

THE ENZYMES RESPONSIBLE FOR THE ADDITION AND MAINTENANCE OF THE
3' OLIGOURIDYLATION OF HISTONE MRNA

Patrick Earl Lackey

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Approved by:

William F. Marzluff

Jeannette G. Cook

Howard M. Fried

Gregory A. Matera

Zefeng Wang

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ABSTRACT

PATRICK EARL LACKEY: The enzymes responsible for the addition and maintenance of the 3' oligouridylation of histone mRNA
(Under the direction of William F. Marzluff)

Histone mRNAs end in a highly conserved 3' stemloop, making them the only known metazoan mRNAs that do not end in a poly(A) tail. They are also tightly cell-cycle regulated, with expression increasing 30-fold at the beginning of S-phase followed by a quick reduction in half-life as S-phase ends. Because of its unique 3' ends, histone mRNAs require a different mechanism to initiate degradation than bulk mRNA. Previous work has revealed that initiation of histone mRNA degradation is mediated by an oligouridylation at the 3' end of the message. Further work showed these oligouridylations vary widely in base position, length, and when they are added to the message. In this thesis, I use a high-throughput sequencing approach to show that the oligouridylation of the 3' UTR of histone mRNA are added by the enzyme TUT7 and that the length and function of the oligouridylations is controlled by the human 3' exonuclease, 3'hExo; during S-phase the U tails are 1-2 nts and function to protect the 3' end of the message from degradation. As S-phase ends, longer U-tails are added and 3'hExo initiates the degradation of the message. From the next-generation sequencing data, I also present evidence that oligouridylation of the open reading frame is added differently than the oligouridylation of the 3' end, likely by a different enzyme.

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TABLE OF CONTENTS

LIST OF FIGURES	x
CHAPTER 1	1
INTRODUCTION	1
The role of histones and chromatin in S-phase.....	1
The canonical histone genes and SLBP.....	3
Histone mRNA transcription and processing	4
Histone mRNA export and translation	6
RNA DEGRADATION.....	6
De-adenylation dependent degradation	7
Other pathways of RNA degradation	9
Nonsense-mediated decay	10
Non-stop decay	11
No-go decay.....	12
Histone mRNA degradation	12
TUTASES AND URIDYLATION	15
TUT1 (mtPAP/hmtPAP, PAPD1)	17
TUT2 (PAPD4, hGLD2)	18
TUT4 (ZCCHC11) and TUT7 (ZCCHC6).....	18
TUT3 (PAPD5, TRF4-2) and TUT5 (PAPD7, TRF4-1).....	19
U6 TUTASE.....	21

SUMMARY.....	22
CHAPTER 2.....	30
INTRODUCTION.....	30
MATERIALS AND METHODS	32
Cell culture and RNA interference	32
Western blotting	33
Northern blotting	33
Preparation of samples for high-throughput sequencing.....	33
Mapping EnD-seq Data	34
RESULTS.....	35
Most histone mRNA is uridylated in some form.....	36
3'hEXO trims to maintain the length of histone mRNA	36
TUT7 contributes to the addition of the U-tails to the 3' end of histone mRNAs	38
Knockdown of TUT4 had minimal effects on uridylation of histone mRNAs	40
Double sihEXO/siTUT7 knockdown provides more evidence that the enzymes work in concert at the 3' end of histone mRNA.....	42
Initiating histone mRNA degradation does not alter the oligouridylation pattern at the 3' end of histone mRNA	42
Knockdown of TUT7 but not TUT4 affects the initial step in degradation of histone mRNA	44
Subsequent 3' to 5' degradation through the coding region of the histone mRNA does not require TUT7	46
DISCUSSION.....	47
TUT7 and 3'hExo may collaborate in the uridylation of histone mRNA.	51
CHAPTER 3.....	90
INTRODUCTION.....	90

MATERIALS AND METHODS	92
In vitro GST pulldown assays	92
siRNA knockdowns.....	92
shRNA lentivirus knockdowns.....	92
EnD-SEQ and AppEND	93
RESULTS.....	93
TUT1 does not have a direct effect on histone mRNA degradation	93
TUT3 interacts with SLBP	95
End-Seq and APPend show unexpected alterations to histone mRNA oligouridylation when TUT3 is knocked down.....	96
DISCUSSION.....	98
TUT3's interaction with SLBP indicates a role in histone mRNA metabolism.....	98
TUT3 is likely not involved in bulk histone mRNA degradation	99
TUT3's alteration of the histone mRNA degradation pattern resembles the exosome and may provide clues to its function in histone mRNA metabolism	100
CHAPTER 4.....	124
INTRODUCTION	124
UPF1 MAY BE CRITICAL FOR THE FIRST STEP OF HISTONE mRNA DEGRADATION	125
TUT7 IS THE PRIMARY TUTASE AT THE 3' END OF HISTONE mRNA	126
TUT7 AND 3'hEXO FUNCTION TOGETHER TO PROMOTE URIDYLATION AND DEGRADATION.....	128
TUT7 AND 3'hEXO COOPERATE TO ACHIEVE PROPER URIDYLATION OF HISTONE mRNA.....	130
IS THERE A ROLE FOR TUT3 IN HISTONE mRNA METABOLISM?.....	131
THE MECHANISM OF DEGRADATION FOR INTERMEDIATES IN THE OPEN READING FRAME REMAINS UNKNOWN	132

SORTING OUT RELATIVE CONTRIBUTIONS OF 5'-3' AND 3'-5' DEGRADATION	133
FINAL CONCLUSIONS	135
REFERENCES	143

LIST OF FIGURES

Figure 1. Histone mRNA has a unique 3' end and is tightly cell cycle regulated.....	24
Figure 2. The TUTases	26
Figure 3. Histone mRNAs are uridylated, likely in concert with the beginning of degradation	28
Fig. 4. EnD-Seq workflow.....	54
Figure 5. Common EnD-Seq products	56
Figure 6. EnD-Seq results for synchronized HeLa cells	58
Figure 7. Knockdown of 3' hEXO changes histone mRNA uridylation patterns	60
Figure 8. Quantification of different histone mRNA 3' ends after hEXO knockdown.....	62
Figure 9. TUT7 knockdown shifts monouridylations to diuridylations	64
Figure 10. Quantification of different histone mRNA 3' ends after TUT7 knockdown.....	66
Figure 11. Stacked bar graphs for TUT7 repeat experiment	68
Figure 12. Pie charts for repeat TUT7 EnD-Seq experiment	70
Figure 13. TUT4 knockdown has no effect on uridylation of histone mRNA.....	72
Figure 14. Quantification of different histone mRNA 3' ends after TUT4 knockdown	74
Figure 15. TUT4/TUT7 double knockdown has a slight stacking effect on the 3' ends of histone mRNA.....	76
Figure 16. 3'hEXO/TUT7 double knockdown results in lengthened, un-uridylated histone mRNA.....	78
Figure 17. Oligouridylation at the 3' end is unaffected by HU treatment.....	80
Figure 18. Quantification of 3' ends for each knockdown after HU treatment.....	82

Figure 19. Degradation intermediates in the HIST2H2AA3 stemloop after HU treatment	84
Figure 20. Degradation intermediates in the 3'UTR (but outside of the stemloop) and open reading frame after HU treatment.....	86
Figure 21. Model of histone mRNA degradation with uridylation carried out by TUT7 and mediated by 3' hEXO trimming.....	88
Figure 22. Splice variants of TUT1	102
Figure 23. <i>In vitro</i> pulldown assays for TUT1 and SLBP	104
Figure 24. Western blots for TUT1 specific isoform knockdowns	106
Figure 25. Northern blot for histone mRNA stability after TUT1 isoform-specific knockdowns	108
Figure 26. GST pulldowns with <i>in vitro</i> translated TUT3 and TUT4 and recombinant SLBP	110
Figure 27. TUT3 interacts with SLBP somewhere between SLBP's amino acids 165 and 200.....	112
Figure 28. SLBP interacts with TUT3 somewhere between TUT3's amino acids 459 and 549.....	114
Figure 29. Mapping the interaction between TUT3 and SLBP	116
Figure 30. EnD-Seq results for TUT3 siRNA knockdown without HU treatment	118
Figure 31. TUT3 knockdown results in accumulation of messages uridylated in the stem-loop in HU-treated samples	120
Figure 32. TUT3 knockdown has little effect on uridylation in the open reading frame	122

LIST OF TABLES

Table 1. siRNA sequences used to target 3'hEXO and TUTases of interest	137
Table 2. Important linker and primer sequences for EnD-Seq/AppEND.....	139
Table 3. shRNA sequences used to target TUT1 isoforms.....	141

CHAPTER 1

INTRODUCTION

The role of histones and chromatin in S-phase

During S-phase, a eukaryotic cell must both replicate its genome and package the newly made DNA of that replicated genome into chromatin. Chromatin is a nucleoprotein complex made up of an octamer of the four canonical histone proteins, H2A, H2B, H3 and H4 (Kornberg 1977), with 146 nucleotides of DNA arranged in a double helix around the octamer (Luger et al. 1997). The linker histone, H1, further stabilizes the nucleosome particle and adds higher-order structure (Hizume et al. 2005). Histone proteins are an important architectural unit of chromatin; without proper levels of histone proteins, the genome is vulnerable to damage via chromosome loss (Meeks-Wagner and Hartwell 1986).

There are two classes of histone genes that encode for these histone proteins. The first is the replication dependent histone genes; this includes the four canonical histones listed (H2A, H2B, H3, and H4) along with the linker histone H1. The genes that encode these histones are the only non-polyadenylated metazoan mRNA, they are tightly cell-cycle regulated to be expressed during S-phase while DNA is being synthesized, and then to be immediately degraded once S-phase ends or DNA synthesis is stopped. These replication-dependent histone genes and their metabolism will be discussed in detail in this thesis. The second type of histone is the replication-independent histone. These are also known as

histone variants; the genes for these histones variants are polyadenylated and cell cycle independent. The histone variants have a wide variety of functions. The centromere-specific H3 replaces H3 in centromere DNA, helping kinetochores to form. Another H3 variant, H3.3, varies from H3 only by four amino acids and seems to function in actively transcribed genes. H2A also has two variants, H2A.Z and H2A.X. H2A.Z is found around transcription start sites of actively transcribed genes and H2A.X is involved in chromatin remodeling (Talbert and Henikoff 2010).

As histone proteins are being rapidly transcribed and translated during S-phase, they must then be deposited on replicating DNA in order to form the nucleosome. This is a complex process that can involve a large number of proteins (Burgess and Zhang 2013), but I will provide a brief outline here. The histones are loaded onto the newly synthesized DNA in stepwise-fashion; first the H3 and H4 histones are deposited on the DNA, then H2A and H2B, and finally H1 (Worcel et al. 1978). H3 and H4 are deposited onto the DNA by a three-protein complex known as Chromatin Assembly Factor 1 (CAF-1), specifically by the interaction between the H3/H4 proteins and two subunits of CAF-1 (Kaufman et al. 1995). This interaction with CAF-1 and H3/H4 is S-phase specific; outside of S-phase a protein complex called HIRA deposits H3.3/H4 onto histone mRNA (Tagami et al. 2004). A similar process takes place to deposit the H2A/H2B dimers onto the chromatin once the H3/H4 tetramer is in place. Nucleosome assembly protein 1 (NAP1) is a chaperone protein that assists in both the import of H2A/H2B into the nucleus, as well as likely having a role in the deposition of the proteins onto the chromatin itself (Mosammaparast and Ewart 2002).

The canonical histone genes and SLBP

Because of the importance of cell cycle regulation to the canonical, replication-dependent histone genes, they have a unique orientation within the genome. In humans, there is one large cluster of histone genes on chromosome 6. This locus, *HIST1*, encodes for 55 histone genes. A second, smaller cluster, known as *HIST2* is located on chromosome 2 and encodes for six genes (Marzluff et al. 2002). As mentioned above, these canonical histone genes are not polyadenylated. Instead they all end in a highly conserved 3' stem-loop (SL). This stem-loop is the cis-element that controls almost all of the elements of the post-transcriptional life of the canonical histone mRNAs. It is required for both the increased expression at the beginning of S-phase and the quick degradation at the end of it (Harris et al. 1991).

There is also a *trans*-element that works in concert with the stem-loop in controlling almost all aspects of histone mRNA metabolism – the stem-loop binding protein (SLBP). SLBP binds to the stem-loop for its entire life-cycle, both in the nucleus after transcription and when the message is being translated on the polyribosome. The interaction between SLBP and histone mRNA is a specific interaction facilitated by SLBP's non-traditional RNA binding domain (Z F Wang et al. 1996). SLBP is subsequently required for every other aspect of histone mRNA metabolism; processing, export from the nucleus, translation, and finally degradation (Z F Wang et al. 1996; Sullivan et al. 2009; Sánchez and Marzluff 2002; Mullen and Marzluff 2008). SLBP is cell cycle regulated during and after its translation (Whitfield et al. 2000). It is degraded at the end of S-phase through a process that is uncoupled from histone mRNA degradation, as inducing histone mRNA degradation during DNA synthesis does not induce SLBP degradation (Whitfield 2004). Instead, SLBP is

phosphorylated at two threonine residues and that phosphorylation is necessary for the degradation of the protein (Lianxing Zheng et al. 2003).

Histone mRNA transcription and processing

The expression of the replication-dependent histone genes is tightly tied to the cell cycle and DNA replication, as depicted in Figure 1. Histone genes are actually constitutively transcribed throughout the cell cycle, though their transcription increases 3-5 fold at the beginning of S-phase (DeLisle et al. 1983). This increase in transcription is regulated by the transcription factor NPAT. During S-phase, the Cyclin E/CDK2 complex phosphorylates NPAT, which upregulates histone mRNA transcription (Ma et al. 2000; Zhao et al. 2000). This is done through interactions between NPAT and a series of co-activators to increase transcription of the individual histone genes (Mitra et al. 2003; Fletcher et al. 1987; Lei Zheng et al. 2003; Gallinari et al. 1989).

This increase in transcription does not account for the total increase in histone message in the cell during S-phase. As mentioned, the increase in transcription is 3-5 fold during S-phase, but the total increase in message is on the order of 35-50-fold (DeLisle et al. 1983). The rest of the increase in histone message is accounted for by the processing of the histone mRNA. Processing is controlled by two cis-elements -- the stem-loop and a loosely conserved histone down-stream element (HDE) -- and well as SLBP and the U7 small nuclear riboprotein (snRNP), which act as trans-elements.

The RNA component of the U7 snRNP is the U7 snRNA, which has some complementarity with the HDE and is capable of binding to it (Mowry and Steitz 1987). Similar to the splicing-associated snRNPs (U1, U2, U4/U6, and U5), U7 snRNA has a binding for the Sm proteins. Instead of the D1 and D2 Sm proteins, however, the U7 snRNP

has two like-SM proteins – Lsm10 and Lsm 11 in their place (Pillai et al. 2001; 2003). This unique binding site and the two U7-specific Lsm proteins are important for histone mRNA processing; if the binding site is mutated to the traditional Sm-binding site and the canonical Sm-ring binds to U7, processing is abolished (Grimm et al. 1993).

The U7 snRNP and its unique Lsm proteins help create a platform for recruiting the rest of the factors required for histone mRNA processing. Lsm11 interacts with FLASH, a protein required for processing, and this interaction proved to be necessary for forming the cleavage complex at the 3' end of histone message (X c Yang et al. 2011; 2012).

Surprisingly, a number of factors are shared between cleavage of polyadenylated message and histone mRNA, despite the differences in the message and the different processing steps. Symplekin, CPSF73, CSTF100, and Fip1 are involved in both processes, with CPSF73 being required for cleavage in both situations (Kolev 2005; Dominski et al. 2005; Eric J Wagner et al. 2007).

Processing and degradation converge at the histone locus body (HLB). Histone genes were first mapped to nuclear loci by Joe Gall in 1981 (Gall et al. 1981) and first described as a distinct nuclear body in 2006, when it was observed as the U7 snRNP concentrated at the histone locus in a nuclear body separate from the Cajal Body (Liu et al. 2006). These HLBs can assemble with only one active histone gene repeat and gene expression is correlated to HLB assembly (Salzler et al. 2013). There is a conserved interaction between the factor that helps activate histone mRNA transcription, NPAT, and FLASH, that works to assemble the required transcription and processing factors together at the HLB (X c Yang et al. 2012).

Histone mRNA export and translation

After transcription, histone mRNA is transported out of the nucleus by TAP via the same mechanism as polyadenylated mRNA (Erkman et al. 2005). SLBP is also required for this process by some unknown mechanism – SLBP knockdown causes histone message to be retained in the nucleus and tethering SLBP to non-histone mRNAs causes them to be immediately exported as well (Sullivan et al. 2009).

Like most aspects of histone mRNA metabolism, translation requires the 3' UTR and the stem-loop, as well as SLBP. Work done in Chinese hamster cells showed that the stem-loop promotes translation, dependent on 3'UTR length and the RNA's 5' cap (Gallie et al. 1996). SLBP is required for translation as well, though notably only its presence is required; when *Xenopus* SLBP-1 was fused to human MS2 and expressed in rabbit reticulocyte with a reporter RNA containing an MS2 binding site, translation was increased (Sánchez and Marzluff 2002). Thus, both the stem-loop and SLBP are required for translation, but the interaction between the two elements is not. Instead, SLBP helps with translation through its interaction with the SLBP Interacting Protein (SLIP1). SLIP1 is also required for translation, and may facilitate the circularization of the message for translation, via its interaction with SLBP and its interaction with eIF4G (Cakmakci et al. 2008).

RNA DEGRADATION

The flow of information in the cell is well-known and well-taught as the Central Dogma of Molecular Biology: DNA is transcribed into RNA, RNA is translated into protein. In practice, of course, the way that DNA blueprints are turned into proteins is a much more complicated and regulated process. Because RNA is transcribed from DNA and translated into protein, it has long been a point of study for the control of gene expression. It can be

controlled at the level of transcription, splicing, processing, nuclear export, translation, and overall stability (Darnell 1982). A recent deep sequencing study of yeast mRNAs showed that different mRNAs have widely varying half-lives and that sometimes even different isoforms of the same gene see widely varying half-lives, along with some data that the 3' UTR is likely responsible for the control of that half-life (Geisberg et al. 2014). This thesis is particularly concerned with the regulation of RNA stability through the half-life of messenger RNA molecules, and with the process by which histone mRNA is degraded. As discussed above, histone mRNA's half-life is variable throughout the cell cycle and it has a unique 3' UTR, which makes it an ideal candidate to try and answer questions about the control of histone mRNA degradation and half-life.

De-adenylation dependent degradation

All messenger RNA (with one notable exception) has both a 5' 7-methylguanosine cap and a 3' poly(A) tail to provide stability to the message after transcription and splicing. Both of these elements must be removed in order for mRNA to be degraded. For bulk eukaryotic mRNAs, the first step in almost all mRNA degradation is the shortening of the 3' poly(A) tail. From this point, the cell can either re-polyadenylate the message, or take irreversible steps to degrade the message either in the 5'-3' direction via decapping, or the 3'-5' direction via the exosome (Garneau et al. 2007).

