.3.5 The Ran Binding domain of the mobile nucleoporin, Nup2, is essential for Ty1 insertion

Nup2 is a mobile Nup that is primarily located on the nuclear basket through a physical interaction with Nup60, but is also present in the nucleoplasm (270,271,274,275). I have shown that Ty1 insertion upstream of tRNA genes is reduced by ~5-fold in the *nup2Δ* mutant whereas removal of the Nup2 FG repeats (Nup2ΔFxFG) did not impact Ty1 insertion (Figures 2.11C, 2.12C, 3.1A and 3.7A). The C-terminus of Nup2 (aa583-720) contains a RBD that binds the Ran small GTPase (276). Ran, which is mostly GTP-bound in the nucleus, mediates release of
nuclear protein cargo that transits through the NPC. The RBD also contributes to the interaction of Nup2 with the NPC as cells lacking the Nup2 RBD have a marked increase of Nup2 in the nucleoplasm (270). I found that a nup2ΔRBD yeast strain, engineered to lack residues 606-720 on Nup2, had a 50-fold reduction in Ty1 insertion compared to wild type (Figure 3.8B). Deletion of the Nup2 RBD did not cause a reduction in Ty1 mRNA expression or Ty1 Gag levels (Figure 3.8C, 3.8D). Although tLEU and tLEU de novo levels were similar to wild type, tGLY tRNA levels were reduced to 56% of wild type which is consistent with the role for Nup2 in docking tRNA genes at the NPC (Figure 3.8E, (82)). The reduction in tGLY expression could explain a partial (50%) defect in Ty1 insertion, however the reduction of Ty1 insertion to almost background levels in the nup2ΔRBD strain suggests that mislocalization of Nup2 into the nucleoplasm, defects in Nup2 mediated nucleo-cytoplasmic transport, or disruption of the Ran-GTP gradient impairs Ty1 targeting.
Figure 3.8 Removing the RBD of Nup2 abrogates Ty1 insertion. (A) Schematic of the Nup2 protein with the RBD shown in yellow (residues 583 to 720). (B) PCR amplification of Ty1 insertion upstream of 16 tGLY (GCC) genes in wild type (WT) and the nup2ΔRBD strains. spt3Δ is a negative control for Ty1 replication. Image lab software was used to quantify Ty1 insertion levels (number and intensity of bands) for each strain relative to WT which was set to 1. PCR amplification of the CPR7 locus is shown for each sample to verify the isolation of PCR quality genomic DNA. (C, E) Relative quantification (RQ) of Ty1 mRNA levels and tLEU, tLEU de novo, tGLY, respectively, relative to a TAF10 control as measured by qPCR in triplicate. RQ values are relative to WT Ty1 mRNA or wild type tLEU, tLEU de novo or tGLY which were set to 1. (D) Bar graph of Ty1 Gag (unprocessed or p49 and processed or p45) protein levels quantified relative to a GAPDH loading control in duplicate by western blot. Whole cell extracts were processed from yeast strains grown to log phase. Quantification values are relative to WT Ty1 Gag and GAPDH levels which was set to 1.0.

3.3.6 Removing the tRNA exportin Los1 abolishes Ty1 insertion upstream of tRNA genes

The nuclear export of tRNAs requires transport factors such as the yeast exportin Los1 protein. Los1, for los of suppression, was identified by a loss of suppression assay as a mutant (los1-1) deficient in tRNA processing (302). Los1 binds tRNAs and mutations of Los1 causes an accumulation of precursor tRNAs in the nucleus (302,303). Los1 has also been shown to physically interact with the Nup2 and Nsp1 NPC proteins as well as the GTP-bound form of Ran (304). Interestingly, Los1 was previously identified in a genetic screen for Ty1 host factors that suppress the hypertransposition phenotype of the rtt101Δ and med1Δ mutants (178). Since Ty1 elements insert upstream of tRNA genes and given the Los1-mediated link between tRNA export and the NPC, we tested a los1Δ mutant yeast strain for Ty1 replication defects.

The los1Δ mutant had a 1.7-fold increase in Ty1 mobility compared to wild type (Figure 3.9A) but near complete loss of Ty1 insertion upstream of SUFI6 and SUP61 (Figure 3.9B and 3.9C). This is the only mutant in this study to have significantly higher Ty1 mobility than wild type but defects in Ty1 insertion. The defects in both SUFI6 and SUP61 Ty1 insertion suggest that both pericentromeric and non-pericentromeric tRNA genes are affected in the los1Δ mutant.
Despite the fact that Ty1 cDNA was previously shown to be reduced in the los1Δ mutant to 0.44-fold of wild type levels, the reduction of Ty1 cDNA is not reflected in the Ty1 mobility data shown here (178) (Figure 3.9A). In fact, our data suggests that in the absence of Los1, Ty1 is inserted in high frequency to genomic locations but not upstream of tGLY genes. Although tRNA export is an essential cellular function, Los1 is not an essential gene, and is therefore likely not the sole tRNA exporter (305). A genome-wide screen searching for novel tRNA exporters found that mutations to CRM1 (Exportin-1), MEX67/MTR2 (TAP, p15), and genes encoding five Nups (NDC1, NUP57, NUP159, NUP170, and NUP192) cause nuclear accumulation of unspliced tRNA, a hallmark of defective tRNA nuclear export (260). These data suggests that in the absence of Los1, Ty1 is inserted in high frequency to other genomic locations.
Figure 3.9 Deleting Los1 increases Ty1 mobility but impairs tRNA targeting. (A) Ty1 mobility was monitored with a URA marked Ty1 plasmid (pTy1-HIS3AI) expressed in a wild type (WT) or los1Δ yeast strain. Strains were grown in SC-URA media for 5 days at 20°C then plated on SC-URA or SC-URA-HIS plates. Plates were incubated at 25°C for 2 days and colonies were counted to calculate transposition frequency. (B, C) Genomic DNA was isolated from WT and los1Δ strains grown for 3 days at 20°C in YPD. Ty1 insertion events upstream of SUF16 (B) or SUP61 (C) were amplified via PCR and quantified (number and intensity of bands) using ImageLab software. Mutant insertion values were compared to WT which was set to 1.0. The spt3Δ yeast strain serves as a negative control for Ty1 replication and the CPR7 locus was PCR amplified for each sample to demonstrate the presence of genomic DNA.

3.3.7 The S. cerevisiae THO/TREX and TREX-2 NPC Associated Complexes can alter Ty1 insertion

I have demonstrated that the NPC nuclear basket impacts Ty1 element targeting upstream of tRNA genes. The nuclear basket has a variety of cellular roles including recruitment of actively transcribed genes to the NPC and coupling gene transcription to nuclear mRNA export.
Therefore, I explored the possibility that complexes that interact with the nuclear basket may have a role in Ty1 element targeting.