Deadenylation is largely carried out by two main cytoplasmic deadenylase complexes: the CCR4/NOT complex and the PAN2/PAN3 complex (Wahle and Winkler 2013). The CCR4-NOT complex is a large protein complex that is responsible for most deadenylation in yeast (Tucker et al. 2001) and is highly conserved from yeast to humans (Dupressoir et al. 2001). The complex has five conserved subunits (Ccr4, Caf1, Not1, Not2,

Not3/5) and two catalytic subunits (Ccr4 and Caf1) (Temme et al. 2004; 2010; Albert et al. 2000; Lau et al. 2009; Morita et al. 2007; Schwede et al. 2008). Both proteins have been crystalized, helping to reveal their mechanisms of action. Ccr4 binds specifically to adenosine residues, though its exact mechanism of cleavage (specifically, whether or not it cleaves into mono or dinucleotides) is currently uncertain (Hui Wang et al. 2010), while . Crystalization and purification of Caf1 revealed that divalent metal ions help determine the enzyme's substrate specificity. (Jonstrup et al. 2007).

The other complex involved in deadenylation is the cytoplasmic poly(A) binding protein (PABPC)-associated nuclease complex, or PAN complex. First identified in yeast (Boeck et al. 1996), the yeast and human complexes both consist of a catalytic subunit (PAN2) that interacts with a regulatory subunit (PAN3) (Uchida et al. 2004). PABPC is essential to its activity; PAN3 interacts with PABPC, which allows it to regulate the activity of PAN2 (Boeck et al. 1996; Uchida et al. 2004).

It is worth noting that there is evidence that the CCR4-NOT and PAN2/PAN3 complexes may complement each other. An experiment by Roy Parker's lab to investigate the role of the two complexes in yeast proved interesting in several aspects; knockout lines for the CCR4-NOT and PAN2-PAN3 complexes slowed down deadenylation and degradation of bulk RNA messages, a double knockdown of both complexes slowed down the degradation substantially more. A follow-up pulse-chase experiment showed that in the absence of both complexes, deadenylation in the cell was almost non-existent, indicating that both of the complexes contribute to general deadenylation in the cell (Tucker et al. 2001).

From this point, post-deadenylation, degradation can be carried out in either direction. In a process that was first discovered in yeast and is conserved in humans, the heptameric

Lsm1-7 ring binds the small remainder of the deadenylated tail, which works to target the mRNA for decapping via a specific interaction with the decapping activator Pat1 and an RNA-dependent interaction with the decapping protein Dcp1 (Tharun et al. 2000; Tharun and Parker 2001). This decapping is carried out by the Dcp1/Dcp2 complex, with the two subunits being discovered independently (Beelman et al. 1996; Dunckley and Parker 1999). In this complex, Dcp2 does the active removing of the cap and while it can act on its own, the presence of Dcp1 and its binding to Dcp2 greatly increases its activity (Steiger et al. 2003). Once decapping occurs, the message is degraded from the 5' end by the exonuclease Xrn1 (Hsu and Stevens 1993). Degradation in the opposite direction, from 3'-5', is carried out by the cytoplasmic version of the exosome (J S Anderson and Parker 1998), a massive complex of proteins that has multiple catalytic subunits and functions in both the nucleus and cytoplasm (Mitchell et al. 1997; Allmang et al. 1999). It is worth noting that these two processes seem to compensate for each other; genome-wide screens done for knockouts of components in both the 5'-3' pathway (He et al. 2003) and 3'-5' pathway (Houalla et al. 2006) did not greatly affect the levels of mRNA in *S. cerevisiae*.

Other pathways of RNA degradation

There are other types of RNA decay that are not dependent upon deadenylation. The endonucleolytic pathway involves an endonucleolytic cleavage in the 3' UTR between the poly(A) tail and the stop codon, allowing for either decapping and 5'-3' decay or 3'-5' decay of the two resulting RNA molecules as discussed above (Wu and Brewer 2012). PMR1 is a well-studied protein with this function – it's targeted to the polyribosome by a phosphorylation that's triggered under stressful conditions in the cell, targeting the message to stress granules for decay (F Yang et al. 2004; 2006).

The cell has also ways to degrade aberrant transcripts through non-traditional pathways. As histone mRNA degradation utilizes some of the proteins in these pathways, I will briefly discuss them now.

Nonsense-mediated decay

Nonsense-mediated decay, or NMD, is the best-studied RNA surveillance pathway. NMD occurs when the cell is able to both recognize and degrade RNA with a premature termination codon, or PTC. The main protein involved in NMD is UPF1. UPF1 binds the 3' UTR of mRNA non-discriminately; it doesn't require a PTC or translation (Kurosaki and Maquat 2013). UPF1 is then able to stimulate translation termination at the PTC by its interaction with two eukaryotic release factors, eRF1 and eRF3 (Czaplinski et al. 1998). Under normal circumstances, this interaction cannot happen. The cytoplasmic poly(A) binding protein (PABPC1) interacts with eRF3 to stimulate translational termination (Cosson et al. 2002). Furthermore, PABPC1 abolishes NMD when tethered downstream of a PTC (Kornberg 1977; Ansmant et al. 2007), providing a mechanism by which a normally terminated RNA message has the release factor eRF3 bound to the poly(A) associated protein PABPC1, therefore making it impossible to interact with UPF1. Premature stop codons, however, create excessive distance between eRF3 and PABPC1, therefore allowing the interaction with UPF1 that stimulates NMD.

Under most circumstances, another early NMD trigger is the stop codon being located upstream of the exon junction complex (EJC). This creates a binding platform for the NMD proteins UPF2 and UPF3 (Luger et al. 1997; Le Hir et al. 2001), which in turn form the core NMD complex with UPF1 (Hizume et al. 2005; J Lykke-Andersen et al. 2000) (Meeks-Wagner and Hartwell 1986; Melero et al. 2012). This is a very tightly regulated process and

likely requires coordination with PAPBC and the release factors; the presence of introns in the 3' UTR or an exceptionally long 3' UTR is not enough on its own to trigger NMD, as there are mRNAs that are not degraded by NMD (Talbert and Henikoff 2010; Singh et al. 2008).

The actual degradation of mRNA during NMD is a very complex process (there are at least four different methods by which this is carried out) that need not be discussed in detail here. In broad strokes, the UPF proteins, the release factors, and the SMG protein kinase complex form a SURF complex (SMG1-UPF1-eRF1-eRF3) at the premature stop codon. In addition to their kinase functions, the SMG complex contains an endonuclease capable of cleaving of the aberrant message so that it can be degraded bidirectionally, though the specifics of this vary depending on the exact manner of premature termination and the mRNA itself (Burgess and Zhang 2013; Søren Lykke-Andersen and Jensen 2015).

Non-stop decay

Messages without stop codons are also unstable in the cell, and are degraded through a different mechanism from NMD and general mRNA decay, as UPF1 and the decapping complexes are not required for this decay pathway (Worcel et al. 1978; Frischmeyer et al. 2002). Instead, these messages are decayed through a process termed Non-Stop Decay, which involves the exosome. In yeast, the exosome-associated protein Ski7p binds to the ribosome's empty A-site once the ribosome reaches the poly(A) tail or the extreme 3' end of the mRNA, bringing the exosome directly to the 3' end for 3'-5' degradation (Kaufman et al. 1995; van Hoof et al. 2002).

No-go decay

The final type of RNA surveillance occurs on transcripts with stalled ribosomes, called No-Go Decay. Studies in yeast indicate that this decay occurs via an endonucleolytic cleavage, requires translation, and requires the proteins Dom34 and Hbs1 (Tagami et al. 2004; Doma and Parker 2006). Dom34 has structural similarities to eRF1 (Mosammaparast and Ewart 2002; Carr-Schmid et al. 2002), while Hbs1 is similar to eRF3 (Marzluff et al. 2002; Carr-Schmid et al. 2002). Together, the two proteins promote the dissociation of ribosomal subunits (Harris et al. 1991; Shoemaker et al. 2010). A structural analysis of the Dom34:Hbs1:80S subunit complex confirms that the two proteins are responsible for this dissociation and provides a model in which the binding of the Dom34:Hbs1 heterodimer to the ribosome is favored over binding to elongation factors during stalled translation (Z F Wang et al. 1996; Becker et al. 2011). Dom34 and Hbs1 are conserved in mammalian cells, and the similarity of Hbs1 to Ski7 allows the heterodimer to function in mammalian non-stop decay as well (Z F Wang et al. 1996; Saito et al. 2013; Sullivan et al. 2009; Sánchez and Marzluff 2002; Mullen and Marzluff 2008).

Histone mRNA degradation

Just like the expression of histone mRNA is greatly up-regulated at the beginning of S-phase, it must be rapidly degraded at the end of S-phase. Degradation is also controlled by the stem-loop. When the last thirty nucleotides of the 3' UTR of a mouse histone gene were fused to human globin and put into mouse cells, those chimeric genes were quickly degraded when DNA synthesis was stopped (Whitfield et al. 2000; Pandey and Marzluff 1987). This degradation is also tied to translation – when histone mRNA translation is disrupted or when the 3'UTR is lengthened to place the stemloop well beyond the stop codon, degradation does

not proceed (Whitfield 2004; Graves et al. 1987). Further work indicated that the reason that degradation did not proceed properly with these long 3' UTRs is that translation termination is what's required to properly degrade message (Lianxing Zheng et al. 2003; Kaygun and Marzluff 2005b). Histone mRNA degradation also requires the NMD protein Upf1 (DeLisle et al. 1983; Kaygun and Marzluff 2005a), although its role in degradation isn't entirely clear.

Most mRNA degradation begins with deadenylation, however histone mRNA has no poly(A) tail to be removed and so degradation must be initiated through a different method. In 2008 it was shown that histone mRNA can be bidirectionally degraded, just like poly(A) mRNA and similarly that decapping, the Lsm1-7 complex, and the exosome are all involved. At the same time, oligo(U) tails were discovered at the 3' end of histone mRNA, both at the cleavage point and further into the stem-loop (Ma et al. 2000; Mullen and Marzluff 2008; Zhao et al. 2000). Further work showed that the Lsm4 – part of the Lsm1-7 ring – binds directly to SLBP and the Lsm1-7/SLBP complex is capable of binding the 3' ends of histone mRNAs that have been oligouridylated (Mitra et al. 2003; Lyons et al. 2013; Fletcher et al. 1987; Lei Zheng et al. 2003; Gallinari et al. 1989).

This oligouridylation is carried out by non-canonical poly(A) polymerases also known as terminal uridylyl transferases, or TUTases. These proteins were initially identified in yeast (DeLisle et al. 1983; Kwak and Wickens 2007) (Mowry and Steitz 1987; Rissland et al. 2007) and have been shown to have a wide variety of functions in the life cycles of snRNAs, miRNAs, snoRNAs, and traditional mRNAs (Pillai et al. 2001; Trippe 2006; Pillai et al. 2003; Heo et al. 2009; Berndt et al. 2012; Heo et al. 2012; Lim et al. 2014). Several different TUTases have been implicated in histone mRNA degradation (Grimm et al. 1993; Mullen and Marzluff 2008; Schmidt, West, and Norbury 2010a), and one of the goals of this

study was to better understand exactly how the TUTases are responsible for uridylyating the 3' end of histone message.

In order to better understand the nature of the oligouridylation of the 3' end of histone mRNA, our lab combined a high-throughput, next-generation sequencing method used to study miRNA (X c Yang et al. 2011; Newman et al. 2011; X c Yang et al. 2012) with a soft-clipping bioinformatics analysis of untemplated 3' additions in order to gain more insight into these additions (Kolev 2005; Slevin et al. 2014; Dominski et al. 2005; Eric J Wagner et al. 2007) (Liu et al. 2006; Welch et al. 2015). Our first experiments with this method expanded our knowledge of 3' end additions to histone mRNA greatly – they show that most histone mRNA is uridylylated in some form, though a great majority of those uridylation are one-nucleotide additions at the 3' end that appear to allow histone mRNA to maintain a consistent length. They also showed that histone mRNA is uridylylated both while capped and still associated with the polysome (Salzler et al. 2013; Slevin et al. 2014).

While much of the focus during histone mRNA degradation is placed on SLBP, a recent crystal structure of the histone mRNA/SLBP RNA-protein complex shows that a third protein, the human 3'-exonuclease (hEXO), is necessary for a stable structure. hEXO appears to function both in the processing of histone message, as well as in degradation. hEXO is specifically responsible for the trimming of two bases off of the 3' end of histone mRNA post-cleavage (X c Yang et al. 2012; Dominski et al. 2003), while a knockout of the protein in mice prevents histone mRNA from degrading entirely. Low-throughput sequencing of the knockout mice's histone mRNA shows the accumulation of longer oligo(U) tails (Erkman et al. 2005; Hoefig et al. 2012).

The goal of this study was to use our high-throughput sequencing and analysis methods (End-Seq and AppEnD) to better understand both the different types of uridylation at the 3' end of histone mRNA, the TUTases responsible for those uridylation, and the role of 3' hEXO in histone mRNA trimming and degradation.

TUTASES AND URIDYLATION

The history of uridylation, both in general and in mammalian cells, is long and wide-ranging. This subject has been well reviewed (Sullivan et al. 2009; Rissland and Norbury 2008), and I will provide a summary here. The earliest detection of uridine incorporation was actually in the late 1950s and early 1960s, when both rat livers (Gallie et al. 1996; CANELLAKIS 1957) and ascites carcinoma cells (Sánchez and Marzluff 2002; L Wang et al. 2002) were found to incorporate radioactive UMP and UTP, respectively. The best early example of a TUTase, though, came from the tobacco plant. In 1975, Brishammer and Juntti found and characterized what they called a poly(U) polymerase in tobacco leaves (Cakmakci et al. 2008; Brishammar and Juntti 1975).

In 2002 a *C. elegans* protein named GLD-2 was found to be the long sought-after cytoplasmic poly(A) polymerase (Darnell 1982; L Wang et al. 2002). This protein was found to be structurally similar to the DNA polymerase β -like superfamily of enzymes, which differ from the canonical polymerases in that they appear to be simpler, less processive enzymes (Geisberg et al. 2014; Holm and Sander 1995; Aravind and Koonin 1999). Cid1, a yeast protein shown to be active at the S-M checkpoint (Garneau et al. 2007; Rissland and Norbury 2008; S W Wang et al. 2000), was also shown to be a cytoplasmic poly(A) polymerase that prefers RNA substrates (Wahle and Winkler 2013; CANELLAKIS 1957; Read et al. 2002). Further *S. cerevisiae* work showed that two topoisomerase related factors, TRF4 and TRF5,

previously identified as part of the pol β -like superfamily and as important proteins for sister chromatid cohesion (Tucker et al. 2001; BURDON and SMELLIE 1961; Z Wang et al. 2000), also had the ability to poly adenylate RNA messages, though they were generally found to function in the nucleus and not the cytoplasm . They were also found to adenylate yeast histone mRNAs (Dupressoir et al. 2001; Holm and Sander 1995; Reis and Campbell 2006; Aravind and Koonin 1999)(as previously discussed, yeast histone mRNAs do not have stem-loops and the relevance of this finding to our own work on mammalian histone mRNA is unclear, though it will be discussed later in this thesis).

Using all of this information about non-canonical poly(A) polymerases across species, a study by Kwak and Wickens in 2007 was able to identify several of these proteins and establish some basic characteristics of this type of enzyme (Temme et al. 2004; Kwak and Wickens 2007; Temme et al. 2010; Albert et al. 2000; Lau et al. 2009; Morita et al. 2007; Schwede et al. 2008). They made several observations: these tails added by these enzymes are added to the 3' end, are non-templated, do not stimulate translation, and are often (but not exclusively) poly(U) tails. This study was unable to determine what caused the enzyme's specificity for uridine, but a recent crystal structure of Cid1 showed that a single histidine residue near the active site is responsible for the uridine specificity and that without that histidine, the enzyme will add adenine preferentially over uridine (Hui Wang et al. 2010; Yates et al. 2012).

Using the structural basis for these enzymes provided by these previous studies, our lab bioinformatically identified seven human proteins with similar domain structures as putative human TUTases (Jonstrup et al. 2007; Mullen and Marzluff 2008). We named them TUT1-TUT7, though most of the enzymes have different names that are often used

interchangeably. The seven human TUTases are depicted in Figure 2. In the last decade, much research has been done on all seven of these proteins, providing us with a much deeper picture of how these non-canonical poly(A) polymerases function across the cell. In the next section of this thesis, I will discuss recent research on each of these enzymes.

TUT1 (mtPAP/hmtPAP, PAPD1)

TUT1 was first identified as the human mitochondrial poly(A) polymerase, or mtPAP/hmtPAP. When the enzyme was knocked down by RNAi, several mitochondrial RNA messages were shown to migrate more quickly on gels, indicating the removal of a 50 nt or longer poly(A) tail (Boeck et al. 1996; Tomecki et al. 2004). Further knockdown studies of the enzyme show that depleting it results in both the loss of mitochondrial mRNA stability and general defects across the mitochondria, ranging from morphological changes to a change in oxygen consumption by the mitochondria (Uchida et al. 2004; Nagaike 2005).

Crystalization of mtPAP revealed several important details about the way that the enzyme functions. In addition to domains found in a canonical poly(A) polymerase, there is also a non-canonical RNA binding domain (RBD, or RNA-recognition motif, RRM) in the N-terminus, though it is still unclear whether this motif is capable of binding RNA. The active site is formed by a pocket between the finger and palm domains, where several of the amino acids π -stack with the adenine in ATP. Despite this interaction, the enzyme appears to be capable of adding any of the four nucleotides to mitochondrial substrates *in vitro*, though it adds ATP and UTP much more efficiently than CTP or GTP. Finally, the structural study revealed that mtPAP dimerizes. When a sextuple mutant was made to prevent dimerization, but to allow a normal monomer to form without changes to the active site, the enzyme's PAP activity was abolished (Bai et al. 2011).

TUT2 (PAPD4, hGLD2)

The human homologue of the *C. elegans* GLD-2 is known as TUT2, PAPD4, or hGLD-2 (Boeck et al. 1996; Kwak et al. 2004; Uchida et al. 2004). Its primary function is in the regulation of miRNAs. It's best-studied function is in the regulation of miR-122. In Gld2 knockout mice, the steady state of miR-22 is lower, despite the levels of pre-miR-122 remaining unchanged (Tucker et al. 2001; Katoh et al. 2009). A second study at the same time reproduced a similar finding in human fibroblast cells and further connects the enzyme to p53 regulation, as miR-122 negatively regulates p53 expression through binding with the cytoplasmic polyadenylation element binding protein (CPEB) (Tharun et al. 2000; Burns et al. 2011; Tharun and Parker 2001). Both studies showed that miR-122 has a monoadenylation that disappears when GLD-2 is not present. A more recent study tied that monoadenylation to the stability of miR-122, and showed the mechanism by which the monoadenylation can protect specific miRNA targets (Beelman et al. 1996; D'Ambrogio et al. 2012; Dunckley and Parker 1999).