The evolutionary conserved THO/TREX (transcription-export) complex is composed of five tightly bound proteins that form the THO subcomplex (Hpr1, Mft1, Tho2, Thp2, Tex1) and interact with the Sub2 and Yra1 export factors to form TREX (Figure 3.10A) (306-309). THO/TREX accompanies elongating RNA Polymerase II to couple mRNA biogenesis with nuclear export and recruits the mRNA export receptor Mex67-Mtr2 to the mRNA (Figure 3.10A) (307,308,310). Mex67-Mtr2 binds to the mRNA to form a mRNP and also binds to the NPC to export the mRNP to the cytoplasm (311). I tested a subset of mutants in the THO/TREX complex for a Ty1 targeting phenotype. The tho2Δ and mft1Δ mutants had a ~9-fold and 2-fold decrease respectively in Ty1 insertion upstream of SUF16 compared to wild type (Figure 3.10B). Conversely, the hpr1Δ mutant had a ~2.8-fold increase in Ty1 insertion upstream of SUF16 compared to wild type (Figure 3.10B). Hpr1 is less stable than the other proteins in the THO/TREX complex and is targeted for degradation which is thought to control THO/TREX formation and mRNA export (312). Since degradation of Hpr1 is thought to promote dissociation of the THO/TREX complex it is not clear why tho2Δ and mft1Δ mutants have the opposite Ty1 insertion phenotype to hpr1Δ mutants. This said, deletion of Hpr1 also causes high frequencies of recombination and chromosome loss suggesting that Hpr1 plays a role in genome stability which could affect Ty1 replication (313,314).
Figure 3.10 Deleting components of the THO/TREX and TREX-2 complexes alters Ty1 insertion levels and patterning. (A) Schematic representation of THO/TREX and TREX-2 complexes interacting with a NPC during transcript processing and export [adapted from (306)]. Nuclear export of a transcript can occur by interactions with transcription export complexes such as THO/TREX and TREX-2. The THO/TREX complex (Tho2, Mft1, Thp2, Hpr1, Tex1, Sub2, Yra1) is shown in blue and complexed to an mRNP ready for export shown in red. A dotted line depicts predicted interactions between the THO/TREX associated mRNP with the mRNP export machinery (Mex67-Mtr2) shown in magenta. The export complex then binds to TREX-2 (Sac3, Cdc31, Sus1, Thp1) which is shown in orange. The TREX-2 complex docks at Nup1, a component of the NPC nuclear basket (Nup1, Nup60, Nup2, Mlp1, Mlp2) shown in green, and the mRNP is poised for export. This process could be a step in ‘transcription coupled export’. Proteins in the same complex have the same colour. (B) Ty1 insertion upstream of the glycine tRNA gene SUFI6 on chromosome III or (C) 16 copies of the tGLY genes (GCC) indicated on the right of the gel. Yeast strains [(THO/TREX complexes (tho2Δ, mft1Δ, hpr1Δ), and TREX-2 complex (sac3Δ, sac3ΔCID, sus1Δ)] were grown for 3 days at 20°C and Ty1 insertions were amplified by PCR and measured by the relative intensity and frequency of Ty1 insertion bands compared to wild type (WT) which was set to 1.0.

The TREX-2 complex also functions to couple transcription with nuclear mRNA export (Figure 3.10A) (155,306,315). In S. cerevisiae TREX-2 is composed of Cdc31, Thp1 and two copies of Sus1 linked to the core Sac3 protein (151,316). TREX-2 is involved in genome stability, DNA replication, transcription coupled mRNA export which includes the relocation of actively-expressed genes such as GAL1 to the NE and binding to the NPC (152,155,245,288,316-318). Specifically, Sac3 binds the mRNA exporter Mex67-Mtr2 and requires Nup1 to tether to the NPC (Figure 3.10A) (151). Sus1 is also a member of the SAGA complex; a large complex involved in chromatin remodeling and transcription activation (317). Sac3 binds to Sus1 and Cdc31 via the CID motif (Sac3 residues 733–860) and deletion of the Sac3 CID domain results in loss of Sus1 and Cdc31 from TREX-2, coupled with a defect in poly(A)+ RNA export and mislocalization of TREX-2 from NPCs (316,319). I acquired TREX-2 mutants from the Foiani lab which are in the W303 strain background and found, similar to the Wente lab strains, that the W303 wild type strain had very low levels of Ty1 insertion when compared to the S288C wild
type strain (Figure 3.10C). However, I was able to detect Ty1 transposition in the TREX-2 mutants because the \textit{sac3Δ} mutant and the \textit{sac3ΔCID} mutant had a 14-fold and ~24-fold increase in Ty1 insertion upstream of \textit{tGLY} genes, respectively (Figure 3.10C). As well, the \textit{sus1Δ} mutant had a ~19-fold increase in Ty1 insertion upstream of \textit{tGLY} genes (Figure 3.10C). Another interesting feature of the TREX-2 mutants was that the pattern of Ty1 insertion differed between the \textit{sac3Δ}, \textit{sac3ΔCID} and \textit{sus1Δ} mutant although I was not able to compare to a wild type strain. My data suggests that the presence of TREX-2 may interfere with Ty1 insertion and that removal of TREX-2 from the NPC may impact chromatin arrangement, similar to what I detected with the Nup1 and Nup60 AH and HR mutants. Another possibility is that the increased Ty1 insertion in the TREX-2 mutants is due to cellular stress caused by genome instability or defects in DNA replication or transcription. Taken together, my analysis of THO/TREX and TREX-2 suggest that neither of these NPC associated complexes are required for Ty1 transposition and their presence may impede the insertion of Ty1 elements into the genome.

3.3.8 Mutating Cdc31 can disrupt both Ty1 mobility and insertion

Cdc31 is a ~19 kDa calcium binding protein associated with both TREX-2 and the SPB which is embedded in the NE and is the microtubule-organizing center of yeast (316,320). Sequence analysis of \textit{CDC31} suggests homology to the calmodulin family of Ca$^{2+}$ regulators (321). Specifically, Cdc31 is located on the cytoplasmic face of the SPB as a component of the half-bridge (320). \textit{CDC31} mutations block SPB duplication which in turn causes G$_1$ phase arrest (322). Cdc31 also plays a role in protein degradation as Cdc31 binds the proteasome and multiubiquitinated proteins (323). Given that Cdc31 is part of the TREX-2 complex and interacts with Nup1 (151), I decided to test its role in Ty1 insertion into the genome.
I acquired three Cdc31 ts mutants (cdc31-1, cdc31-2, cdc31-5) from the Boone lab ts mutant collection (235) for analysis of Ty1 mobility and integration defects. The cdc31-1 mutant was originally described in 1973, and yeast cells carrying this mutation (A48T) were found to have normal nuclear and cellular morphologies at the permissive temperature (23°C), but had elongated nuclear and cellular morphologies indicative of late nuclear division arrest at the restrictive temperature (36°C) (324). In 1981, Schild et al. generated the cdc31-2 mutant and found that at 36°C the cdc31-2 (E133K) mutation causes cell cycle arrest, and at 30°C, causes increased ploidy (number of chromosome sets) (325). The cdc31-5 (P94S) ts mutant yeast strain was originally generated by Vallen et al. in 1994 but was not studied in-depth as it was not a suppressor of KAR1, their gene of interest (322).

Interestingly, the three CDC31 ts mutants had unique Ty1 mobility phenotypes but all three had impaired Ty1 insertion (Figure 3.11). The cdc31-2 mutant had wild type Ty1 mobility whereas Ty1 mobility in the cdc31-1 mutant was significantly decreased from wild type by ~82% (Figure 3.11A). The cdc31-5 mutant also had significantly decreased Ty1 mobility but mobility was marginally reduced by ~40% compared to wild type (Figure 3.11A). Ty1 insertion upstream of tGLY was impaired by 97% in the cdc31-2 mutant when compared to wild type and Ty1 upstream of SUF16 was completely abolished in the cdc31-1 mutant (Figure 3.11B, 3.11C). The cdc31-5 mutant also had a 33-fold decrease in Ty1 insertion upstream of tGLY relative to wild type (Figure 3.11B). Altered Ty1 insertion in CDC31 mutants could potentially be caused by changes to TREX-2 tethering at NPCs that in turn affect the role of TREX-2 in coupling mRNA transcription to nuclear export that affect Ty1 replication.
Figure 3.11 Cdc31 is required for Ty1 mobility and insertion. (A) Ty1 mobility measured in cdc31-1, cdc31-2, cdc31-5 mutant yeast strains compared to wild type (WT). Yeast strains carrying a Ty1-HIS3AI plasmid were grown for 5 days at 20°C in SC-URA media after which cells were plated on SC-URA and SC-HIS plates and grown for 2 days at 25°C. Colonies were counted to calculate transposition frequency of each strain. (B) Quantification of Ty1 insertion event upstream of 16 glycine tRNA genes, collectively termed tGLY, in cdc31-5 and cdc31-2 mutant strains compared to WT which was set to 1.0. (C) Ty1 insertion upstream of the glycine tRNA gene SUF16 in a cdc31-1 mutant compared to WT which was set to 1.0. spt3Δ was used as a negative control in (B) and (C) and amplification of the CPR7 locus (bottom panels) was performed to ensure the presence of PCR quality genomic DNA.

3.3.9 Ty1 insertion is retargeted to sub-telomeric regions in nuclear basket mutants

The nup1Δ and nup2Δ mutants have wild type Ty1 mobility yet reduced Ty1 targeting upstream of tGLY genes suggesting that Ty1 insertion is occurring elsewhere in the genome. It has previously been shown that Ty1 elements are redirected to telomere-proximal regions when the S. cerevisiae Rpc40 protein is substituted for the S. pombe Rpc40 protein which no longer
interacts with Ty1-IN (7). I tested if Ty1 elements are mis-targeted to telomere-proximal regions in nuclear basket deletion mutants with primers that hybridize to Ty1 insertion sites identified in the Bridier-Nahmias et al. study that are near chromosome ends (7). The insertion sites are located in genes with nearly identical homologues (SNZ2/SNZ3 and HXT15/HXT16) therefore each set of PCR reactions amplifies Ty1 elements at two sub-telomeric regions (8). As a control, I induced endogenous Ty1 transposition in the S. cerevisiae RPC40 and S. pombe RPC40 strains which have previously been shown to accumulate Ty1 elements at telomere-proximal regions only in the S. pombe RPC40 strain (Figure 3.12 (230,231)). When compared to the S. cerevisiae RPC40 strain the yeast strain carrying the S. pombe RPC40 had ~33-69 times higher Ty1 insertion at the SNZ2/SNZ3 loci (Chr XIV left end/Chr VI left end) and ~18-51 times higher Ty1 insertion at HXT15/16 loci (Chr IV left end and Chr X right end) (Figure 3.12). I found that Ty1 elements are indeed mis-targeted to the chromosome ends in nup1Δ, nup2Δ, mlp1Δ and mlp2Δ mutants but not in a wild type strain (Figure 3.12A, 3.12B). When compared to wild type or spt3Δ strains which both have minimal insertion at sub-telomere regions, mlp1Δ strains had a ~33-fold and mlp2Δ strains had a ~25-fold increase in Ty1 insertion at SNZ2/SNZ3 loci and an even higher increase in Ty1 insertion at HXT15/HXT16 loci (Figure 3.12). Removal of Nup1 caused a more modest increase in Ty1 insertion (~6-8 times) whereas the nup2Δ strain had a ~22 to 39-fold increase in Ty1 insertion at the sub-telomere regions (Figure 3.12). Even though nup60Δ mutants express Ty1 elements at low levels (Figure 2.3A), I also detected mis-targeting to the SNZ2/SNZ3 gene and a few insertion events at the HXT15/HXT16 locus (Figure 3.12A). In summary, my data suggests that the nuclear basket is required for targeting of Ty1 elements upstream of tRNA genes and for preventing insertion at chromosome ends.
Figure 3.12 Ty1 is targeted to sub-telomeres in the absence of nuclear basket proteins. PCR amplification of Ty1 insertion events from genomic DNA extracted from (A) control strains [S. cerevisiae RPC40, S. pombe RPC40, wild type (WT), spt3Δ] and nuclear basket deletion strains (mlp2Δ, nup60Δ) or (B) control strains (S. cerevisiae RPC40, S. pombe RPC40, spt3Δ) and nuclear basket deletion strains (nup1Δ, nup2Δ, mlp1Δ) grown for 3 days at 20°C. Each PCR contained a Ty1 element primer and a sub-telomere-proximal primer; the sub-telomere-proximal primers hybridize to two identical genomic regions at SNZ3/SNZ2 or HXT15/HXT16 loci. CPR7 control PCR is shown to demonstrate the presence of genomic DNA in each sample. Ty1 insertion levels were quantified based on the number of bands and band intensity for the S. pombe RPC40 positive control relative to the S. cerevisiae RPC40 which was set to 1.0 (denoted as 1.0). Each Nup basket deletion strain was compared to WT or spt3Δ, which were set to 1.0 (denoted as 1.0). Chr, chromosome, R, right; L, left.

3.4 Discussion

I focused my study on the NPC nuclear basket proteins, which are reported in numerous studies to interact with chromatin (147,268,269). Ty1 insertion into the genome was nearly abolished in the nup60Δ mutant, likely due to the low levels of Ty1 mRNA and Gag expression (Figures 2.3, 2.8C, 2.9C). I acquired mutants in two Nup60 N-terminal helical domains from the Kohler lab in an attempt to identify a NUP60 mutant that did not interfere with Ty1 mRNA expression. The Kohler lab previously demonstrated that removal of the Nup60 AH (nup60Δ1-47) or Nup60 HR (nup60Δ48-162) led to a partial detachment of Nup60 from the nuclear basket, and the deletion of both HRs (nup60Δ1-162) caused nearly complete mislocalization of Nup60 (280). Unlike the nup60Δ yeast strain, the Nup60 AH and HR mutants generated wild type levels of Ty1 mRNA, Ty1 Gag and tRNA genes which allowed us to test for Ty1 element targeting in the Nup60 AH and HR mutants (Figure 3.2). The fact that the nup60Δ1-162 mutant, which almost completely detaches from the NPC, expresses Ty1 mRNA at wild type levels, also raised the question of whether or not recruitment to the NPC is necessary for Ty1 mRNA expression (280). I found that Ty1 elements are able to insert upstream of tGLY genes in all three Nup60 AH and HR mutants, however the pattern of Ty1 targeting is altered (Figure 3.2B). Docking of
tRNAs at the NPC requires Nup60 (82) and I found that nup60Δ mutants have a 50% reduction in tLEU and tGLY gene expression (Figure 2.4). Hence, there may be changes in the chromatin and nucleosome positioning upstream of tRNAs in the Nup60 AH and HR mutants that causes changes in Ty1 targeting, although expression of tLEU, tLEU (de novo) and tGLY are not affected in these mutants.