TUT4 (ZCCHC11) and TUT7 (ZCCHC6)

The two zinc-finger non-canonical poly(A)polymerases (TUT4 and TUT7, or ZCCHZ11 and ZCCHC6) are also involved in microRNA regulation. TUT4, specifically, is involved in the regulation of let-7 via the intermediary protein Lin28, a well-known pluripotency factor. Lin28 mediates the interaction between TUT4 and let-7, which leads to uridylation of the pre-microRNA and eventually degradation (Steiger et al. 2003; Heo et al. 2009). Further studies into this topic show that this is not the only type of uridylation involved in the life of Group II miRNAs such as let-7. In fact, the presence of lin28 appears to determine the context of the uridylation. Without lin28, the pre-miRNAs are still

uridylated, but they are monouridylated instead of a longer tail. This monouridylation can be carried out by TUT4, TUT7, or hGLD2. This monouridylation creates an overhang for Dicer to process the pre-miRNA into a mature miRNA (Hsu and Stevens 1993; Heo et al. 2012).

The two zinc-finger TUTases have also been implicated in the stability of bulk mRNAs. Next-gen sequencing of a number of mRNA targets shows that mRNA with short poly(A) tails accumulate oligo(U) tails that are diminished with depletion of TUT4 and TUT7 by siRNA. *In vitro* assays confirm that this uridylation happens preferentially on messages with short poly(A) tails, and that the poly(A) binding protein (PABP) inhibits this uridylation. The mRNAs affected by this uridylation show a longer half-life when the two TUTases are knocked down, connecting this uridylation with global mRNA decay (J S Anderson and Parker 1998; Lim et al. 2014).

TUT3 (PAPD5, TRF4-2) and TUT5 (PAPD7, TRF4-1)

The earliest studies on the Trf proteins in *S. cerevisiae* implicated the protein as a DNA polymerase (Pol κ) involved in sister chromatid cohesion (Mitchell et al. 1997; Z Wang et al. 2000; Allmang et al. 1999). In 2004, it was shown to poly-adenylate tRNA messages in the nucleus to mark them for degradation via the nuclear exosome (He et al. 2003; Kadaba 2004). Further work the Trf proteins as the transferase enzymes in the nuclear polyadenylation complex known as the TRAMP complex, which works in tandem with the exosome to prevent traditional polyadenylation and promote degradation (Houalla et al. 2006; LaCava et al. 2005).

In humans two non-canonical poly(A) polymerases are analogues, of the yeast Trf4, TUT3 and TUT5. TUT3 is part of the human nuclear exosome complex, although it's not part of the nuclear exosome targeting (NEXT) complex, so it's unclear if its function in

nuclear surveillance is similar to its yeast homologue (Wu and Brewer 2012; Lubas et al. 2011; Sloan et al. 2012). Earlier results do indicate that TUT3 is involved in polyadenylating prematurely terminated RNA pol I transcripts and marking them for degradation by the exosome (F Yang et al. 2004; Shcherbik et al. 2010; F Yang et al. 2006), so it seems likely to be involved in the degradation of some targets in the nucleus.

An in-depth study was done on TUT3 in 2011, revealing several aspects of its function. It works as a poly (A) polymerase *in vitro* and it works without a co-factor. This is because it's able to bind its RNA target with a non-traditional RNA binding domain at its C-terminus. Using a cross-linking technique that utilizes photo-activatable ribonucleosides to facilitate an RNA immunoprecipitation (PAR-CLIP), TUT3 was also shown to target ribosomal RNA (Kurosaki and Maquat 2013; Rammelt et al. 2011).

TUT3 has also been shown to have at least two other functions separate from marking transcripts for degradation in the nucleus -- it's also involved in both snoRNA and microRNA maturation. The H/ACA class of snoRNAs are substrates for the poly(A) specific ribonuclease (PARN), which are adenylated to make them targets for the enzyme. This adenylation is provided by TUT3, which allows for the PARN trimming to create the snoRNA. There is also some evidence that this trimming is done by Rrp6, which is part of the nuclear exosome (Czaplinski et al. 1998; Berndt et al. 2012).

TUT3 has also been implicated in some of the same microRNA processing as other TUTases. The same study that identified TUT2 as a potential factor for adenylating miR-122 also indicated that TUT3 may be involved to a lesser degree (Cosson et al. 2002; Burns et al. 2011). It has also been shown to again couple with PARN in the regulation of miR-21, adenylating it to mark it for degradation by PARN (Boele et al. 2014). PARN, miR-21, and

miR-122 all function together in the regulation of tumor suppressor p53, which makes TUT3 an interesting future target to study.

There is not nearly as much known at this point about the other Trf4 analogue in humans, TUT5 (also known as PAPD7). Recently, however, an active isoform was discovered that may lead to new discoveries regarding the enzyme's function (Ogami et al. 2013).

U6 TUTASE

The longest-studied non-canonical poly(A) polymerase in humans is most likely the U6 TUTase. Its activity was first identified in 1987, when HeLa cell extracts were shown to uridylylate the 3' end of the U6 snRNA (Reddy et al. 1987). The responsible enzyme was identified as what is now known as the U6 TUTase (also known, confusingly, as TUT1) (Trippe et al. 1998). Some functional analysis of the enzyme was done to reveal a nucleolar localization and that it does not target U6atac RNA, even though it's a functional homolog of the U6 snRNP. The protein is not well-studied as compared to the other, previously discussed noncanonical poly(A) polymerases, though, as its an essential cellular protein and knockdown by RNAi results in lethality in HeLa cells (Trippe 2006).

The U6 TUTase is also the controversial poly(A) polymerase "Star-PAP" (Speckle-Targeted PIPK α -regulated Poly (A) Polymerase). Star-PAP was initially identified as a PIPK α -interacting protein that also interacted with the cleavage and polyadenylation stimulating factor-73 (CPSF-73) (Mellman et al. 2008). Further work indicated that Star-PAP stimulates the interaction between CPSF-73 and its pre-mRNA targets, thus making it essential for the cleavage that precedes polyadenylation (Laishram and Richard A Anderson 2010) and that there is a novel phosphorylation event controlled by PIPK α that makes it

specific for its pre-mRNA targets (Mohan et al. 2015). As mentioned above, this work is considered to be controversial as all of the research on the U6 TUTase as Star-PAP has come from one lab.

SUMMARY

Histone mRNA is a unique mRNA molecule; it has a 3' stem-loop in place of a poly(A), no introns, and is very tightly cell cycle regulated. The lack of poly(A) tail and very short half-life at the end of S-phase creates an interesting question about the method by which it is degraded; bulk mRNA degradation is initiated by deadenylation, and histone mRNA can't be deadenylated. Our lab has worked over the years to learn more about this process, and our research has revealed a number of requirements for histone mRNA degradation. We know that histone mRNA degradation requires both the stemloop and SLBP, and that it requires proper translation termination.

More recent work showed that histone mRNA is degraded bidirectionally using the same degradation complexes as bulk mRNA; Xrn1 carries out the 5' to 3' degradation after decapping, the exosome degrades the message 3' to 5', and the Lsm 1-7 complex is involved in degradation. A low-throughput sequencing assay revealed the existence of oligouridylations on some histone messages, including partially degraded messages, indicating that oligouridylation may be the method by which the cell initiates the degradation of histone mRNA in the absence of deadenylation.

We also identified seven putative human TUTases and developed a high-throughput sequencing method and bioinformatics work-flow to look at the oligouridylation of histone mRNA with great detail. Our earliest high-throughput sequencing experiments showed that oligouridylation is much more prevalent in histone mRNA than we initially anticipated; in

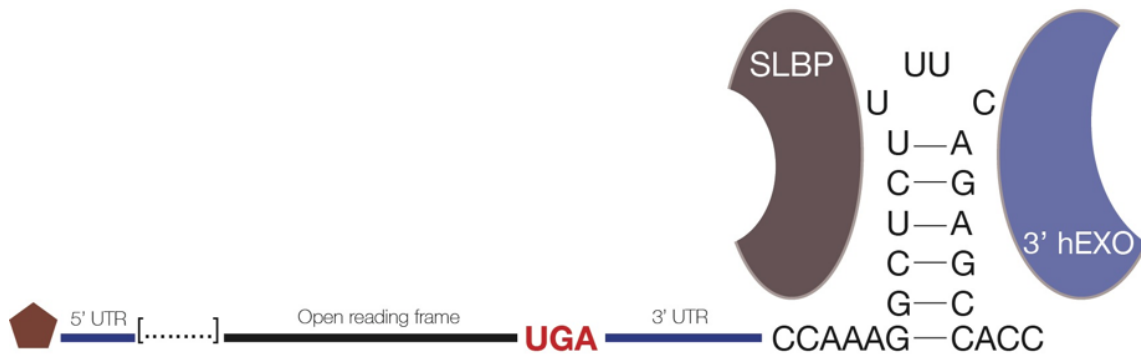
some genes, more than half of histone mRNA is oligouridylated and many of the oligouridylation marks look quite different from each other.

In Chapter 2 of this thesis, I focus on two main questions: Which enzyme or enzymes oligouridylate histone mRNA, and what function do these oligouridylation marks serve as related to both the degradation and general metabolism of histone mRNA? Using high-throughput sequencing and a bioinformatics analysis, I have been able to give these questions much better answers than we had in the past. In Chapter 3, I focus on the TUTases initially identified as uridylyating histone mRNA for degradation. Using data I've gathered over my time in grad school, I was able to rule these enzymes out for that particular role, though other evidence still indicates that at least one of them may be involved in a different part of the histone mRNA lifecycle. Chapter 4 of this thesis summarizes the work done in Chapters 2 and 3 and looks forward. While my thesis work has answered some questions and (hopefully) settled some controversies over the role of TUTases in histone mRNA degradation, it has raised many more questions about the role of oligouridylation in the histone mRNA lifecycle for future grad students to address.

Figure 1. Histone mRNA has a unique 3' end and is tightly cell cycle regulated.

A. The highly conserved histone 3' stem-loop and its two omnipresent (at least, in the cytoplasm) *trans*-factors, SLBP and 3' hEXO. B. Histone mRNA is tightly cell cycle regulated, with a combinationatorial change in transcription and processing resulting in an increase of histone message at the beginning of S-phase and a rapid reduction in half-life at the end.

A



B

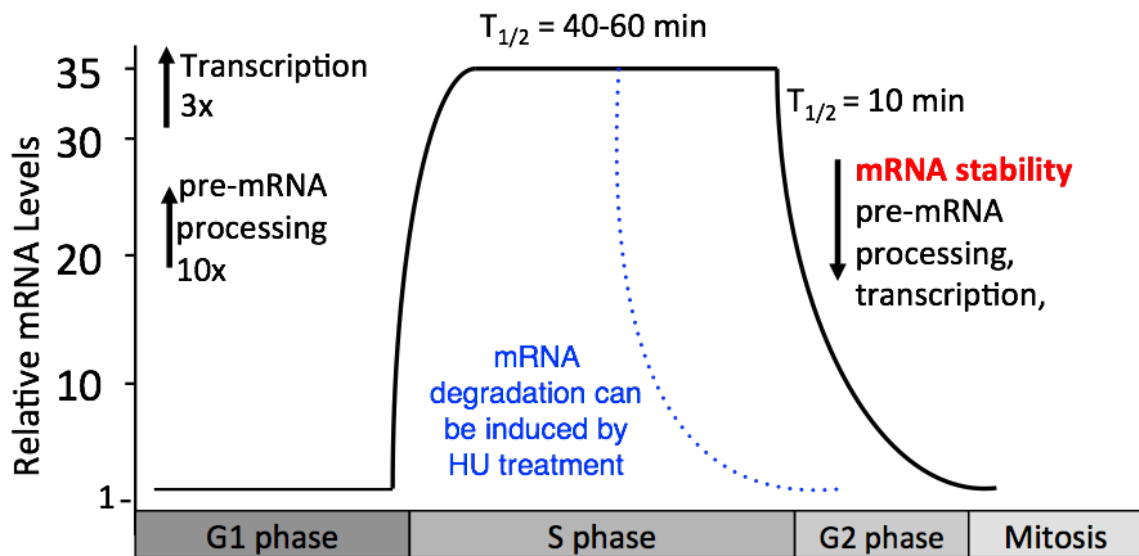


Figure 2. The TUTases

Seven non-canonical poly(A) polymerases have been identified as putative TUTases. Here they are depicted with approximate size and important domain structure. (Adapted from (Heo et al. 2012)).

TUT1 (mitochondrial TUTase)



TUT2 (GLD2)



TUT3 (PAPD5, TRF4-2)



TUT4 (ZCCHC11)



TUT5 (PAPD7, TRF4-1)



TUT6 (U6 TUTase)



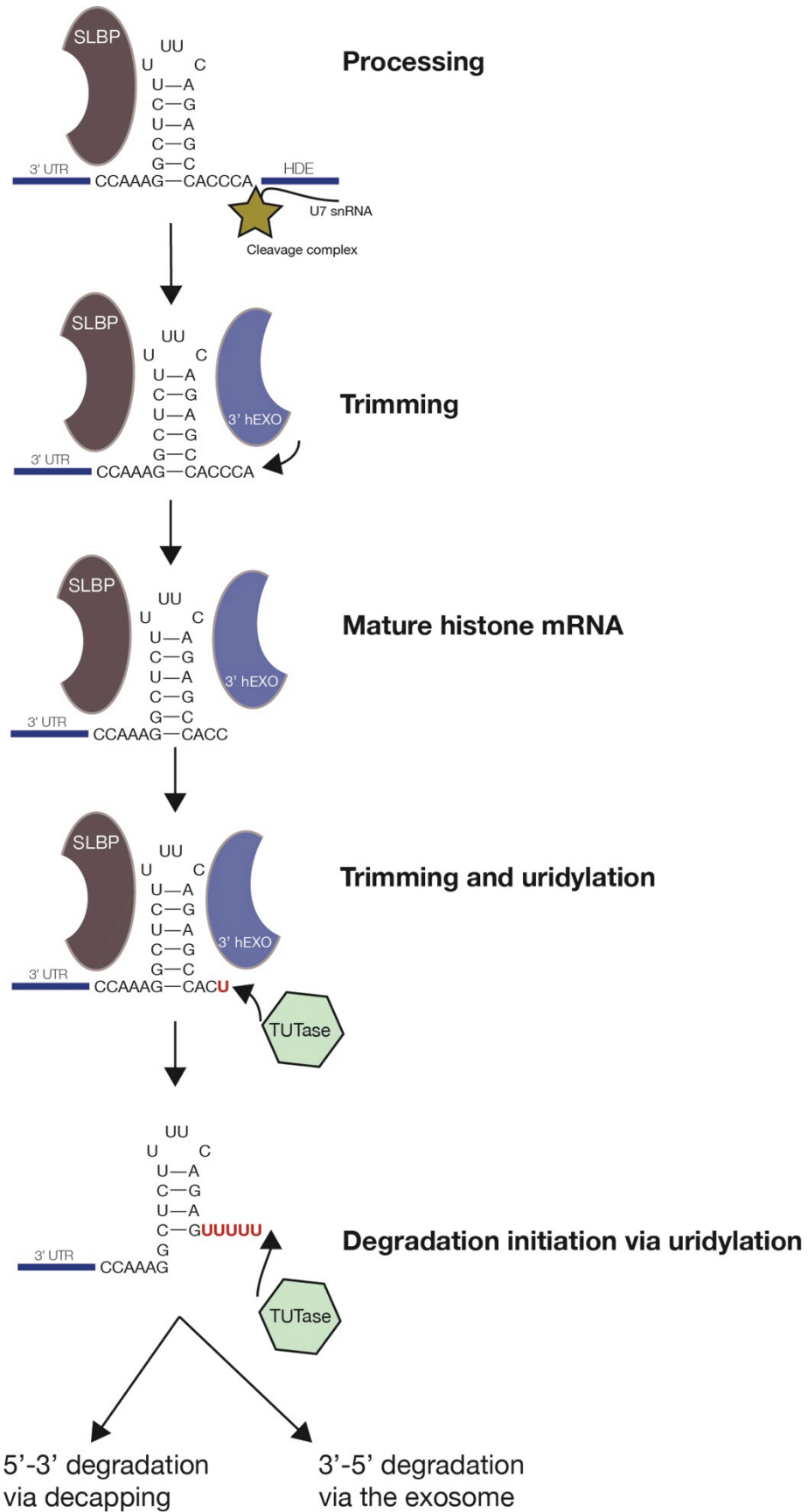
TUT7 (ZCCHC6)



Figure 3. Histone mRNAs are uridylated, likely in concert with the beginning of degradation

Histone mRNA is degraded bidirectionally like bulk mRNA, but its unique 3' end requires a number of unique steps before getting to that point. This is the best understanding of the life of histone mRNA prior to the work done in this thesis.

A



CHAPTER 2

TUT7 IS RESPONSIBLE FOR BOTH SHORT URIDYLATIONS OF STEADY-STATE HISTONE mRNA AS WELL AS THE LONGER URIDYLATIONS THAT INITIATE HISTONE mRNA DEGRADATION

INTRODUCTION

After our lab's initial discovery of the uridylation of histone mRNA (Mullen and Marzluff 2008), we set out to find a way to create a high-throughput sequencing method to detect these uridylations with much greater resolution than the circular PCR used in our early experiments. Mike Slevin, a post-doc in our lab, was able to adapt an existing method for deep-sequencing pre-miRNAs using a pre-adenylated linker (Newman et al. 2011). The advantage of this pre-adenylated linker is that it ligates to the 3' end of RNA without needing ATP, preventing an accidental circularization of the message during the ligation process. It also provides an anchor for reverse transcription of the ligated RNA, from which we can then specifically target and amplify the histone genes for deep sequencing. This is possible because of the high conservation in the open reading frames of histone genes; one primer is able to target most of the (for example) H2A genes (Marzluff et al. 2002) (see Figure 4 for a graphic representation of this sequencing method).

These first experiments to deep sequence histone mRNA (Slevin et al. 2014; Welch et al. 2015) left us with many questions about the nature of the oligouridylation and its role in

histone mRNA metabolism and degradation. The presence of a large number of messages that retained the length of a mature histone mRNA with one or two uridine residues at the 3' end was entirely unexpected. These studies also identified oligouridylations in the open reading frame for the first time. Combined with the longer uridylations in and around the stem-loop that we had prior knowledge of (Mullen and Marzluff 2008), this created several different types of uridylation and no real answer as to their functions, beyond the observation that the longer tails in the stem-loop tend to accumulate after HU treatment during degradation.

The question of which enzyme or enzymes are responsible for the uridylation was both unanswered and further complicated by these studies. Several candidate enzymes had been identified both by our lab and by others (Mullen and Marzluff 2008; Schmidt, West, and Norbury 2010b), but our deep sequencing experiments brought us to the realization that the previous looks at these enzymes via northern blots and RT-PCR was an insufficient picture of how these knockdowns affect degradation. As such, we decided to step back and take a wider view of the issue.