Similar to Nup60, Nup1 also has an AH and HR domain in its N-terminus that interacts with the inner nuclear membrane to promote curvature (280). I found that the Nup1 AH (nup1Δ1-32) and HR (nup1Δ85-123) mutants had Ty1 mRNA, Ty1 Gag and tRNA levels similar to wild type but a change in the Ty1 insertion pattern upstream of tGLY (Figure 3.1). The nup1Δ1-32 mutant that lacks the AH, had ~11-fold increased Ty1 insertion at the second nucleosome position upstream of tGLY genes whereas the nup1Δ85-123 mutant that lacks the HR had ~1.8-fold increased Ty1 insertion targeted approximately five nucleosomes upstream of the tRNA gene (Figure 3.1B). Both the AH and HR regions contribute to Nup1 NPC localization as removing the AH (nup1Δ1-32) causes a slight mislocalization of Nup1 into the nucleoplasm which is increased when removing the HR (nup1Δ85-123) (280). The Ty1 targeting patterns in Nup1 and Nup60 AH/HR mutants point to a role for NPC proximal membrane curvature in the accurate targeting of Ty elements likely due to the role of Nup60, and probably Nup1, in recruitment of tRNA genes to the NPC.

Monoubiquitylation of Nup60 plays a role in tethering both Nup60 and Nup2 to the NPC (281). Nup60 is also SUMOylated however the function of the Nup60 SUMO modification is not yet known (281). I found that Ty1 insertion in the Nup60 ubiquitin-deficient mutant [nup60-K(105-175)R] is reduced to 58% of wild type level which may be because this mutant displays increased dissociation from the NPC (Figure 3.3) (281). Interestingly, Ty1 insertion was reduced
to 34% of wild type levels in the Nup60 SUMOylation mutant [nup60-K(440,442,505)R] (Figure 3.3B). Detection of Nup60 SUMOylation requires mutation of the Ulp1 protease and the SUMO ligases Siz1 and Siz2 are required for Nup60 SUMOylation in the ulp1 background (281). I tested deletions of the three mitotic E3 SUMO ligases (Siz1, Siz2, Mms21) and found that the mms21Δ mutant had the greatest reduction in Ty1 insertion (Figure 3.3F). Mms21 is a component of the Smc5/6 complex that functions in DNA repair and genome integrity, particularly at the ribosomal DNA locus (326,327). SUMOylation of RNA Pol III subunits is important for Pol III assembly and recruitment to tRNA genes but the E3 ligase responsible for Pol III SUMOylation has not yet been identified (328,329). Therefore SUMOylation is likely to affect multiple proteins involved in Ty1 targeting to the genome, including Nup60 and possibly other NPC and RNA Pol III proteins.

I next explored if other regions of the nuclear basket proteins are involved in Ty1 targeting. Nup1, Nup2 and Nup60 all contain FG repeats that bind cargo for facilitated transport across the NPC (109,298). I did not detect a defect in Ty1 insertion upstream of tGLY genes in any single FG repeat mutant or even the triple nup1ΔFxFG nup2ΔFxFG nup60ΔFxFG mutant (Figure 3.5). Therefore, the FG repeat cargo binding capacity of the nuclear basket is not required for Ty1 element targeting into the genome. Ty3 transposition is also not affected in nup1ΔFxFG, nup2ΔFxFG and nup60ΔFxFG single mutants (35). Instead, Ty3 Gag interacts with Nup GLFG repeats to allow docking of the Ty3 VLP onto the NPC (185). Whether or not Ty1 VLPs also dock onto the NPC remains to be determined.

Unlike the nuclear basket proteins, Nup2 is transiently associated with NPCs, sharing the roles of NPC component and soluble transporter (272-274,295). Upon binding near the promoter regions of genes, Nup2 transports active genes to NPCs (122,133,160,245). Nup2, along with the
tRNA exporter Los1, also participates in recruitment of tRNA genes to the NPC (21). I found that when Nup2 is deleted, Ty1 mobility remains normal but Ty1 insertion upstream of tRNA genes is decreased (Figures 2.1B, 2.11C, 2.12C, 2.13). Given that Ty1 mRNA levels are normal in the nup2Δ yeast strain, the decreased Ty1 insertion at tRNA genes could be partially due to the reduced transcription of tRNA genes (Figure 2.13). Nup2 contains a RBD and localization of Nup2 to the nuclear face of the NPC requires Ran-GTP binding (89). I found that removing the RBD of Nup2 reduced Ty1 element insertion upstream of tGLY genes by 50-fold but did not affect Ty1 mRNA or Gag levels and only reduced tGLY levels by ~50% (Figure 13B). Nup2 could be acting as a gene-NPC tether or mediator in this process given the potential role of Nup2 in nuclear export of tRNAs as Nup2 interacts with the tRNA exportin, Los1 (304). Consistent with the hypothesis that Nup2 is a tRNA gene-NPC tether, deletion of Los1 resulted in a dramatic loss of Ty1 insertion upstream of two tRNA genes but not a loss of Ty1 mobility (Figure 3.9A-C). Removing or altering Nup2, and other nuclear basket Nups, could affect the cell cycle as the NPC-tRNA interaction occurs more frequently during M phase (82).

Furthermore, perturbing the interaction of NPC-gene tethering could result in changes to chromatin organization or the loss of complexes localizing to the nuclear periphery both of which could have broad cellular effects on processes such as transcription and nucleocytoplasmic transport.