For the TUTases, we focused on TUT3, TUT4, and TUT7. As mentioned in the introduction, TUT3 is a human Trf4 analogue and has been implicated in the degradation of prematurely terminated RNA polymerase I transcripts via polyadenylation (Shcherbik et al. 2010) and the adenylation of snoRNAs for processing by PARN (Berndt et al. 2012). Both of these are nucleolar functions, where TUT3 is known to be part of the nuclear exosome targeting, or NEXT, complex (Lubas et al. 2011; Sloan et al. 2012), although it's currently unclear if its activity is limited to the nucleolus or even the nucleus. TUT3 will be further discussed in Chapter 3. TUT4 and TUT7 are zinc-finger TUTases that have implicated in

various parts of microRNA biogenesis (Heo et al. 2009; 2012; Kim et al. 2015) as well as uridylyating bulk mRNAs for degradation (Lim et al. 2014).

We also wanted to focus our efforts on the human-3'-exonuclease, or 3'hEXO. Recently, Matthias Heissmeyer's group knocked 3'hEXO out in mice and saw wide-ranging effects on histone mRNA degradation that we had previously been unable to detect via knockdowns and Northern blots; both the trimming back of the processed histone message from ACCCA to ACC and the rapid degradation of histone mRNA was completely abolished in these knockouts, and as a result the oligo(U) tails changed positions in these samples significantly (Hoefig et al. 2012). These results were accomplished with low-throughput sequencing, however, and so they encouraged us to try our higher-throughput methods to learn more about how 3'hEXO affects both uridylation and degradation.

MATERIALS AND METHODS

Cell culture and RNA interference

RNA interference experiments were done in HeLa cells using Invitrogen's Lipofectamine RNAiMAX for reverse transfections. In a six-well dish, 5 μ L of the Lipofectamine reagent and 100 pmol of siRNA were mixed in 500 μ L of serum-free OPTI-MEM media for 20 minutes. After 20 minutes, 200,000-250,000 cells were plated directly onto the transfection mix, a concentration that resulted in them being approximately 70% confluent the next day. The day after transfection, the cells were trypsinized and re-plated on 10 cm² plates so that they would be below 50% confluency for the ensuing HU treatment. A single plate was used for each time point. 48 hours after transfection, the cells were treated with 7.5 mM HU and RNA was harvested from the cells via TRIzol extraction (Ambion). A parallel plate was

harvested to make lysates for protein analysis by Western blotting. The siRNAs used are collected in Table 1.

Western blotting

Protein lysates were resolved by electrophoresis on 8% SDS-polyacrylamide gels and transferred to 0.45 μ m nitrocellulose membrane (BioRad). Westerns were carried out with a 3' hEXO antibody previously described (Dominski et al., 2003), Bethyl Labs' antibody to TUT4 (product number A302-636), and Proteintech's antibody to TUT7 (product number 25196-1-AP).

Northern blotting

1 μ g of total RNA harvested from HeLa cells per sample was loaded onto a 6% acrylamide/8 M urea gel. The gel was transferred to a positively charged Nylon membrane (GE Healthcare) and probed in either QuikHyb (Stratagene) or RapidHyb (GE Healthcare) buffer. Probes were generated by random primed, α^{32} P-dCTP labeling (PrimeIt II Kit, Stratagene) of PCR products generated to the ORF of cloned histone genes.

Preparation of samples for high-throughput sequencing

Forty-eight hours after siRNA transfection, the cells were treated with 7.5 mM hydroxyurea to stop DNA synthesis. RNA was harvested before HU addition and 15, 30, and 45 minutes. The amount of degradation was analyzed by Northern blotting as described above, mixing probes for histone H2a mRNA and 7SK RNA as an internal control (Mullen and Marzluff, 2008) to determine the amount of degradation. Two timepoints from each experiment were chosen for high-throughput sequencing: before HU treatment and usually the 30 minute timepoint, when about half of the histone mRNA had been degraded. The

remaining total RNA was treated with RQ1 DNase (Promega) and precipitated. Libraries specific for histone H2a and H2b mRNAs were prepared from this DNase treated RNA as previously described (Slevin et al., 2014; Welch et al., 2015); I will briefly summarize the method here. First, ~1 µg of preadenylated linker was ligated to 1.25 µg of the DNase treated total RNA in a 10-20 µL reaction using T4RNL2 K227Q (New England BioLabs) at 14°C overnight. RNA was phenol-chloroform extracted from this ligation and ethanol precipitated. The resulting pellet was used directly for first-strand synthesis using SuperScript III (Invitrogen). Approximately one-fifth of the 25 µL first-strand synthesis reaction was used for the first round of PCR. This consisted of 15 cycles of PCR carried out with hot-start Q5 high fidelity DNA polymerase (New England BioLabs) with primers specifically targeting both H2A and H2B genes. The first round of PCR was purified using magnetic HighPrep PCR beads (MagBio) and roughly quantified using the Nanodrop. Approximately 75 ng of the first round of PCR was used for a second round PCR reaction, again with 15 cycles and this time using primers with Illumina's adapter sequences specific for the first round primers. These libraries were quantified using a Qubit and a quality-control check was done with an Agilent 2100 BioAnalyzer. The Qubit reading and BioAnalyzer traces were used to calculate the concentration of the PCR libraries, which were then analyzed on a MiSeq using 125 nt paired end sequencing. All of the primers and linkers used in this protocol are collected in Table 2.

Mapping EnD-seq Data

Bioinformatic analysis on the samples was carried out by Joshua Welch. He mapped EnD-seq data to the hg19 to determine, for each sequencing read, the precise 3' terminus of transcription and the presence, length, and sequence composition of any untemplated 3'

additions. The data were analyzed using AppEnD as previously described (Welch et al., 2015). Briefly, AppEnD aligns reads to the genome using bowtie2 in --local mode, then identifies soft-clipped portions of the reads, corresponding to bases that do not match the genome. Untemplated 3' additions (if any) and the precise 3' terminus of transcription are then located using dynamic programming alignment of 3' linker sequence and the soft-clipped portion of the read.

RESULTS

The life cycle of histone mRNA is outlined in Fig. 3. The only processing step in histone mRNA biosynthesis is co-transcriptional cleavage of the nascent transcript to form the 3' end of histone mRNA, which occurs 5 nts after the stemloop in mammalian cells (Scharl and Steitz 1994). The mRNA is trimmed by 3'hExo, likely right after processing, removing 2 or 3 nts from the histone mRNA, which is the histone mRNA found in the cytoplasm. When DNA replication is inhibited, histone mRNA is rapidly degraded. The initial step in degradation is addition of an oligo(U) tail, which binds Lsm1-7, and then 3'hExo degrades the histone mRNA 3-4 nts into the stem. This degradation intermediate accumulates and is uridylated, resulting in rapid subsequent degradation of the histone mRNA by the exosome. When degradation stalls, the resulting intermediate may be uridylated to allow further 3' to 5' degradation.

We examined two points in the histone mRNA lifecycle: 1) The exonucleolytic trimming and uridylation of histone mRNA in the cytoplasm, and 2) uridylation of degradation intermediates during 3' to 5' degradation of the histone mRNA. We used our high-throughput sequencing strategy (EnD-Seq) and a bioinformatics analysis pathway (AppEnD) to identify untemplated 3' additions to histone mRNAs (Slevin et al., 2014; Welch

et al., 2015) (Figure 4) to analyze changes in the 3' ends of histone mRNAs, and in mRNA degradation intermediates that resulted from knockdown of different TUTases and 3'hExo. Examples of the most commonly detected uridylation products are shown in Figure 5. In all of our sequencing experiments, the HIST2H2AA3 gene was the most common read (it is also the most highly expressed histone gene (Graves et al. 1985), so this was not an unexpected result), and so most of the experiments shown here refer to this gene unless otherwise noted.

Most histone mRNA is uridylated in some form

When we synchronize HeLa cells to S-phase and make EnD-Seq libraries, we find that the majority of HIST2H2AA3 3' ends extend 3 nts beyond the stem, ending in ACC, ACU or (rarely) AUU (Figure 6). The ACU and AUU result from non-templated additions of uridines after shortening of the histone mRNA. The sequencing results are displayed in stacked bar plots, showing the positions of the untailed RNAs (see Figure 5 for a diagram of the numbered histone mRNA stem-loop), RNAs with a single non-templated U, two non-templated U's or more than 2 non-templated U's.

3'hEXO trims to maintain the length of histone mRNA

We first examined the mono-and-di uridylation at the 3' end of histone mRNA that create these ACU and AUU endings under most normal circumstances in HeLa cells. These were the most unexpected results in our lab's prior studies on histone mRNA, and they raised the possibility that histone mRNA uridylation has a wider utility than just initiating degradation. Because these uridylation exist solely at the 3' end of the message and because they appear in messages that are functionally the same length as "normal" histone mRNA, we started by knocking down 3'hEXO with siRNA (Figure 7A), then sequenced and mapped the 3' end of histone mRNAs using EnD-Seq and AppEnD. Note that while this level of

knockdown does not significantly affect the overall rate of histone mRNA degradation (Mullen and Marzluff, 2008), a knockout of 3' hExo stabilizes histone mRNA, and results in cytoplasmic histone mRNAs that ends 5 nucleotides (nts) after the stemloop (Hoefig et al., 2013). However, the knockdown of 3'hExo does perturb the overall distribution of 3' ends of histone mRNAs (Fig. 7B).

The knockdown resulted in several changes in the 3' ends of histone mRNA compared with control siRNA, consistent with less 3' trimming due to the decreased levels of 3' hEXO. There is small amount of histone mRNA that is untrimmed (ending in ACCCA), and a large increase in the histone message that is trimmed back by 1 nt to ACCC, and by 2 nt to ACC (Fig. 7B). There are also fewer mRNAs that were trimmed back further and uridylated to restore the original length of 3 nts. We quantified the changes in histone mRNA 3' ends in the pie charts in Fig. 8. In control cells only about 15% of the HIST2H2AA3 message ends in ACC, compared to 35% of mRNA in the 3'hExo knockdown cells. We also examined HIST1H2AG, an H2A with a shorter 3' UTR than HIST2H2AA3. We saw a similar change in this gene (Fig. 7C, 8B) with an increase in message ending in ACC, from ~33% to 45%.

The 3' hExo knockdown also affected the number and position of the one and two nucleotide U-tails added to the 3' end of histone mRNA. In control cells the majority of the 3' ends have a short U-tail replacement of the cytosine residues, ending in ACU or, less commonly, AUU. The unmodified ACC is present in lower amounts than the ACU. In the HIST2H2AA3 gene from control cells, 86% of the mRNAs end 3 nts after the stem loop, 6% extend beyond that point, and 7% are trimmed shorter but not uridylated. A similar distribution is seen in the HIST1H2AG gene, with ACC being more prevalent than in the

HIST2H2AA3 mRNA, but with ACU still the major 3' end. In the 3'hExo knockdown, there was a 4-5 fold increase in the number of mRNAs that ended more than 3 nts after the stemloop in both HIST2H2AA3, (28% compared with 6%) and in HIST1H2AG, (41% compared to 10%) (Fig. 7B-C, 8A-B). This increase resulted in fewer "mature" histone mRNAs that end three nucleotides after the stem (78.5%, down from 87.8%) and fewer "short" messages ending two or fewer nucleotides after the stem (2.4%, down from 6.4%), as seen in Table 1. Again, most (72%) of the HIST2H2AA3 molecules have been uridylated, with the uridylation often resulting in a 4 nt extension beyond the stemloop comprised of both templated and untemplated nts.

Because there are multiple non-allelic mRNAs for each core histone (Marzluff et al., 2002), we further analyzed HIST1H2AJ (Figure 8C, 8E) and HIST1H2AC (Figure 8D, 8E). Each gene had a reproducible and characteristic pattern of uridylation that varied slightly from each other mRNA, but the direction of the changes remained consistent; the hEXO knockdown always results in more long histone messages, both uridylated and un-uridylated, and a decrease in the shorter, partially degraded messages. In all, we saw these effects replicated in 17 histone genes (data not shown).

These results suggest that the one and two nucleotide tails that make up a large number of the reads at the 3' end in our control experiment and under normal conditions result from a balance of uridylation and exonucleolytic trimming and not from addition of specific number of nts by a TUTase.

TUT7 contributes to the addition of the U-tails to the 3' end of histone mRNAs

The other obvious question is the source of the oligo(U) tails at the 3' end of histone mRNA. To answer this question, we knocked down TUT7 >80% by siRNA (Figure 9A).

This knockdown had a similar efficiency to that of the 3'hExo knockdown, although it is once again worth noting that it was not a complete knockdown and there was still residual enzyme present in the cells. The knockdown had two major effects. There was nearly a 4-fold increase in the proportion of H2AA3 mRNAs in the TUT7 knockdowns that were shorter than the ACC/ACU/AUU 3' ends (from 5% in the control to 19% in the knockdown) (Fig. 9B), suggesting that 3'hEXO shortened the 3' end but the cell has a reduced ability to uridylate the 3' end and properly restore the length of histone mRNA. In the control siRNA cells, about 75% of the HIST2H2AA3 mRNAs had non-templated uridines at the 3' end. When TUT7 was depleted, that number dropped to 61%. There was a similar effect in H2AG mRNAs (Figure 9C), and in all the histone H2a and H2b mRNAs we analyzed. Thus, knockdown of TUT7 resulted in effects that are opposite of the effect of knocking down 3'hEXO.

As with the hEXO knockdowns, we quantified the HIST2H2AA3 and HIST1H2AG knockdowns in pie charts and a table in Figure 10 (HIST2H2AA3 in 10A, HIST1H2AG in 10B, the table in 10E) and compared them with the effects of the TUT7 knockdown on HIST1H2AJ and HIST1H2AC. Similar to the hEXO knockdown, we saw slightly different patterns of uridylation in each histone gene, but the same trend in each: the absence of TUT7 results in more diuridylation (AUU) and more short messages trimmed further in than the three nucleotides beyond stem. Taken together with the evidence shown for hEXO in Figures 8 and 9, this indicates a slower uridylation response by the cell when TUT7 is depleted.

Because this result was unexpected, we repeated the TUT7 knockdown experiment to replicate our data. As Figure 11 shows, even though the sequence results are slightly different than expected for the TUT7 replicate (there are more reads that end in ACCC vs. ACC in

both the control and the knockdown, which could be explained by a number of small environmental or incremental changes to the HeLa cells themselves), the uridylation pattern shifts in the exact same fashion for both HIST2H2AA3 and HIST1H2AG in the replicates as they did in the original experiment; there are more reads that end in AUU, and there are more reads slightly trimmed back but not uridylated back to the three nucleotide-beyond-the-stem point. We repeated the pie-chart analysis for this experiment in Figure 12, and came to the same conclusion; that our repeat experiment gave us very similar results to the first experiment.

There is one interesting observation to be made in the repeat experiment that is not obvious in the original round of sequencing on TUT7 knockdowns; in each of the four genes observed in Figure 12, there is a higher proportion of reads ending in ACC in the TUT7 knockdown reads than there is in the control. Although it's not immediately apparent from the sequences themselves, this may indicate that hEXO trimming is actually dependent on an initial uridylation by TUT7. This is likely a very transient uridylation, given hEXO's ubiquitous presence at the 3' end of histone mRNA, so this hypothesis may be difficult to validate.

Knockdown of TUT4 had minimal effects on uridylation of histone mRNAs

TUT4 is a very similar protein to TUT7, both structurally and functionally. It has been previously suggested that TUT4 and TUT7 can compensate for each other in uridylation of miRNAs and pre-miRNAs (Thornton et al. 2012). When we knocked down TUT4 (Fig. 13A), there was little effect on the 3' end of H2AA3 mRNAs when compared to the siRNA control. Neither the length of the mRNAs nor the pattern of uridylation was changed in HIST2H2AA3 or HIST1H2AG (Fig. 13B, 13C). As with the previous experiments, we

quantified these results in Figure 14 and added HIST1H2AJ and HIST1H2AC for comparison's sake. No significant effect was found in any of the genes analyzed.

However, when we knocked down both TUT4 and TUT7 (Fig. 15A) we saw a slightly larger effect on histone mRNA than knocking down TUT7 alone (Fig. 9). There was an increase in the number of shorter histone mRNAs, and a decrease in the number ending in ACU. When we knocked down TUT7 alone, we saw an increase in the reads ending in AUU such that the percentage of reads ending in ACU and AUU almost matched in the TUT7 knockdowns compared to the control. For example, for HIST2H2AA3 in our first TUT7 knockdown experiment (Figure 10) resulted in 57.6% of HIST2H2AA3 reads ending in ACU/AUU vs. 63.5% in the control. The second TUT7 experiment (Figure 12) saw those two numbers even closer together; 67.9% vs 72% in the control. The observation carried to HIST1H2AG, as well, with 40.6% of experimental reads ending in ACU/AUU vs. 48.4% in the control in our first experiment (Figure 10) and a 33.%/45.1% split in the second (Figure 12). In the double knockdown, however, a much lower percentage of reads are uridylated to restore the length of this 3' end. In HIST2H2AA3 (Figure 12C), 49.1% of the double knockdown reads end in ACU/AUU vs. 66.6% in the control. In HIST1H2AG (Figure 12D) the split is 33.9%/50.1%.

Since the TUT4 knockdown doesn't have this effect on its own (Figures 13-14), this seems to indicate that TUT4 may be capable of compensating for TUT7 when TUT7 is depleted.

Double sihEXO/siTUT7 knockdown provides more evidence that the enzymes work in concert at the 3' end of histone mRNA

We also knocked down TUT7 and 3'hEXO simultaneously by pooling the two siRNAs (Fig. 16A). The results were consistent with the conclusions drawn from the individual knockdowns. The 3' hEXO knockdown resulted in an increase in long reads in both HIST2H2AA3 (Fig. 16B, D) and HIST1H2AG (Fig. 16C, E), but most of those reads are untailed due to the absence of TUT7 (compare back to the long tails remaining in Figure 7, which included a significant number of reads in the two and three positions with longer oligouridylations).

Initiating histone mRNA degradation does not alter the oligouridylation pattern at the 3' end of histone mRNA

Armed with a better understanding of how histone mRNA is uridylated and how those oligouridylations are maintained, we moved our focus towards degradation. Specifically, we were first curious if these one-and-two-U oligouridylations at the 3' end of histone mRNA functioned to prime the message for degradation or to “restore” the 3' end of the message to a particular length to protect it from 3'hEXO until degradation begins.

We treated cells with HU to inhibit DNA replication and initiate histone mRNA degradation. The knockdowns of TUT4 and 3'hExo had minimal effects on the overall kinetics of histone mRNA degradation when DNA replication was inhibited (Fig. 16A), and the TUT7 knockdown showed a slight reduction in the rate of degradation at about 30 minutes after the HU treatment (Fig. 17A). Previously, we had analyzed knockdown of 3'hExo, TUT4 and TUT7, and found no significant effect on the overall rate of histone mRNA degradation (Dominski et al. 2003; Mullen and Marzluff 2008), although a

subsequent knockout of 3' hExo in mice demonstrated that it is essential for histone mRNA degradation (Hoefig et al. 2012). We reasoned that although the small amount of residual enzyme was sufficient to promote degradation, that RNAi would alter the pattern of degradation intermediates if we reduced the concentration of the enzyme. To determine the effect of knockdown of 3'hExo, TUT4 or TUT7 on the pathway of histone mRNA degradation, we analyzed the spectrum of degradation intermediates found during histone mRNA degradation by high-throughput sequencing when about 50% of the histone mRNA had been degraded as analyzed by Northern blotting.