I tested mutants in both the THO/TREX and TREX-2 complexes for Ty1 targeting upstream of tGLY genes (Figure 3.10B, 3.10C). For the THO/TREX complex, I tested tho2Δ, mft1Δ and hpr1Δ and found that deleting Tho2 or Mtf1 caused an 89% and 51% decrease in Ty1 insertion upstream of the SUF16 tRNA gene, respectively, as compared to wild type whereas the hpr1Δ strain had a 2.8-fold increase in Ty1 insertion (Figure 3.10B, 3.10C). Given that the three
TREX mutants tested here had different Ty1 insertion phenotypes, it is difficult to identify the role of TREX in Ty1 replication. The ~50% decrease and the ~3-fold increase in Ty1 insertion in the mft1Δ and hpr1Δ strains respectively could be due to defects in TREX-mediated transcription elongation or mRNA export of Ty1 mRNA. I also acquired three TREX-2 CDC31 ts mutants (cdc31-1, cdc31-2, and cdc31-5) and found that Ty1 insertion was abolished in all three mutants but Ty1 mobility was significantly reduced in the cdc31-1 and ccd31-5 strains but normal in the cdc31-2 strain (Figure 3.11). The TREX-2 complex tethers to the nuclear basket of NPCs (319), therefore mutating CDC31 could result in mislocalization of TREX-2 that changes the chromatin landscape at NPCs. Loss of TREX-2-NPC tethering could also affect genomic processes such as transcription and mRNA export as TREX-2 functions in coupling transcription of mRNAs to nuclear export. I also acquired TREX-2 mutants (sac3Δ, sac3ΔCID, sus1Δ) from Dr. Marco Foiani (288) and found that the wild type strain (W303 strain background) had negligible levels of Ty1 insertion under conditions that we typically use for S288C (Figure 3.10C). However, deletion of SAC3 or SUS1 in the W303 strain background resulted in an increase in Ty1 insertion upstream of tGLY genes (Figure 3.10C). The sac3ΔCID mutant, which causes loss of Sus1 and Cdc31 from TREX-2 and mislocalization of TREX-2 from the NPC (288) no longer interacts with Nup1 also had greatly increased Ty1 insertion upstream of tGLY genes (Figure 3.10C). Therefore in the W303 background, TREX-2 is inhibitory to Ty1 insertion.

Although nup1Δ and nup2Δ cells had wild type Ty1 mobility as well as Ty1 mRNA and Gag levels, Ty1 insertion upstream of tGLY was reduced (Figures 2.1B, 2.11C, 2.12C, 2.13). It has previously been demonstrated that a strain expressing S. pombe Rpc40 that no longer interacts with Ty1-IN targets Ty1 elements to subtelomeric regions (230). I found that Ty1 elements are mis-targeted to the same subtelomeric regions in nup1Δ and nup2Δ mutants (Figure
3.10). I also detected Ty1 mis-targeting to subtelomeric regions in mlp1Δ and nup60Δ mutants that have low Ty1 mobility and the mlp2Δ mutant that has increased Ty1 mobility (Figure 2.1B, 3.10). Notably, the subtelomeric regions do not contain Pol III genes and my wild type strain does not target Ty1 elements to chromosome ends (Figure 3.10). I suspect that in the absence of nuclear basket proteins, another NPC protein is directing Ty1 elements into the genome such as Nup170 which interacts with subtelomeric chromatin (128).
Chapter 4:

Conclusion

4.1 Chapter Summaries

In Chapter 2, I tested 15 non-essential deletion and 4 essential ts Nup mutant yeast strains for significant changes in Ty1 mobility. Ty1 mobility was quantified by monitoring the formation of HIS+ colonies in NPC mutant yeast strains carrying a plasmid (pBDG922) with a HIS3 marked Ty1 element (Figure 2.1A). Of the 19 nup mutants that I tested 11 (mlp1Δ, mlp2Δ, nup53Δ, nup60Δ, nup120Δ, nup159-1, nup170Δ, nup188Δ, nup192-15, pom34Δ, sec13-1) had significant changes in Ty1 mobility (Figure 2.1B). I monitored Ty1 mRNA, Ty1 Gag, Ty1 cDNA and tRNA levels in these mutants to determine if the observed Ty1 mobility changes were due to differences in the availability of transposition intermediates (Figure 2.3, 2.4, 2.5 Table 2.2). I found that removing Nup60 caused a decrease in Ty1 mRNA levels, expression of the Ty1 Gag protein, and Ty1 cDNA (Figure 2.3, 2.5A). The nup159-1 ts mutant generated circa half of wild type Ty1 mRNA levels, and had reduced Ty1 Gag protein and Ty1 cDNA levels (Figure 2.3, 2.5A). Nup159 is on the cytoplasmic side of the NPC and could impact Ty1 mRNA export, Ty1 VLP docking at the pore or Ty1 PIC import. Most of the remaining Nup mutants did not have greatly impaired Ty1 mRNA or Ty1 Gag protein levels; the exceptions were nup170Δ and nup188Δ which had no defects in Ty1 mRNA levels but produced Ty1 Gag at 53% and 40% of wild type respectively (Figure 2.3). Importantly, the reduction in Ty1 insertion in the nup170Δ and nup188Δ mutants was much less than predicted by lower Ty1 Gag levels (Figure 2.8, 2.9).
Also, the panel of Nup mutants tested here maintained nuclear localization of Ty1-IN suggesting that no single NPC protein is required for Ty1-IN nuclear import (Figure 2.6).

I then used PCR to measure Ty1 insertion levels upstream of the glycine tRNA (SUF16 locus) and the serine tRNA SUP61 in my panel of Nup mutants. When compared to the wild type strain, Ty1 insertion upstream of both the SUF16 and SUP61 tRNA genes was higher in nup120Δ and almost negligible in the 8 Nup mutant strains with reduced Ty1 mobility: nup53Δ, nup60Δ, nup159-1, nup170Δ, nup188Δ, nup192-15, pom34Δ, and sec13-1. The mlp2Δ yeast strain, which had significantly higher Ty1 mobility compared to wild type, had twice as much Ty1 insertion upstream of SUF16 as compared to wild type but had a ~40% decrease in Ty1 insertion upstream of SUP61 (Figure 2.1B, 2.8A, 2.9A). The mlp1Δ strain, which had a significant defect in Ty1 mobility compared to wild type, had only a 17% decrease in Ty1 insertion upstream of SUF16 compared to wild type but a 80% decrease in Ty1 insertion upstream of SUP61 (Figure 2.1B, 2.8A, 2.9A). The complex Ty1 insertion phenotype of the Mlp deletion strains are possibly due to the pericentromeric location of the SUF16 gene on chr III compared to the location of SUP61 on the right arm of chr III. It would be interesting to test the Ty1 insertion phenotype of a mlp1Δ mlp2Δ double mutant strain which would remove all coiled-coiled proteins that extend from the nuclear basket.

I next quantified Ty1 insertion in Nup deletion strains that had wild type levels of Ty1 mobility (nup84Δ, nup133Δ, ndc1-4, nup42Δ, nup100Δ, pom152Δ, nup59Δ and nup2Δ). Interestingly, the mutants all showed altered Ty1 insertion upstream of SUF16 and SUP61 suggesting that Ty1 is mistargeted to other regions of the genome in these Nup mutant strains. Ty1 insertion upstream of the SUF16 gene favoured the first nucleosome in the nup133Δ strain whereas an extra Ty1 insertion was detected in the nup84Δ strain (Figure 2.11). Nup133 and
Nup84 are part of the Nup84 Y shaped complex that forms the outer ring and interacts directly with the nuclear basket (Kim 2018). The nucleosome positioning upstream of tRNA genes may be affected in these mutants or the ability of Ty1-IN to access the chromatin may be impaired.