After HU treatment there was no change in the distribution of 3' ends (nts 1-4 after the loop) compared to the same cells analyzed before HU treatment (Fig. 17B.-F, with the data quantified in Fig. 18, compared to Figs. 7, 9, 11, 13, and 15). This was true for the control cells, as well as the TUT7, 3'hExo and TUT knockdown cells. That is to say that the distribution of ends ending in ACC, ACU, AUU, longer ends, or shorter ends, was the same in the TUT7 knockdown cells (or 3'hEXO knockdowns, or TUT4 knockdowns, or double knockdowns, etc.) both before and after HU treatment. This result suggests that there is not a preference for initiating degradation among the histone mRNAs ending in AUU, ACU or ACC. Thus the monouridylation and oligouridylation that maintain the histone mRNA at the same length as the "normal," trimmed ACC message are not degradation intermediates, but rather mature histone mRNAs with slightly different 3' ends, which are formed by the activity 3'hEXO and TUT7.

Knockdown of TUT7 but not TUT4 affects the initial step in degradation of histone mRNA

The initial intermediate in histone mRNA that accumulates is a result of degradation into the 3' side of the stem by 3'hEXO (Hoefig et al. 2012)(Fig. 19A). In Fig. 19B-E, we show the degradation intermediates present at nts 5-20 in each of the siRNA knockdowns, removing the remaining mature histone mRNAs from the analysis. In control cells, the bulk of the intermediates are found at positions 7-9 from the 3' end, partway into the stem (Slevin et al. 2014), with the most abundant intermediate at position 7, the A 3 nts into the stem. These intermediates are extensively uridylated (Fig. 19A) and have a large fraction of longer tails (4-6 nts), with tails extending to >10 nts (Fig. 19F). In control cells, very few intermediates were detected further into the stem loop, suggesting that once degradation of this intermediate is initiated, degradation proceeds rapidly through the rest of the stemloop. These uridylated intermediates are subsequently degraded by the exosome (Slevin et al. 2014).

Knockdown of both TUT7 and 3'hExo individually altered the distribution and uridylation of these intermediates. When TUT7 was knocked down, there were many fewer uridylated RNAs, and the average length of the tails was shorter (Fig. 19B, 19F). There was also a change in distribution of the intermediates, with a substantial increase in intermediates that had been degraded further into the stem-loop, most of which were not uridylated. Surprisingly, knockdown of 3'hExo also affected uridylation of the intermediates (Fig. 19E). 3'hExo knockdown resulted in both a decrease in the number of tails (Fig. 19E) as well as in the length of the tails (Fig. 19F), which was strikingly similar to the effect of TUT7 knockdown. A difference between the 3'Hexo knockdown and the TUT7 knockdown was

that in the 3'hExo knockdown there was not further degradation into the stemloop – the degradation intermediates accumulated in the 7-9 nucleotide region, like in the controls. When we knocked down both TUT7 and 3'hExo, we saw a further decrease in the number of tails, as well as fewer molecules degraded further into the stemloop compared with the TUT7 knockdown. This result is consistent with the further degradation resulting from additional digestion by 3'hExo. Taken together these results suggest that 3'hExo and TUT7 work together in degrading the histone mRNA into the stemloop (see Discussion).

In contrast to TUT7 knockdown, knockdown of TUT4 did not change the distribution of intermediates or affect their uridylation (Fig. 19C). However, the double knockdown of TUT4 and TUT7 resulted in a further enhancement of the effect of TUT7 knockdown. There was a further reduction in the total number of U-tails, and a much more dramatic increase in the number of degradation intermediates located further into the stem (Fig. 19D). These results are consistent with TUT7 being the primary enzyme that adds the U-tails to these degradation intermediates, with TUT4 being able to partially compensate for TUT7 when TUT7 was knocked down.

There is a small peak of RNAs at position 20, the last base of the stemloop. This is present in all samples, but is most prominent in the TUT7 knockdown and TUT4/TUT7 double knockdowns. The U-tails at this position are similar in length to those at positions 7-9 in the stem, 4-6 nts long in the control and TUT4 knockdown, and are much shorter in the TUT7 and TUT4/TUT7 knockdowns. Thus they are likely added by TUT7. Since Upf1 is required for histone mRNA degradation and bound to the 3' UTR of histone mRNAs before the stemloop (Zünd et al. 2013; Brooks et al. 2015), these intermediates could result from a

pause in degradation due to the presence of Upf1 on the partially degraded RNA (see discussion).

Subsequent 3' to 5' degradation through the coding region of the histone mRNA does not require TUT7

Degradation of histone mRNA 3' to 5' can be divided into two stages: 1) Degradation into the stemloop by 3'hExo and subsequent uridylation of the degradation intermediates, and 2) rapid degradation to just 3' of the ribosome by the exosome, followed by pausing and uridylation of the degradation intermediates, which continues as the exosome goes through the coding region. There are very few degradation intermediates detected in control cells between the 3' side of the stemloop and a position in the 3' UTR about 15 nts from the termination codon (Fig. 20A), which we have previously shown is likely due to arrest of degradation by the terminating ribosome (Slevin et al. 2014). The relative proportion of these two degradation intermediates reflects the flux of intermediates through the pathway. In control cells treated with HU, the relative amounts of the two types of intermediates (those in the stemloop and those in the 3'UTR and coding region) are constant in multiple experiments. A change in proportion of the two populations of intermediates will occur when there is inhibition or acceleration of one of the steps. For example, when the exosome component, Pml/Sc100 was knocked down, there was an increased accumulation of uridylated intermediates in the stemloop (Slevin et al. 2014), consistent with degradation through the 3'UTR and coding region being catalyzed by the exosome.

In the TUT7 knockdown there was also a change in the relative proportion of these two classes of intermediates. There are many fewer uridylated intermediates in the stemloop, although an increase in the total intermediates in the stemloop. There are also fewer

intermediates in the coding region (Fig. 20B). This is consistent with TUT7 playing a critical role in the accumulation and ultimate degradation of the intermediate in the stemloop, as a result of uridylation of the intermediate. The generation of the second class of intermediates is slowed, and they may be removed by the exosome in a TUT7-independent manner. Hence they are more rapidly removed by the degradation machinery (exosome) which has not been affected by the TUT7 knockdown. This effect was not seen with the TUT4 knockdown, where the relative amounts of the two classes of intermediates remained similar to the control, consistent with it not playing a major role in any of the steps in degradation. There was also no effect of TUT7 or TUT4 knockdown on the proportion of the intermediates with U-tails or in the length of the U-tails (Fig. 20D), in contrast to the dramatic effect of TUT7 knockdown on the number and length of U-tails in the stemloop (Fig. 19).

We conclude that TUT7 plays a major role in histone mRNA metabolism. It is both responsible for the uridylation of the 3' end of the histone mRNA to maintain the proper length of histone mRNA when the mRNA is relatively stable, and for the uridylation of degradation intermediates during the initial steps of degradation by 3'hExo into the stem. In both these steps, TUT7 and 3'hExo likely work together.

DISCUSSION

A major regulatory step in histone mRNA metabolism is regulation of histone mRNA half-life to maintain the coupling of histone mRNA levels with the rate of DNA replication. There is coordinate degradation of the family of replication-dependent histone mRNAs, which is mediated through the stemloop at the 3' end of the mRNA, and the proteins bound to the mRNA, SLBP and 3'hExo. Degradation of histone mRNA requires that the mRNA be

actively translated (Graves et al., 1987; Kaygun and Marzluff, 2005b), and recruitment of Upf1 is an early step in triggering degradation (Kaygun and Marzluff, 2005a). Histone mRNA degradation requires many of the same factors required for degradation of polyadenylated mRNAs, including Lsm1-7 and the exosome. Knockdown of Upf1, Lsm1-7 or the exosome has a greater effect on degradation than knocking down components of the 5' to 3' decay pathway, consistent with a major pathway of histone mRNA degradation proceeding 3' to 5'. Uridylation of the 3' end of histone mRNA plays a prominent role in histone mRNA metabolism. Following 3' end formation in the nucleus, SLBP and 3'hExo form a stable complex on the 3' end of histone mRNA (Yang et al., 2006; Tan et al., 2013), and 3'hExo trims 2 nts off histone mRNA, resulting in the major cytoplasmic histone mRNA that ends 3 nts after the stem loop (Mullen and Marzluff, 2008; Hoefig et al., 2013).

The length of cytoplasmic histone mRNA is maintained by uridylation of the mRNA; if the mRNA is trimmed to shorter than 3' nts after the stemloop, uridines are added to restore the length. In S-phase, the most abundant 3' ends have a nontemplated uridine on the 3' end. In the 3'hExo knockout, histone mRNAs end 5 nts after the stemloop (Hoefig et al., 2013). Thus, there are likely cycles of 3'hExo degradation followed by uridylation that create the final population of cytoplasmic histone mRNAs in growing cells. Our results confirm this hypothesis; knockdown of hEXO results in an increase in long histone mRNAs, and a slowing of the cell's uridylation response by TUTase knockdown results in a shift of these short uridylation one nucleotide further back, suggesting that hEXO is trimming longer tails to length and not that the TUTases are specifically adding a certain length tail based on context.

Initiation of histone mRNA degradation requires oligouridylation of histone mRNA, and oligouridylated full-length (5 nts after the stemloop) histone mRNA accumulates in the 3'hExo knockout (Hoefig et al., 2013), suggesting that uridylation of the 3' end is a critical step in initiating degradation (Su et al., 2013). The initial intermediate that accumulates during degradation results from partial degradation into the stemloop (Ross et al., 1986;Hoefig et al., 2013;Slevin et al., 2014) by 3'hExo and this intermediate is extensively uridylated (Mullen and Marzluff, 2008;Hoefig et al., 2013;Slevin et al., 2014). Since cytoplasmic histone mRNAs end in a stemloop with only 2-3 nts after the stem, they cannot bind Lsm1-7 without the addition of >5 nts to the cytoplasmic mRNA (Lyons et al., 2014). The requirement for Lsm1-7 in degradation of histone mRNAs results from the addition of an oligo(U) tail either to the 3' end and/or to the initial degradation intermediate in the stem, providing a binding site for Lsm1-7. The tails that accumulate on this intermediate are close to the size needed to bind Lsm1-7, suggesting that once they get long enough to bind Lsm1-7, further degradation can take place.

Previous studies using RNAi knockdown of the enzymes potentially involved in histone mRNA degradation (3'hExo, different TUTases) showed at best modest effects on the overall rate of histone mRNA degradation (Dominski et al., 2003;Mullen and Marzluff, 2008;Schmidt et al., 2011). . Much more significant effects are observed with knockdown of factors also required for degradation of many other mRNAs (Upf1, Lsm1, and exosome components (Kaygun and Marzluff, 2005a;Mullen and Marzluff, 2008;Slevin et al., 2014). The results we obtained with knockdown of 3'hExo illustrate this most clearly since very effective knockdown of this enzyme (>90%) had no significant effect on the rate of histone mRNA degradation, as detected by Northern Blotting (Dominski et al., 2003;Mullen and

Marzluff, 2008). Only when 3'hExo was knocked out was histone mRNA degradation severely affected (Hoefig et al., 2013). The knockout cells contained histone mRNA that was not trimmed (had 5 nts after the stem), and was uridylated at the 3' end (but not degraded) demonstrating a critical role for 3'hExo in both forming the 3' end of cytoplasmic histone mRNA and initiating histone mRNA degradation (Hoefig et al., 2013).

We and others have obtained similar inconclusive results in RNAi knockdowns of the putative TUTases, with at best modest effects on histone mRNA degradation (Mullen and Marzluff, 2008; Schmidt et al., 2011). The development of methods to analyze degradation intermediates by high-throughput sequencing has allowed us to look for potential changes in the distribution of intermediates in mRNA degradation as a result of knockdown of different factors. Since we have identified discrete intermediates in histone mRNA degradation, we were able to assess changes in the relative rates in different steps of histone mRNA degradation, which are reflected in the relative proportion of different intermediates. For example, we previously showed that when the exosome subunit Pml-Sc100 is knocked down, the relative proportion of uridylated intermediates in the stem increased (Slevin et al., 2014) [Table 1], consistent with their being the initial targets of the exosome.

The experiments reported here show that knockdown of TUT7 and 3'hExo result in significant changes in the uridylation of the 3' end of histone mRNA to maintain the normal length of mature histone mRNA and in the distribution of mRNA degradation intermediates. These results strongly suggest that TUT7, and not TUT4, plays the major role in uridylation of histone mRNA, both to maintain the normal length of the 3' end in S-phase cells, and to uridylate prominent degradation intermediates.

TUT4 and TUT7 are similar proteins, which were first reported to uridylate both pre-miRNAs and mature miRNAs. In some studies they have been reported to act redundantly and/or compensate for one another. In a recent study, Narry Kim and coworkers showed that both TUT4 and TUT7 add the U-tails present on some oligoadenylated mRNAs after deadenylation (Lim et al., 2014; Chang et al., 2014), marking them for degradation. These enzymes seem to be largely interchangeable for this uridylation, as shown by RNAi and *in vitro* uridylation experiments. The enzymes have also been reported to have specific functions, as well. TUT7 has been shown to add a single uridine to specific miRNA precursors by Kim and workers (Heo et al., 2012). They also reported that TUT4 specifically uridylates let-7 pre-miRNA through an interaction with Lin-28 (Heo et al., 2009), although it has also been reported by Richard Gregory's lab that TUT7 can compensate for this function of TUT4 (Thornton et al., 2012).

Knockdown of TUT7 dramatically changed the uridylation pattern of histone mRNAs, both in growing cells and after inhibition of DNA replication. Knockdown of TUT4 had no detectable effect on either process. However, knockdown of TUT4 together with TUT7 has a larger effect on uridylation of histone mRNA than knockdown of TUT7 alone, suggesting that TUT4 can participate, albeit inefficiently, in the uridylation of histone mRNA. Thus, we conclude that TUT7 is the major enzyme in the cell that uridylates histone mRNA, both on the stem loop to maintain the proper length of the 3' end and in the stem after initial degradation by 3'hExo to trigger further degradation of histone mRNA.

TUT7 and 3'hExo may collaborate in the uridylation of histone mRNA.

Two aspects of our data suggest that TUT7 and 3'hExo may function together in maintaining the levels of histone mRNA uridylation. Knockdown of 3'hExo unexpectedly

altered the pattern of uridylation on the degradation intermediates in the stemloop, reducing both the number and length of the tails, although there was no change in the amount of TUT7. The number and length of the U-tails on the intermediates were reduced after HU treatment in the 3'hEXO knockdown cells, to the same extent that they were reduced in TUT7 knockdown cells. Knockdown of 3' hExo also altered the uridylation of the 3' end of histone mRNA in growing cells, with an increase in histone mRNAs ending in ACCU and ACUU, suggesting that TUT7 adds a non-specific number of uridines to the histone mRNAs that are then trimmed back by 3'hExo. These results are consistent with the possibility that 3'hExo and TUT7 function together to both maintain the normal length of the 3' end in S-phase cells, and to uridylate the 3' end of histone mRNAs, as well as the degradation intermediates in the stemloop as part of the pathway degradation.

The TUT7 knockdown and the TUT4/TUT7 double knockdown also show slight increases in messages ending in ACC with no uridylation. This suggests that the initial exonuclease activity of 3'hEXO may require some uridylation at the 3' end of histone mRNA. We do see a number of messages ending in ACCU in the 3'hEXO knockdowns, which could be interpreted as a "primed" substrate for 3'hEXO to begin its exonuclease activity.

Fig. 21 shows our current model for histone mRNA degradation. TUT7 uridylates the 3' end of histone mRNA to compensate for chewing of the 3' end by 3'hEXO, until a change in the histone mRNP allows for more chewing by hEXO. Once hEXO chews into the stem-loop, TUT7 puts a longer oligo(U) tail on the partially degraded stem-loop, which marks the message for degradation.

An early step in histone mRNA degradation is likely the recruitment of Upf1 to the histone mRNP as a result of inefficient translation termination (Kaygun and Marzluff, 2005b). Upf1 binds to the histone mRNP by binding to the 3' UTR (Zund et al., 2013; Brooks, III et al., 2015) and to SLBP (Kaygun and Marzluff, 2005a) after DNA synthesis is inhibited. How the initial degradation into the stem by 3'hExo is activated or how the TUTase is recruited is not known, but it likely requires the helicase activity of Upf1 which may perturb the binding of SLBP to the stemloop, allowing initial degradation by 3'hExo into the stem. Since 3'hExo and SLBP both interact with the C-terminal tail of Lsm4 in Lsm 1-7 (Lyons et al., 2014), TUT7 and 3'hExo together may also interact with the Lsm1-7 complex to promote recruitment of this complex to the 3' end of the degradation intermediates in the stemloop, which may then result in subsequent degradation by the exosome.

The initial step in degradation of polyadenylated mRNAs is deadenylation leaving an oligoA-tail which can bind Lsm1-7. Recent studies found that a fraction of the shortened A tails have one or two uridines added to the 3' end, both in fission yeast (Rissland and Norbury, 2009) and mammalian cells (Lim et al., 2014), suggesting that oligouridylation may participate in degradation of polyadenylated mRNAs as well, although as yet there are no examples of specificity for this uridylation for specific subsets of mRNAs.

Fig. 4. EnD-Seq workflow

A diagram of the ligation, reverse transcription, and PCR steps used in EnD-Seq before sequencing on the MiSeq (adapted from (Slevin et al. 2014)). At the bottom of the figure is a more detailed look at the Read 1 sequence, which detects the untemplated additions to the 3' end of histone mRNA.