In Chapter 3 I focused on the role of the nuclear basket (Nup1-Nup2-Nup60-Mlp1-Mlp2) in Ty1 replication given its well-defined role in genomic processes and its proximity to chromatin. First, I confirmed that nup2Δ, nup60Δ, mlp1Δ and mlp2Δ have defects in Ty1 insertion upstream of all 16 tGLY genes (Figure 3.1A). Next, I discovered that a nup1Δ mutant has a reduction in Ty1 tGLY insertion but wild type Ty1 mobility, similar to what I noticed with the nup2Δ strain (Figure 3.2). For the remainder of this Chapter, I worked with a panel of nuclear basket mutants, generously provided by the yeast community, that had small deletions or point mutations instead of complete gene deletions that could impact a variety of NPC processes. I identified nuclear basket mutants that had wild type levels of Ty1 mRNA and Ty1 Gag levels, but a change in the pattern of Ty1 insertion which was the first indication that the nuclear basket could impact where Ty1 elements insert into the genome.

Removing either the AH and/or HR domain on Nup1 or Nup60 changes the Ty1 insertion pattern upstream of tGLY genes relative to the wild type strains but importantly does not dramatically affect Ty1 mRNA or Ty1 Gag levels. Interestingly, removing Nup1 or Nup60 AH or HR domains not only affects Ty1 insertion levels but also interrupts the Ty1 insertion band pattern when compared to wild type suggesting that nucleosome positioning upstream of tRNA genes could be disturbed in these mutants.

I studied the role of Nup60 ubiquitylation on Ty1 targeting by utilizing a Nup60 Ub deficient mutant [nup60-K(105-175)R], generated by the Dargemont lab. The Dargemont lab found that ubiquitylation of Nup60 was important for controlling the association/dissociation rate
of Nup60 with the NPC and regulating the DNA damage response and telomere repair (281). In
the nup60-K(105-175)R mutant, the efficiency of Ty1 insertion upstream of tGLY genes was
~58% that of a wild type strain. The Dargemont lab also provided us with a Nup60 SUMO
mutant [nup60-K(440, 442, 505)R] that I found to have a ~3-fold reduction in Ty1 insertion
compared to wild type. Nup60 SUMOylation is independent of Nup60 ubiquitylation and no
previous function for Nup6p SUMOylation has been described (281).

The RBD of Nup2 is important for NPC localization of Nup2 as well as Nup2’s
functional role in transport (270). I found that deletion of the Nup2 RBD did not impair Ty1
mRNA or Ty1 Gag proteins levels but completely abolished Ty1 insertion upstream of tGLY
(Figure 3.8). I also found that deletion of components of the NPC-interacting complex
THO/TREX such as Tho2 and Mft1 impaired Ty1 targeting to tGLY genes (Figure 3.10B). Also,
mutations of Cdc31, a component of multiple complexes including TREX-2 and the SPB,
abolished Ty1 insertion upstream of tRNA genes (Figure 3.11B, 3.11C). Interestingly, the three
mutants tested (cdc31-1, cdc31-2, cdc31-5) had different Ty1 mobility phenotypes: cdc31-2 had
wild type Ty1 mobility, cdc31-5 had Ty1 mobility levels at circa half of wild type, and Ty1
mobility was severely impaired in the cdc31-1 mutant (Figure 3.11A). On the contrary,
mutations of the Sac3 and Sus1 components of TREX-2 resulted in increased Ty1 insertion
upstream of tGLY (Figure 3.10C), however, the strain background was different from the CDC31
mutants. At this point it is not known if the role of Cdc31 in Ty1 insertion is related to its
function in TREX-2, at the SPB or in proteasomal degradation.

The Los1 protein functions in nuclear export of tRNAs in yeast and is involved in
tethering tRNA genes to NPCs as deletion of Los1 was shown to impair the association of Nup60
with three different tRNA genes (82). This suggests that tRNA transcription and/or processing
could be linked to nuclear export of tRNAs at NPCs. I found the deletion of Los1 increased Ty1 mobility compared to wild type but Ty1 insertion upstream of SUF16 and SUP61 was completely abolished (Figure 3.9). One reason for this could be that Ty1 is targeted elsewhere in a los1Δ strain given that tRNA export is likely impaired in this mutant. The requirement of Los1 for accurate targeting of Ty1 element upstream of tGLY genes suggests that Los1 could be an important link between Pol III-transcribed genes and the NPC.

One of the most significant findings of Chapter 3 is that Ty1 elements mistarget into chromosome ends in all nuclear basket deletion mutants (Figure 3.12). Ty1 insertion was targeted to chromosome ends in nup1Δ, nup2Δ, and mlp2Δ yeast strains which have at least wild type Ty1 mobility and even the mlp1Δ and nup60Δ strains which both have significantly decreased Ty1 mobility compared to wild type (Figure 2.1B). The mistargeting of the Ty1 elements to the chromosome ends suggests that nuclear basket Nups are required directly or indirectly, perhaps as global architects or regulators of chromatin organization, to orchestrate Ty1 targeting upstream of Pol III-transcribed genes.

### 4.2 General Discussion

In my thesis, I studied the role of NPCs in Ty1 replication with particular interest in the role of Nups in Ty1 integration. My hypothesis, partially based on Chen et al.’s finding that tRNA genes are tethered to NPCs during M-phase (82), was that Nups could influence Ty1 integration site selection. My results indicate that components of the nuclear basket (Mlp1, Mlp2, Nup1, Nup2, Nup60), couple tRNA expression, Ty1 expression and Ty1 integration. Further work will be required to establish if there is a direct interaction between the nuclear basket and the Ty1 machinery or an indirect interaction due to impacting other cellular processes such as
nucleo-cytoplasmic transport. An interaction between Ty1-IN and nuclear basket proteins is likely transient, because in previous work done by my lab, purification of Ty1-IN, followed by MS analysis, did not identify an enrichment of Nup proteins whereas Pol III subunits were identified (231). However, for these purifications, we did not perform a solubilization step, therefore proteins associated with the NE may have been pelleted after cell lysis. Instead, my lab identified five RNA Pol III subunits by MS (Rpc25, 34, 40, 53, 82) that co-purified with Ty1-IN from two independent purifications (231). Furthermore, Bridier-Nahmias et al. discovered an interaction between Ty1-IN and the Rpc40 subunit of RNA Pol III using a yeast two-hybrid assay that was confirmed by co-IP analysis between HA-tagged Rpc40 and Ty1-IN (230). Future structural studies of Ty1-IN binding to RNA pol III will likely be needed to understand precisely how this interaction takes place. To detect any potential interactions between Ty1-IN and the NPC, a modification of the Ty1-IN immunoprecipitation protocol should be performed that includes a membrane protein solubilization step.