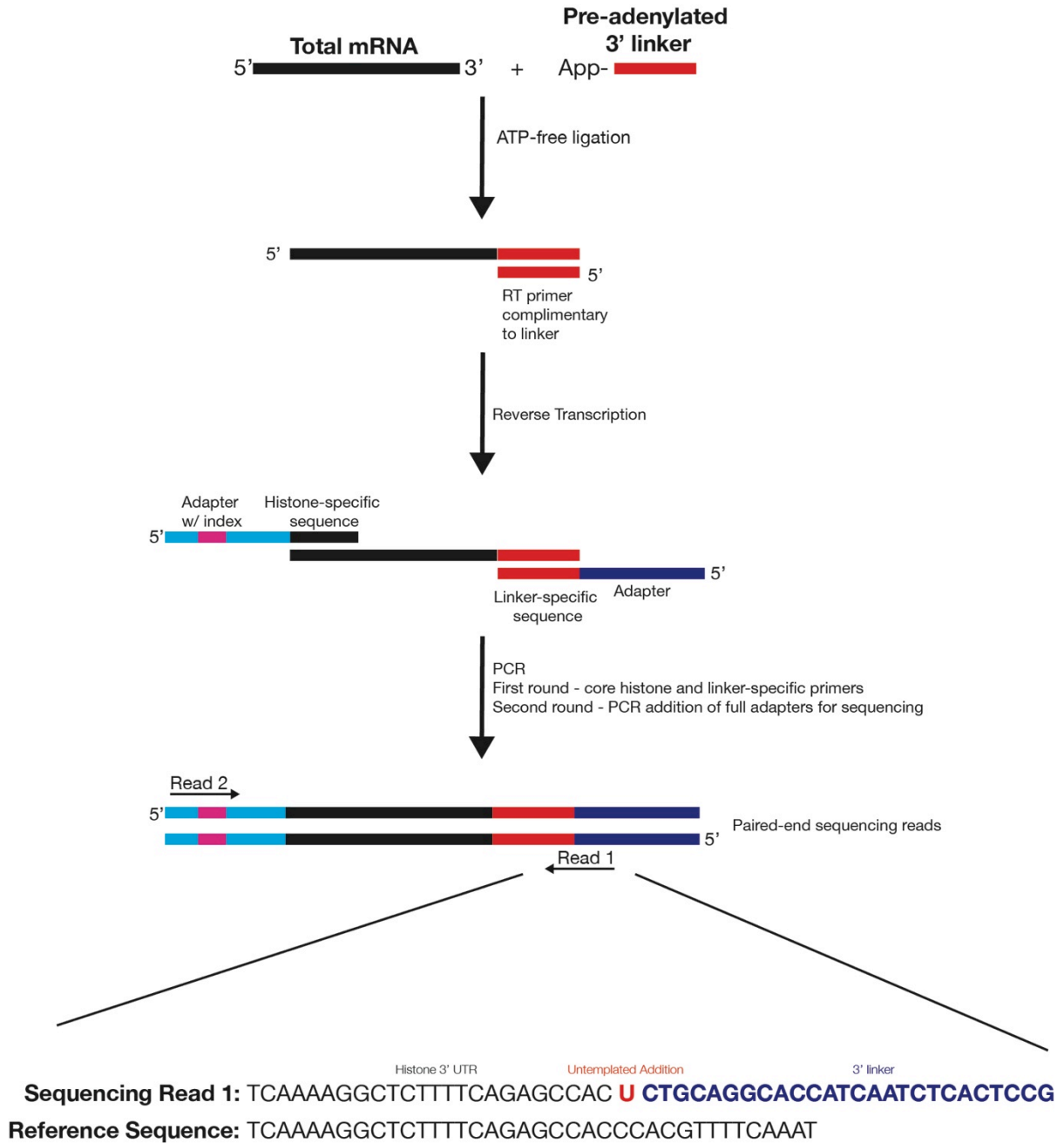
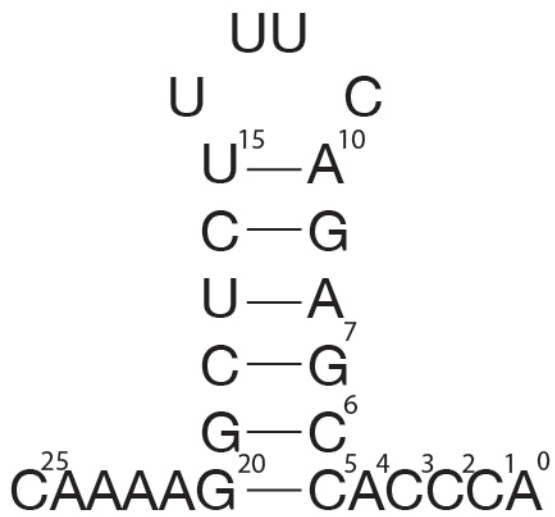


Figure 5. Common EnD-Seq products

The major 3' ends of histone mRNAs in exponentially growing cells, ending in either ACC (templated), ACU or AUU with uridine additions restoring the length of the mRNAs. The stemloop shown is HIST2H2AA3, the most commonly sequenced gene in our EnD-Seq experiments.



⁵ ⁴ ³ ²
 CACC
Mature 3' ends: CACU
 CAUU

⁵ ⁴ ³
 CAC
Short 3' ends: CAU

⁵ ⁴ ³ ² ¹ ⁰
 CACCCA⁶
Long 3' ends: CACCC
 CACCU
 CACUU

Figure 6. EnD-Seq results for synchronized HeLa cells

This stacked bar graph is representative of HIST2H2AA3 uridylation under the most favorable circumstances for histone mRNA (that is, during S-phase, when histone message is most highly expressed and has its longest half-life). The stacked bar graph represents the length of uridylation at each nucleotide position at the 3' end.

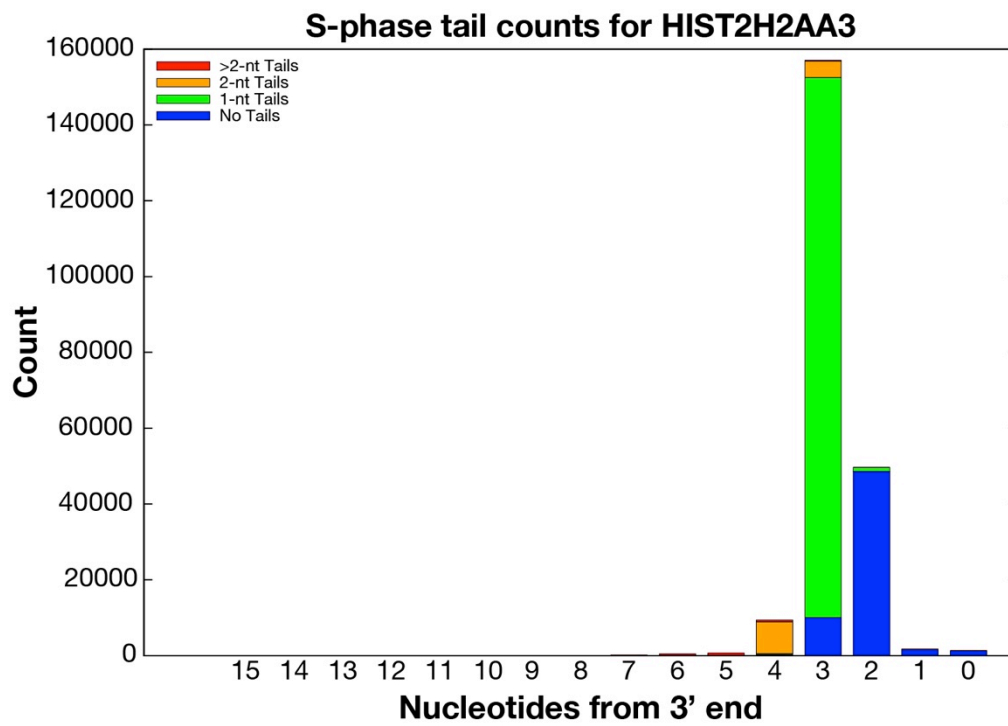


Figure 7. Knockdown of 3' hEXO changes histone mRNA uridylation patterns

A. Western blot showing 3' hEXO knockdown by siRNA. B.-C. Stacked bar graph for our high-throughput sequencing experiments showing the last eight nucleotides of HIST2H2AA3 (B) or HIST1H2AG (C) for the hEXO knockdowns. For both experiments, control panels are on the left and experimental on the right.

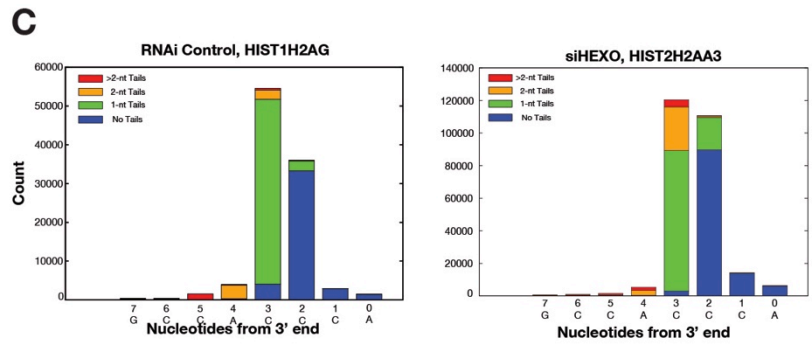
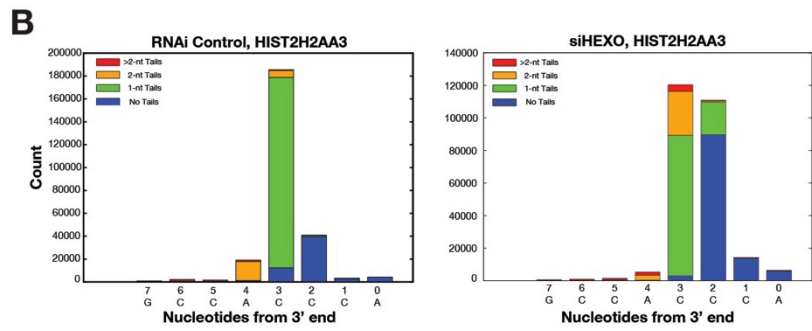
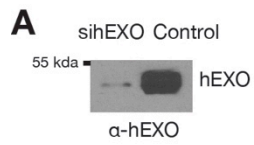
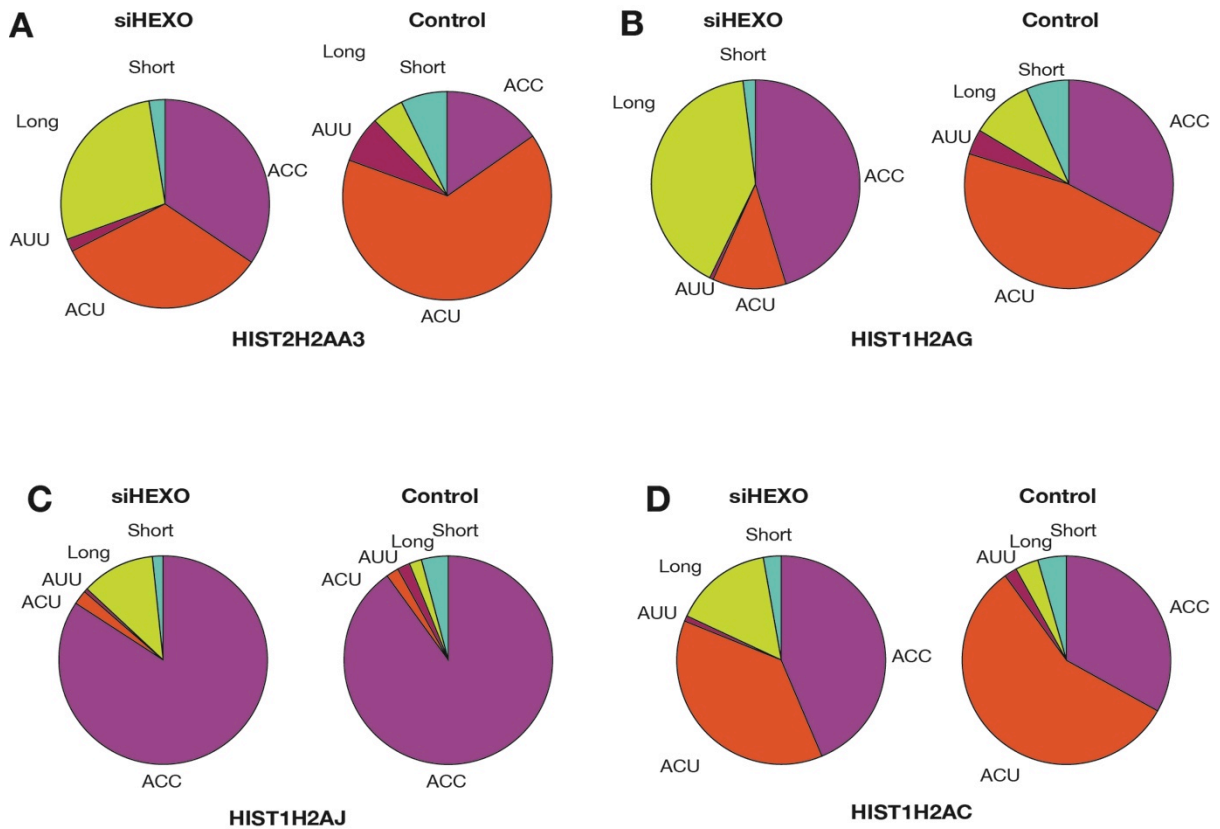


Figure 8. Quantification of different histone mRNA 3' ends after hEXO knockdown

A.-D. Pie graphs quantifying the different species of histone mRNA 3' ends shown in Figure 7, and how they change with 3' hEXO knockdown. This analysis was done for HIST2H2AA3 (A), HIST1H2AJ (B), HIST1H2AJ (C), and HIST1H2AC (D). The percentages for the graphs are shown in (E).



E

		ACC	ACU	AUU	Long	Short
HIST2H2AA3	siHEXO	34.5%	33.2%	1.9%	27.9%	2.4%
	control	15.4%	64.6%	7.0%	5.9%	7.1%
HIST1H2AG	siHEXO	45.4%	11.5%	0.4%	40.9%	1.8%
	control	32.9%	47.1%	3.6%	9.8%	6.6%
HIST1H2AJ	siHEXO	84.4%	2.0%	0.7%	11.4%	1.5%
	control	90.3%	1.8%	1.9%	2.0%	4.0%
HIST1H2AC	siHEXO	43.7%	37.5%	0.8%	15.4%	2.7%
	control	33.2%	56.9%	1.9%	3.6%	4.4%

Figure 9. TUT7 knockdown shifts monouridylations to diuridylations

A. Western blot showing TUT7 knockdown by siRNA. B.-C. Stacked bar graph for our high-throughput sequencing experiments showing the last eight nucleotides of HIST2H2AA3 (B) or HIST1H2AG (C) for the TUT7 knockdowns. For both experiments, control panels are on the left and experimental on the right.

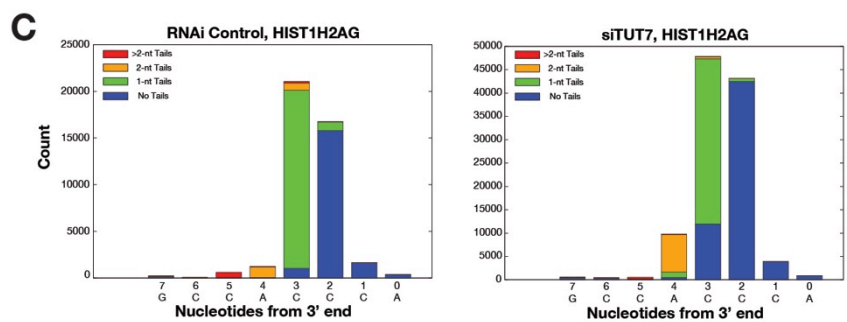
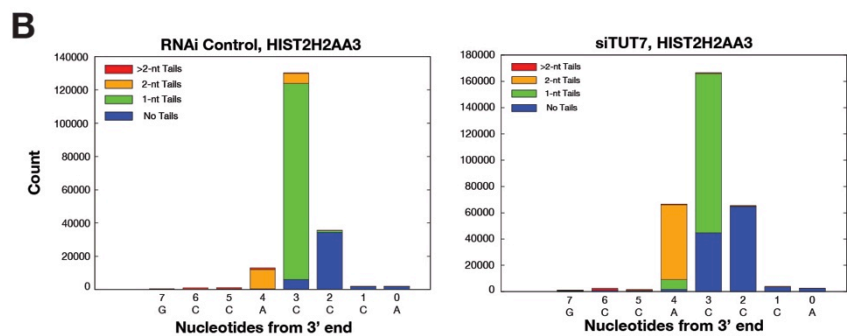
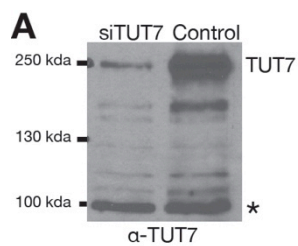
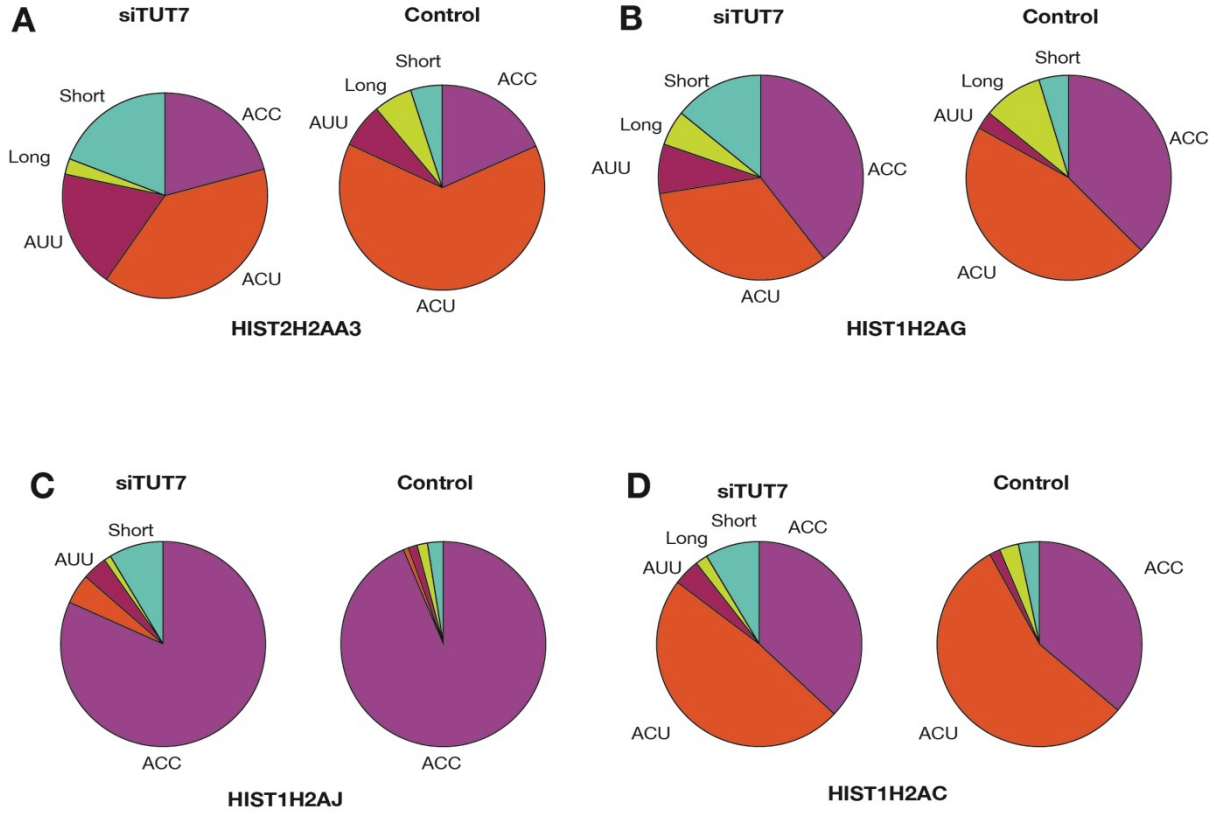


Figure 10. Quantification of different histone mRNA 3' ends after TUT7 knockdown

A.-D. Pie graphs quantifying the different species of histone mRNA 3' ends shown in Figure 9, and how they change with TUT7 knockdown. This analysis was done for HIST2H2AA3 (A), HIST1H2AJ (B), HIST1H2AJ (C), and HIST1H2AC (D). The percentages for the graphs are shown in (E).