A study in *Caenorhabditis elegans* (*C. elegans*) has found that the NPP-13 NPC component, which is the orthologue of vertebrate Nup93 and yeast Nic96, is required for processing of Pol III-snoRNA and tRNA transcripts (318). Ikegami and Lieb also showed that the NPC proteins NPP-13 and NPP-3, which is homologous to yeast Nup192, physically interact with RNA pol III transcribed genes using ChIP followed by tiling microarray and ChIP-seq (330). It was also demonstrated that components of RNA pol III machinery (RNA pol III, TBP, TFIIC proteins) interact directly with NPP-13 and NPP-16, a component of the basket portion of the nuclear pore, by co-IP (330). Taken together, these data suggests that suggest that in *C. elegans* NPC subunits coordinate transcription and processing of Pol III transcripts.
Whether Nups interact directly with Ty1-IN or act as a bridge between Ty1-IN and other factors such as subunits of the Pol III complex remains to be determined. I propose a model that the nuclear basket provides a scaffold for tRNA transcription and Ty1 insertion (Figure 4.1A). Chromatin at actively transcribed tRNA genes is tethered to the NPC by an interaction between the tRNA exporter Los1 and RNA Pol III, which allows tRNA transcription and export to be coupled for efficient production of tRNAs (82) (Figure 4.1A). The Ty1 PIC is imported into the nucleus where it first encounters genes, such as tRNA genes, tethered to the nuclear basket. Ty1 IN then physically interacts with RNA Pol III, targeting the Ty1 PIC to the genome (Figure 4.1A). In this scenario, both the tRNA gene target and the Ty1 PIC complex would be in close physical proximity to undergo Ty1-IN-dependant DNA joining reactions. When nuclear basket proteins are removed, tRNA genes are still transcribed by RNA Pol III but are no longer tethered at NPCs and as a result Ty1 no longer inserts upstream of tRNA genes (Figure 4.1B). There are at minimum two potential models for the fate of Ty1-IN in the absence of nuclear basket proteins. One scenario is that Ty1-IN is released from both Pol III and the NPC and interacts with an unknown complex that targets Ty1 cDNA to transcriptionally silent chromosome ends (Figure 4.1B). The second scenario is that Ty1-IN is maintained at the NPC but released from Pol III and NPC proteins allow Ty1 insertion at sub-telomeres (Figure 4.1C). Whether or not Ty1-IN still interacts with RNA Pol III in the absence of Nup proteins is an important question that could help resolve between these two models. Also, as mentioned above, evidence for a direct interaction between Ty1-IN and NPC proteins, or Los1, is still lacking. Mutation of nuclear basket proteins could also alter chromatin organization at the nuclear periphery, resulting in more condensed, transcriptionally silent chromatin near the nuclear basket. In this scenario,
transcriptionally silenced chromatin is first encountered by the Ty1 PIC and targeted for Ty1 insertion.
Ty1 integration occurs at the nuclear basket. (A) A tRNA gene (grey, with the promoter A and B boxes shown in red and blue respectively) is transcribed by RNA Pol III (orange) and interacts with the nuclear basket (blue) via Los1 (yellow) for efficient tRNA transcription and export. The Ty1 PIC (red) enters the nucleus, encounters genes transcribed at the nuclear basket which include tRNA genes, and directly interacts with RNA Pol III for genomic insertion upstream of RNA Pol III transcribed genes. (B) RNA Pol III transcribed genes no longer tether to the nuclear basket upon mutation of nuclear basket proteins, for example Nup1, and the Ty1 PIC does not first encounter RNA Pol III. Instead, the Ty1 PIC interacts with an unknown protein complex (purple) that directs Ty1 insertion to chromosome ends. (C) In the absence of nuclear basket proteins, such as Nup1, the Ty1 PIC no longer interacts with RNA Pol III but remains at the NPC for insertion of Ty1 into sub-telomeres. In (B) and (C) transcription of tRNA genes by RNA Pol III still occurs but not at the nuclear basket. The Ty1 VLP capsid shell is shown in green.

The evolution of 29 Nup-encoding genes in S. cerevisiae was studied by gathering nucleotide sequences from six divergent Saccharomyces species (S. cerevisiae, S. paradoxus, S. mikatae, S. kudriavzevi, S. arboriculus and S. bayanus) (331). DNA alignments of the genes were constructed and fitted into two different models of codon evolution using bioinformatics tools (331). From their analysis, Rowley et al. found that NUP84, NUP1, NUP116 and NUP82 had accumulated elevated levels of non-synonymous sequence changes (331). Next, they tested the functional consequence of Nup84 sequence changes on Ty1 mobility by replacing NUP84 in the S. cerevisiae genome with NUP84 from diverse Saccharomyces species. Swapping out S. cerevisiae NUP84 with other Saccharomyces NUP84 genes resulted in species-specific changes to Ty1 mobility with one replacement (S. kudriavzevi) causing an increase and the other two (S. mikatae and S. bayanus) causing a decrease in Ty1 mobility compared to wild type (331). A similar result was found when they tested NUP82 using the same approach providing evidence that adaptive changes have occurred to both Nup genes studied in depth (331). All tested housekeeping functions of NUP84 and NUP82 remained equivalent across species suggesting
that evolutionary sequence changes to these Nups could have occurred due to co-evolution with TEs such as Ty1.

It has been well-established that Ty1 elements inserts upstream of RNA pol III-transcribed genes such as tRNA genes (9,22,30-32). I found that in the absence of nuclear basket proteins Ty1 was able to insert at chromosome ends. Although Ty1 elements are not normally found at chromosome ends, Ty1 has been shown to incorporate at eroded telomeres in cells lacking telomerase, an enzyme required for lengthening telomeres at the ends of chromosomes (332). Ty1 inserts at telomeres in the absence of telomerase likely because these regions become unstable in the absence of telomerase and need to be repaired by genomic rearrangements (332).

Bridier-Nahmias et al., discovered a physical interaction between Ty1-IN and the Rpc40 subunit of RNA Pol III (230). To disrupt the interaction of Ty1-IN with RNA Pol III without reducing Pol III transcription, Bridier-Nahmias et al., made clever use of a previous observation that the S. pombe Rpc40 subunit (Rpc40sp) can functionally replace the S. cerevisiae Rpc40 subunit (333). When Rpc40 was replaced with Rpc40sp, the interaction with Ty1-IN and Ty1 element targeting upstream of Pol III genes was disrupted (230). Interestingly, overall Ty1 mobility was not impaired in the Rpc40sp strain and genome-wide mapping revealed that Ty1 elements were preferentially targeted to telomere proximal regions (230). The mechanism that Ty1 uses to insert at telomere regions when the S. cerevisiae Rpc40 is replaced with Rpc40sp or in the absence of Nups remains unknown. The yeast retrotransposon Ty5 inserts at silenced regions at the ends of chromosomes such as telomeres by a direct interaction between Ty5-IN and Sir4 (51). Therefore, an interaction between Ty1-IN and complexes associated with chromosome ends such as the Sir silencing proteins could target Ty1 to this region in the absence of Nups. Another possible explanation could be that chromatin organization at the nuclear periphery in the
absence of Nups could direct Ty1 to chromosome ends. This is plausible given the known role of NPC proteins in the tethering of silenced regions at the ends of chromosomes, such as telomeres and *HM* loci, to the nuclear periphery (119-125). The loss of telomere tethering at NPCs, as a result of deleting nuclear basket proteins, likely causes changes to chromatin organization at telomere-proximal regions where I saw Ty1 insertions in my nuclear basket mutant yeast strain.

Functional conservation of Ty1-IN with HIV-1 IN suggests that studies of Ty1-IN protein interactions and targeting in yeast will be applicable to the mechanism of HIV-1 propagation. Indeed, three independent genome-wide siRNA screens for HIV-1 host factors identified Nups involved in PIC trafficking and integration, suggesting a model of “import-coupled integration” (211-213). Follow-up work has shown that the HIV-1 capsid interacts with human Nups, such as Nup153, and when this interaction is inhibited HIV-1 infectivity is significantly reduced (334-336). Interestingly, HIV-1 integration has been shown to predominantly occur in chromatin located at the outer edge of the nucleus, in close proximity with nuclear pores (215,216).

Moreover, two groups found that HIV-1 associates with the NE in the cytoplasm and preferentially integrates at chromatin close to the NE (218,219). These studies, along with my own, highlight a role for NPCs in dictating integration of yeast retrotransposons or mammalian retroviruses either through direct PIC interactions or as a result of indirect effects on chromatin organization.

### 4.3 Future Directions

In this thesis I have explored the role of NPCs in Ty1 replication. The work presented in this thesis has many avenues for further research.
4.3.1 Mapping genome-wide Ty1 insertions in NPC mutant strains

To identify new genomic locations targeted by Ty1 in the absence of Nups high resolution deep sequencing should be used to analyze the efficiency and specificity of Ty1 integration at tRNA genes in Nup mutant strains that have normal Ty1 mobility levels but impaired Ty1 insertion upstream of tRNA genes (Figure 3.12). My lab has acquired a Ty1 transposon plasmid expressed from a GAL promoter and carrying a 25bp synthetic tag (ssb) engineered into the 3’-Ty1 LTR (pJEF2365) from Dr. Jef Boeke that has been previously used to map genome-wide Ty insertion sites (31). After induction of Ty1 mRNA expression and reverse transcription, both the 3’ and 5’ LTRs of the Ty1 cDNA and newly transposed elements will carry the ssb tag. Yeast strains carrying Nup deletions or mutations along with wild type strains carrying pJEF2365 should be induced for Ty1 transposition, yeast genomic DNA extracted, PCR amplified, and a library could be constructed for sequencing using an Illumina high resolution deep sequencer. If the tested Nups are required for accurate targeting of Ty1-IN, I expect to see a reduction in the frequency of integration at tRNA genes (integration efficiency), or a change in the targeting pattern of Ty1-IN (integration specificity) in the mutant strains as compared to the wild type strains. I already know that in the absence of nuclear basket Nups at least some Ty1 insertions occur in subtelomeric regions so I would expect to find this region, and perhaps other nearby regions, of interest for Ty1 insertion mapping in Nup mutant yeast strains.

4.3.2 Ty1 IN purifications

The synthesis of tRNA genes peaks in M phase of the cell cycle and the association of tRNA genes with the NPC also peaks in M phase (82). In the previous Ty1 IN purifications performed in the Measday lab, an enrichment of NPC associated proteins was not detected,
however the purification were performed in logarithmic phase cells and no Triton was used to increase solubilization of membrane-bound proteins (231). In the future, Ty1 IN could be purified in cells arrested in G2 phase by treatment with nocodazole (Nz) followed by MS analysis with increasing amounts of Triton to increase solubilization of NPC proteins. Cell division cycle mutants could also be used to arrest cells in G2 phase (such as a GAL-CDC20 shut off strain) and purify Ty1 IN. Following identification of Ty1 IN co-purifying peptides, GFP-tagged versions of these co-purifying proteins could be tested directly for interaction with Ty1 IN using co-IP analysis with epitope tagged nuclear pore proteins.

4.3.3 Nup1 and Nup60 AH and HR

I obtained Nup1 and Nup60 AH and HR deletion mutants from Dr. Köhler and tested for Ty1 insertion upstream of tGLY genes. Removal of the Nup60 AH (nup60Δ1-47), Nup60 HR (nup60Δ48-162) or both (nup60Δ1-162) changed the pattern of Ty1 insertion, but unlike the nup60Δ mutant, did not affect Ty1 mRNA levels or tGLY expression. Removal of the Nup1 AH and HR domains also changed the pattern of Ty1 insertion. Genome-wide mapping studies have shown that Ty1 elements target nucleosomes upstream of tRNA genes and insert every ~70bp near the H2A/H2B interface (31,33). Since removal of Nup1 or Nup60 AH and HR domains alters the pattern of Ty1 insertion one hypothesis is that NPC basket-induced bending of the inner nuclear membrane may affect tRNA nucleosome positioning (although, tRNA gene expression is normal). An alternative hypothesis is that the AH and HR domains or an alternative region of Nup1/60 may interact with Ty1 IN. To determine if tRNA nucleosomes are mislocalized, I propose to map nucleosome positions by deep sequencing of micrococcal nuclease resistant fragments.
4.3.4 Host factors targeting Ty1 IN in the absence of Nups

In the absence of nuclear basket proteins (Nup1-Nup2-Nup60-Mlp1-Mlp2) Ty1 is targeted to chromosome ends as opposed to Pol III-transcribed genes. A tandem purification MS approach, that my lab has previously used to identify Ty1-IN interacting proteins, could be utilized to identify potential host factors involved in targeting Ty1 to chromosome ends in the nuclear basket mutant strains. First, a Ty1 element should be overexpressed from an inducible plasmid in yeast cells lacking nuclear basket Nups. Ty1-IN should then be purified using the 8b11 monoclonal anti-IN antibody followed by MS to identify Ty1-IN co-purifying proteins. As in section 4.3.1, prior to pull-down of Ty1 IN, cells will be lysed with increasing amounts of Triton to increase solubilization of NPC proteins. As well, GFP-tagged versions of identified co-purifying proteins could be tested directly for interaction with Ty1 IN using co-IP analysis with epitope tagged nuclear pore proteins.

There is currently a great interest in understanding how retroviruses integrate into genomes. In my thesis, I used the budding yeast *S. cerevisiae* as a model organism to study the role of NPCs on the genomic integration landscape of the Ty1 retrotransposon. TEs, such as Ty1, are global regulators of genome stability, and NPCs are not only cellular gateways but genomic hubs for a variety of functions (chromatin silencing, gene recruitment, transcriptional memory, DSB repair and mRNA surveillance). In my thesis, I show that NPCs are also involved in Ty1 genomic integration and specifically that nuclear basket proteins influence Ty1 integration site selection. The work presented in this thesis has helped to further understand the functional diversity of NPCs and the complexity of retrotransposon targeting.
Bibliography