E

		ACC	ACU	AUU	Long	Short
HIST2H2AA3	siTUT7	20.9%	39.0%	18.6%	2.4%	19.0%
	control	18.6%	63.5%	6.8%	6.2%	4.9%
HIST1H2AG	siTUT7	39.7%	33.0%	7.6%	5.6%	14.1%
	control	37.6%	45.5%	2.9%	9.4%	4.7%
HIST1H2AJ	siTUT7	81.7%	4.8%	3.9%	1.2%	8.4%
	control	93.7%	0.8%	1.4%	1.8%	2.2%
HIST1H2AC	siTUT7	37.1%	48.4%	4.1%	1.8%	8.6%
	control	36.3%	55.7%	1.9%	3.0%	3.1%

Figure 11. Stacked bar graphs for TUT7 repeat experiment

A. Western blot showing TUT7 knockdown by siRNA. B.-C. Stacked bar graph for our high-throughput sequencing experiments showing the last eight nucleotides of HIST2H2AA3 (B) or HIST1H2AG (C) for the TUT7 knockdowns. For both experiments, control panels are on the left and experimental on the right.

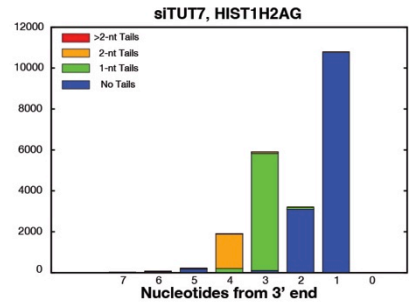
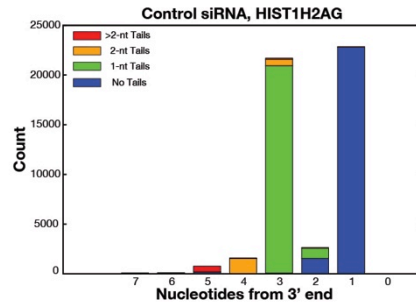
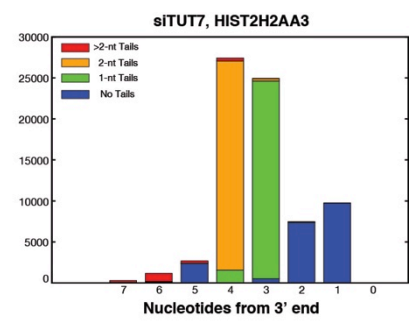
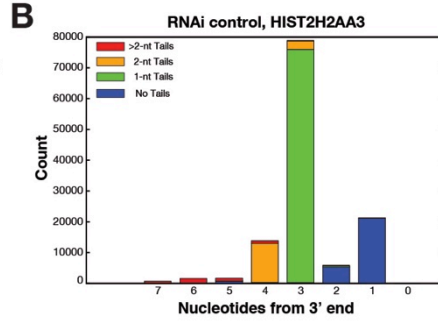
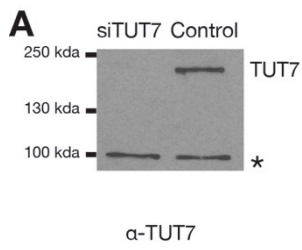
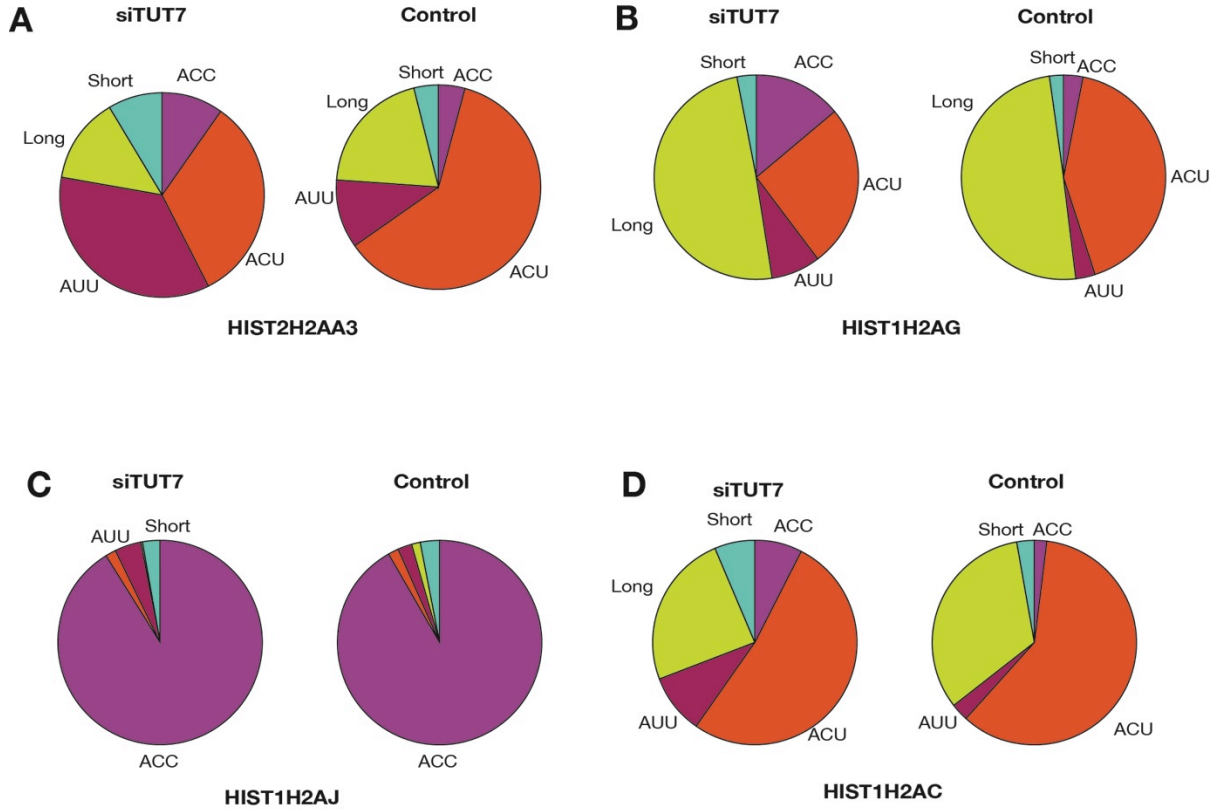


Figure 12. Pie charts for repeat TUT7 EnD-Seq experiment

A.-D. Pie graphs quantifying the different species of histone mRNA 3' ends shown in Figure 11, and how they change with the repeated TUT7 knockdown. This analysis was done for HIST2H2AA3 (A), HIST1H2AJ (B), HIST1H2AJ (C), and HIST1H2AC (D). The percentages for the graphs are shown in (E).



		ACC	ACU	AUU	Long	Short
HIST2H2AA3	siTUT7	10.0%	32.8%	35.1%	13.8%	8.4%
	Control	4.3%	61.1%	11.0%	19.9%	3.7%
HIST1H2AG	siTUT7	14.0%	25.8%	7.7%	49.5%	2.9%
	Control	3.1%	41.9%	3.1%	49.6%	2.2%
HIST1H2AJ	siTUT7	91.2%	1.9%	4.1%	0.3%	2.5%
	Control	91.7%	1.7%	2.2%	1.5%	2.9%
HIST1H2AC	siTUT7	7.7%	52.1%	9.4%	24.4%	6.3%
	control	2.0%	59.8%	2.9%	32.6%	2.7%

Figure 13. TUT4 knockdown has no effect on uridylation of histone mRNA

A. Western blot showing TUT4 knockdown by siRNA. B.-C. Stacked bar graph for our high-throughput sequencing experiments showing the last eight nucleotides of HIST2H2AA3 (B) or HIST1H2AG (C) for the TUT4 knockdowns. For both experiments, control panels are on the left and experimental on the right.

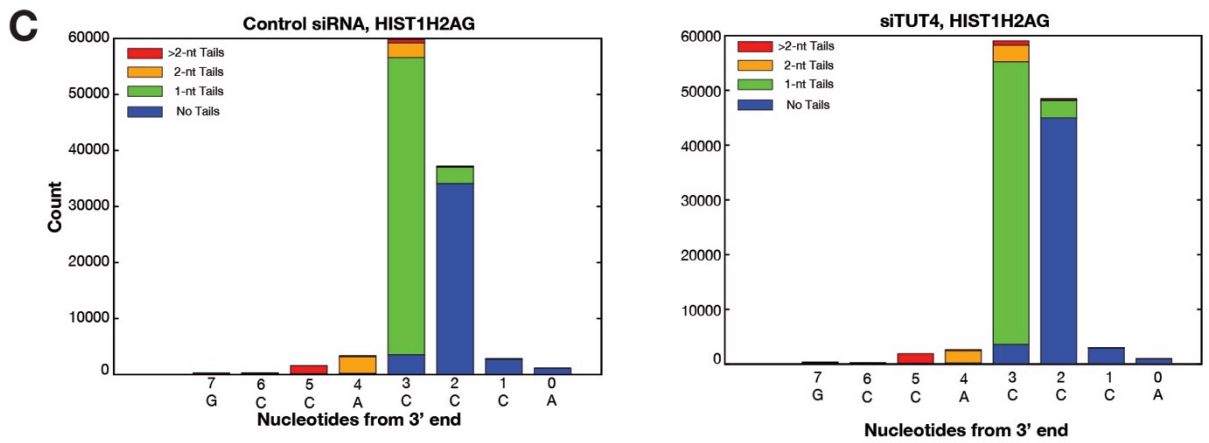
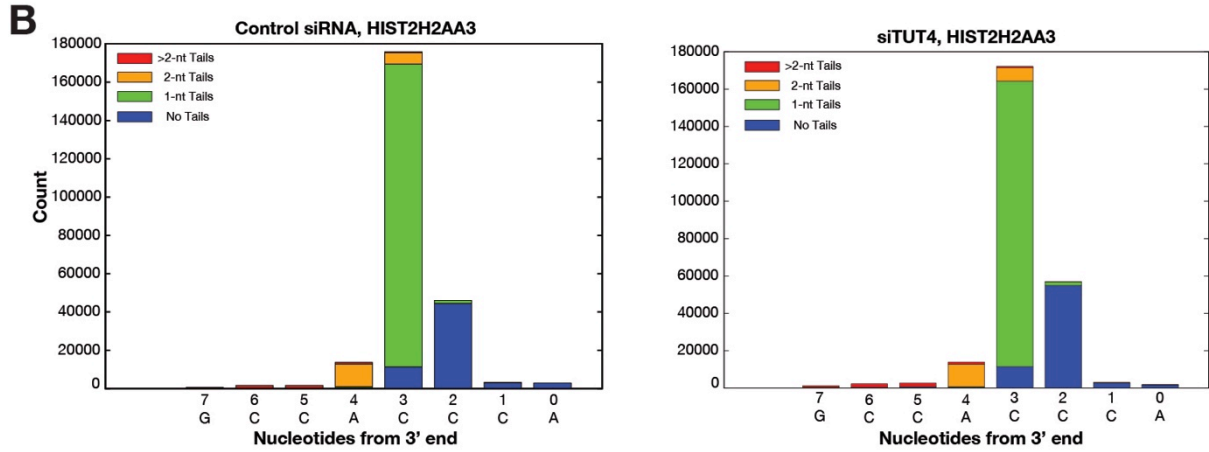
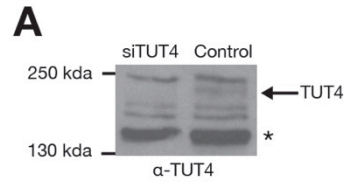
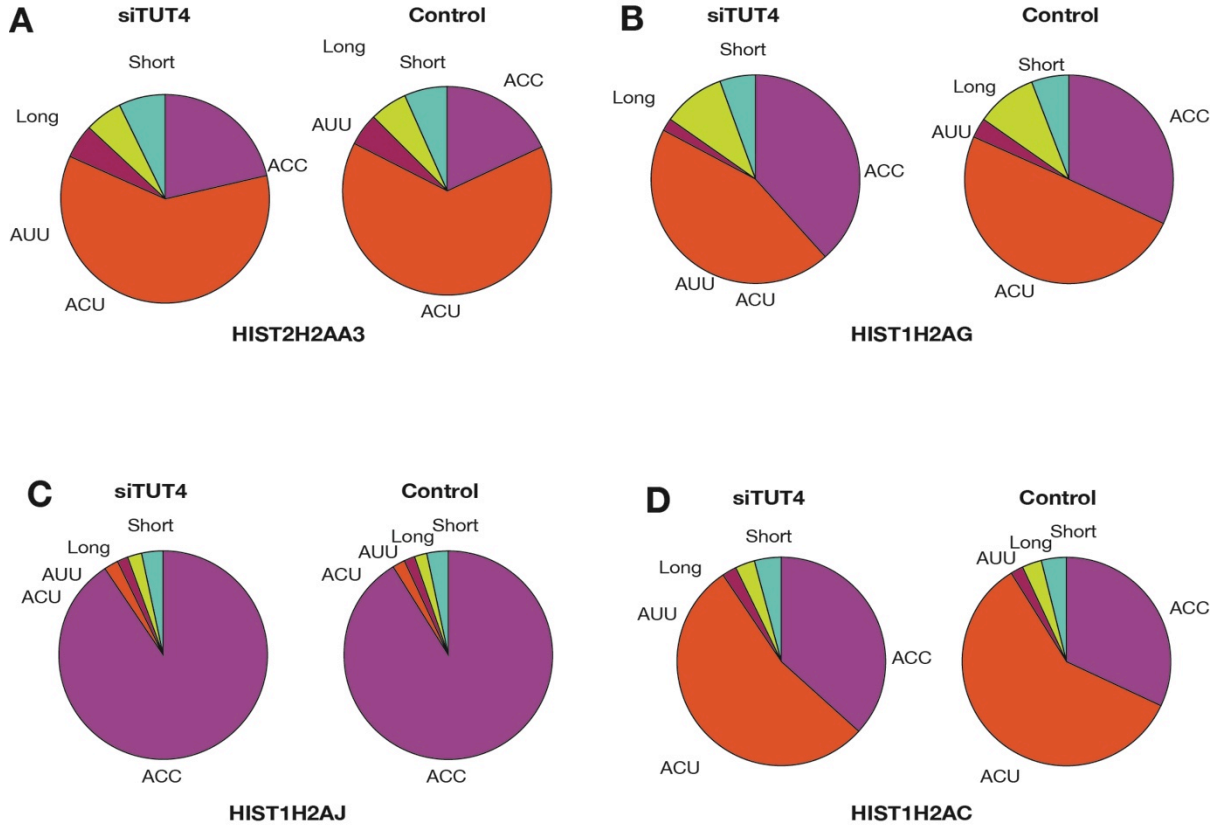


Figure 14. Quantification of different histone mRNA 3' ends after TUT4 knockdown

A.-D. Pie graphs quantifying the different species of histone mRNA 3' ends shown in Figure 5, and how they change with TUT4 knockdown. This analysis was done for HIST2H2AA3 (A), HIST1H2AJ (B), HIST1H2AJ (C), and HIST1H2AC (D). The percentages for the graphs are shown in (E).



		ACC	ACU	AUU	Long	Short
HIST2H2AA3	siTUT4	21.6%	60.3%	5.2%	5.7%	7.2%
	Control	18.1%	64.4%	5.2%	5.6%	6.6%
HIST1H2AG	siTUT4	38.5%	44.3%	2.1%	9.7%	5.4%
	Control	32.0%	49.8%	2.9%	9.6%	5.6%
HIST1H2AJ	siTUT4	90.8%	2.2%	1.7%	2.2%	3.1%
	Control	91.4%	1.8%	1.6%	2.1%	3.1%
HIST1H2AC	siTUT4	36.7%	54.0%	2.2%	3.2%	3.9%
	control	32.0%	59.3%	1.9%	3.1%	3.7%

Figure 15. TUT4/TUT7 double knockdown has a slight stacking effect on the 3' ends of histone mRNA

A. Western blot showing TUT4/TUT7 double knockdown by siRNA. B.-C. Stacked bar graph for our high-throughput sequencing experiments showing the last eight nucleotides of HIST2H2AA3 (B) or HIST1H2AG (C) for the double knockdown. For both experiments, control panels are on the left and experimental on the right. D.-E. Pie chart quantification of double knockdown experiment for HIST2H2AA3 (D) and HIST1H2AG (E).

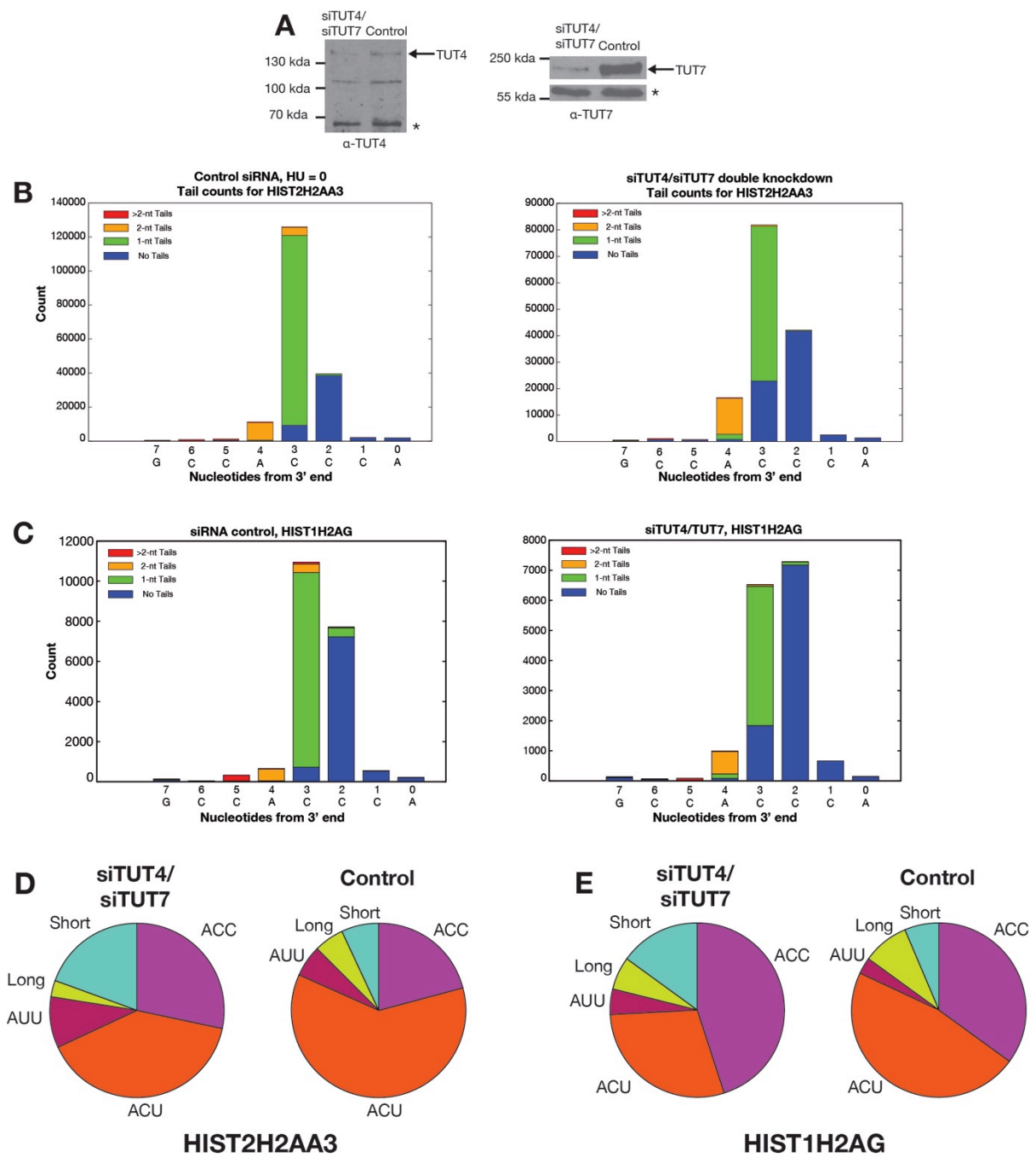


Figure 16. 3'hEXO/TUT7 double knockdown results in lengthened, un-uridylated histone mRNA

A. Western blot showing 3'hEXO/TUT7 double knockdown by siRNA. B.-C. Stacked bar graph for our high-throughput sequencing experiments showing the last eight nucleotides of HIST2H2AA3 (B) or HIST1H2AG (C) for the double knockdown. For both experiments, control panels are on the left and experimental on the right. D.-E. Pie chart quantification of double knockdown experiment for HIST2H2AA3 (D) and HIST1H2AG (E).

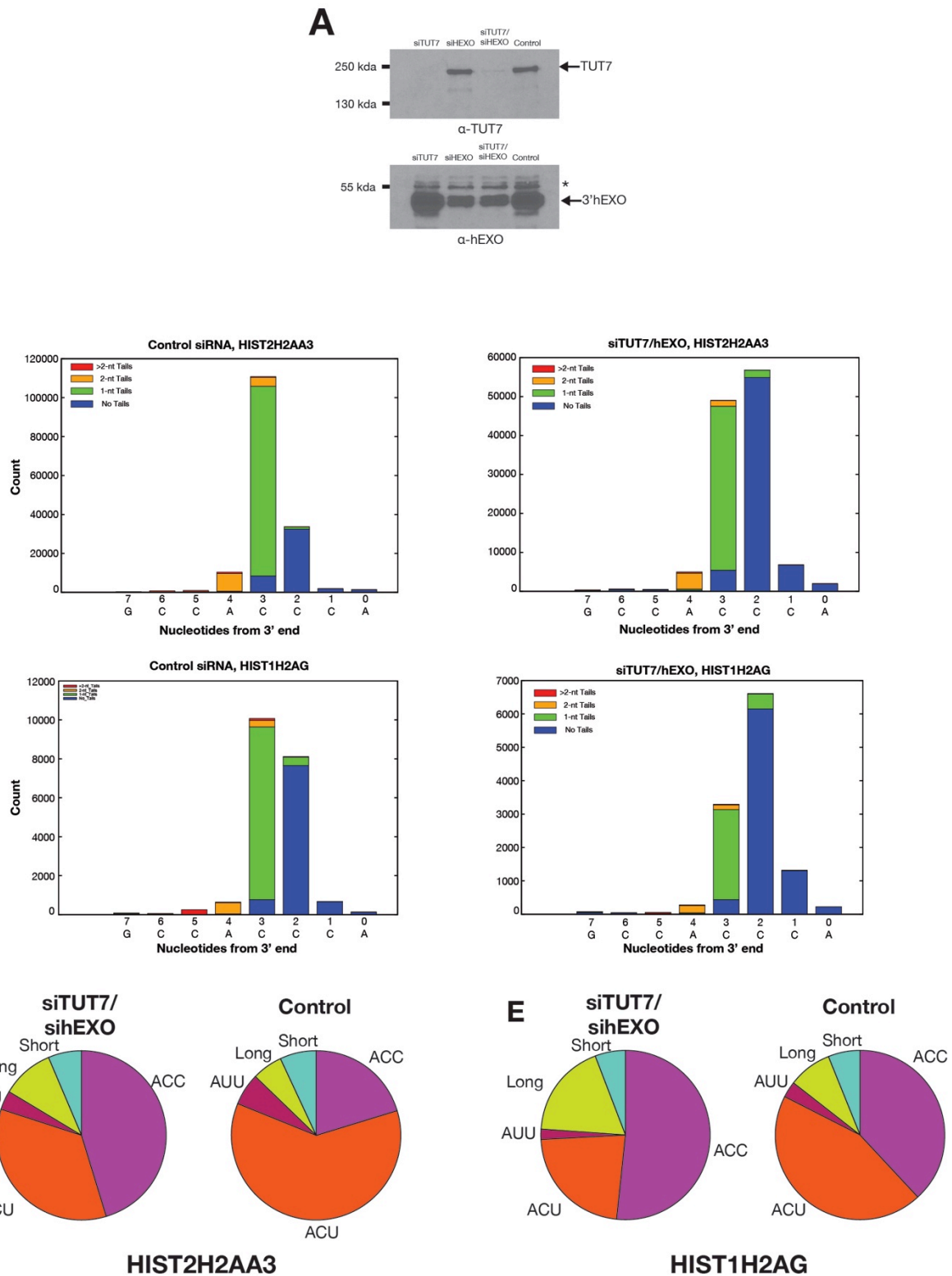


Figure 17. Oligouridylation at the 3' end is unaffected by HU treatment

A. Northern blots showing how knockdowns of 3'hEXO, TUT7, TUT4, and the TUT4/TUT7 double knockdown affect histone mRNA degradation. B.-F. Stacked bar plots for these knockdowns at a point at which approximately half of the histone mRNA has been degraded (45 minutes for the hEXO knockdown, 30 minutes for each other sample). One control experiment has been chosen here to be representative of each sample, though individual controls were performed with each sequencing run (Figure 18).

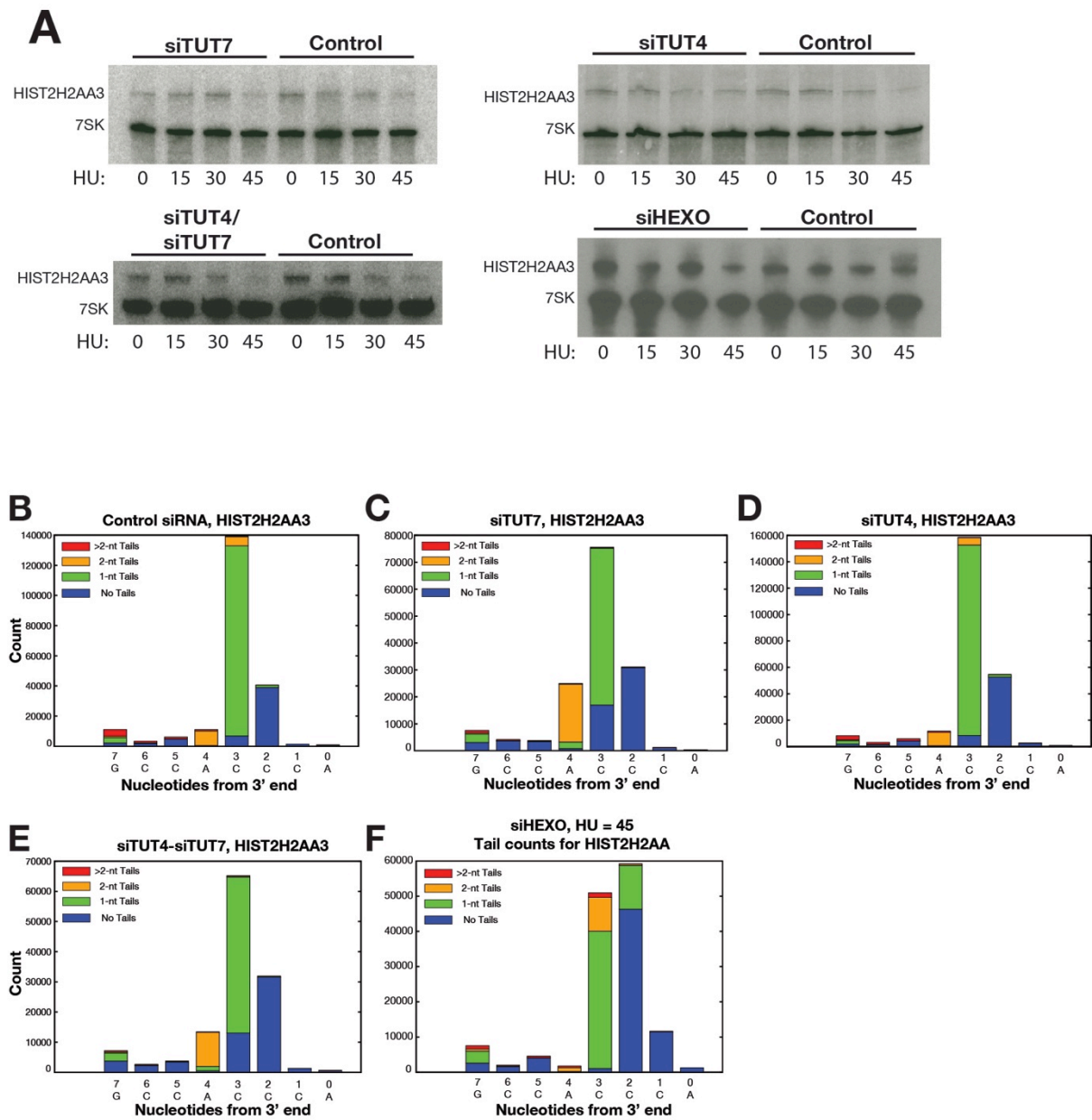
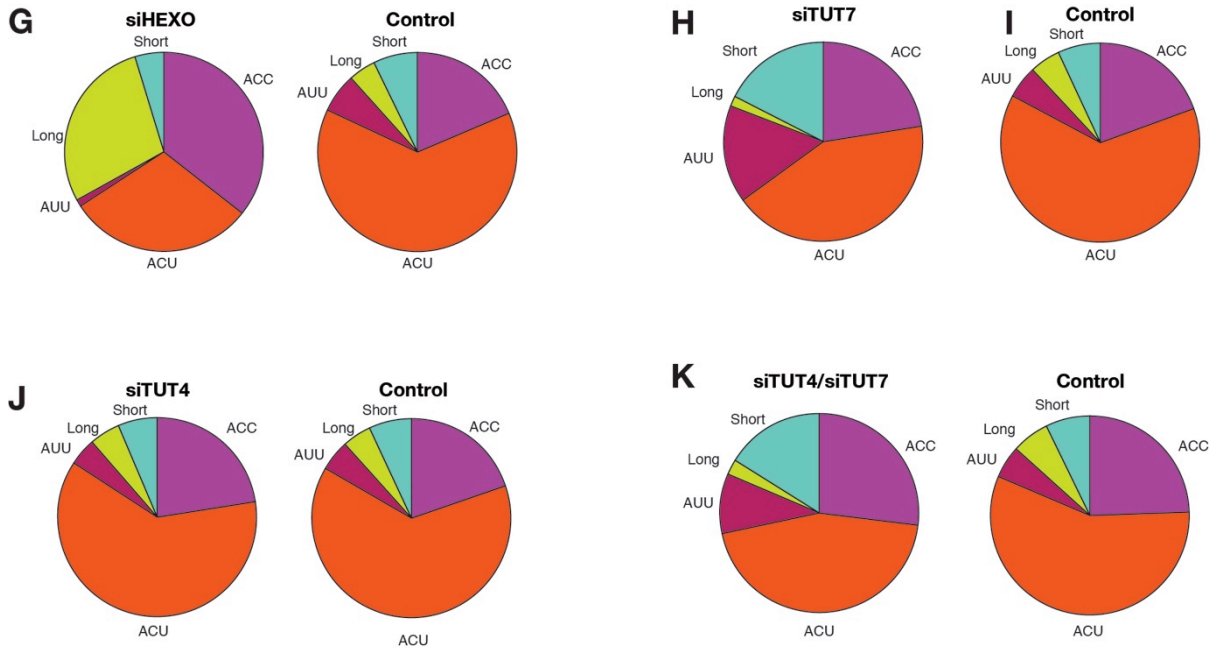


Figure 18. Quantification of 3' ends for each knockdown after HU treatment

A.-D. Pie graphs quantifying the different species of HIST2H2AA3 histone mRNA 3' ends shown in Figure 17. The percentages for the graphs are shown in (E). Unlike the prior pie charts, these charts only quantify the last five nucleotides of HIST2H2AA3 instead of the last seven, as including the last seven begins to include degradation intermediates after HU treatment.



E

	ACC	ACU	AUU	Long	Short
siHEXO	31.2%	26.3%	7.1%	21.3%	14.1%
Control	17.2%	58.3%	5.7%	4.2%	14.5%
siTUT7	20.7%	39.2%	14.6%	1.4%	24.0%
Control	18.3%	59.0%	4.9%	4.8%	13.0%
siTUT4	21.5%	58.8%	4.5%	4.6%	10.7%
Control	19.1%	60.8%	4.6%	4.7%	10.9%
siTUT4/TUT7	25.1%	41.1%	9.1%	2.1%	22.6%
control	22.6%	52.3%	4.9%	5.5%	14.6%

Figure 19. Degradation intermediates in the HIST2H2AA3 stemloop after HU treatment

A.-F. Stacked bar plots showing the distribution of uridylation in the stemloop but beyond the five nucleotides that make the mature and complete 3' ends for a control experiment (again, one control experiment has been chosen to be representative)(A), TUT7 knockdown (B), TUT4 knockdown (C), TUT4/TUT7 double knockdown (D), 3'hEXO knockdown (E), and 3'hEXO/TUT7 double knockdown (F). G. The percentage of all reads in the stemloop that have a given tail length for each experiment. One nucleotide tails have been omitted to avoid flattening of the graph. H. Northern blot showing histone mRNA degradation after HU treatment in cells with both siTUT7 and 3'hEXO simultaneously knocked down.

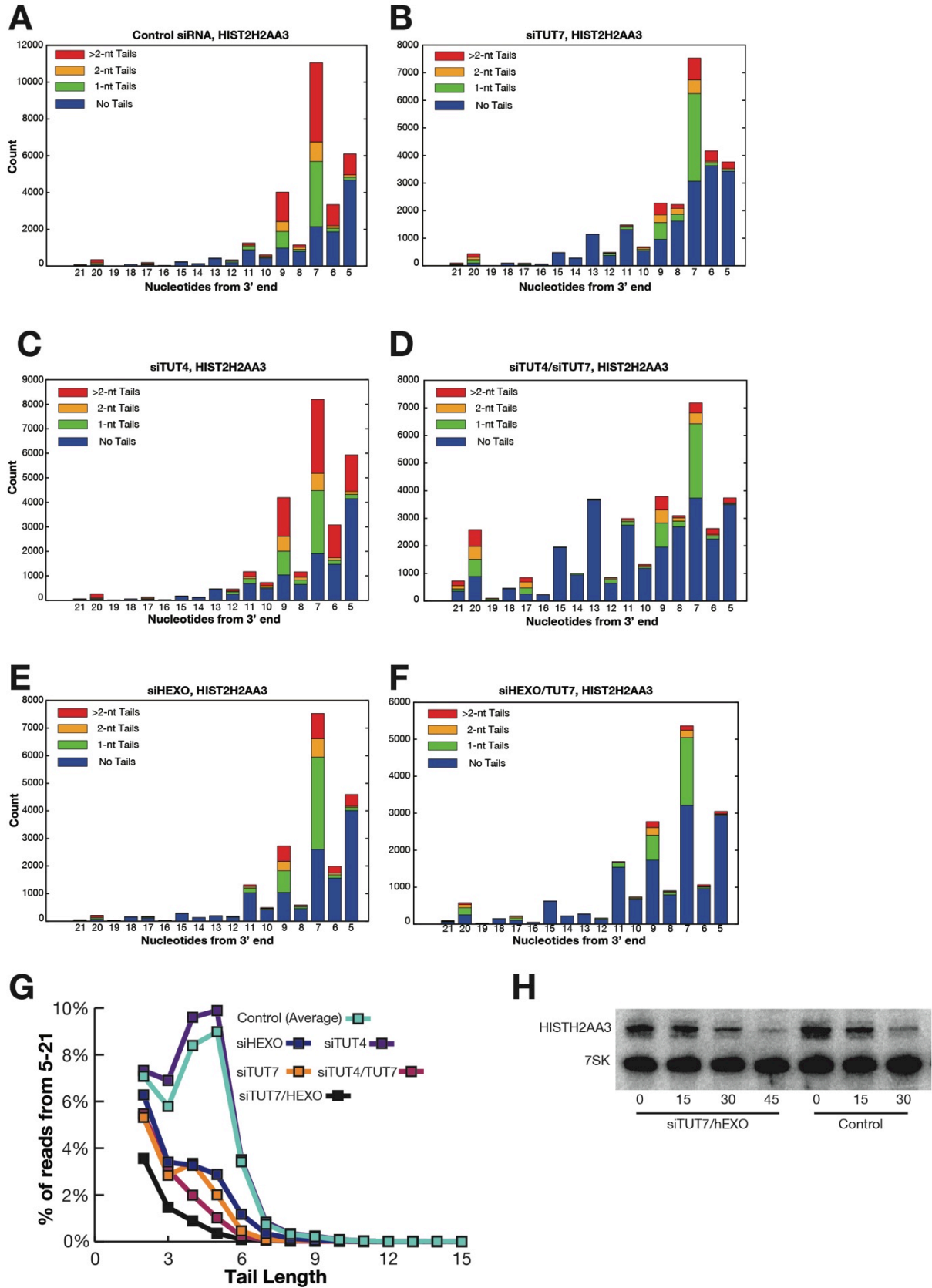


Figure 20. Degradation intermediates in the 3'UTR (but outside of the stemloop) and open reading frame after HU treatment

A.-C. Stacked bar plots showing the distribution of uridylation in the stemloop but beyond the five nucleotides that make the mature and complete 3' ends for a control experiment (again, one control experiment has been chosen to be representative)(A), TUT7 knockdown (B), and TUT4 knockdown (C), D. The percentage of all reads in the stemloop that have a given tail length for each experiment. One nucleotide tails have been omitted to avoid flattening of the graph.

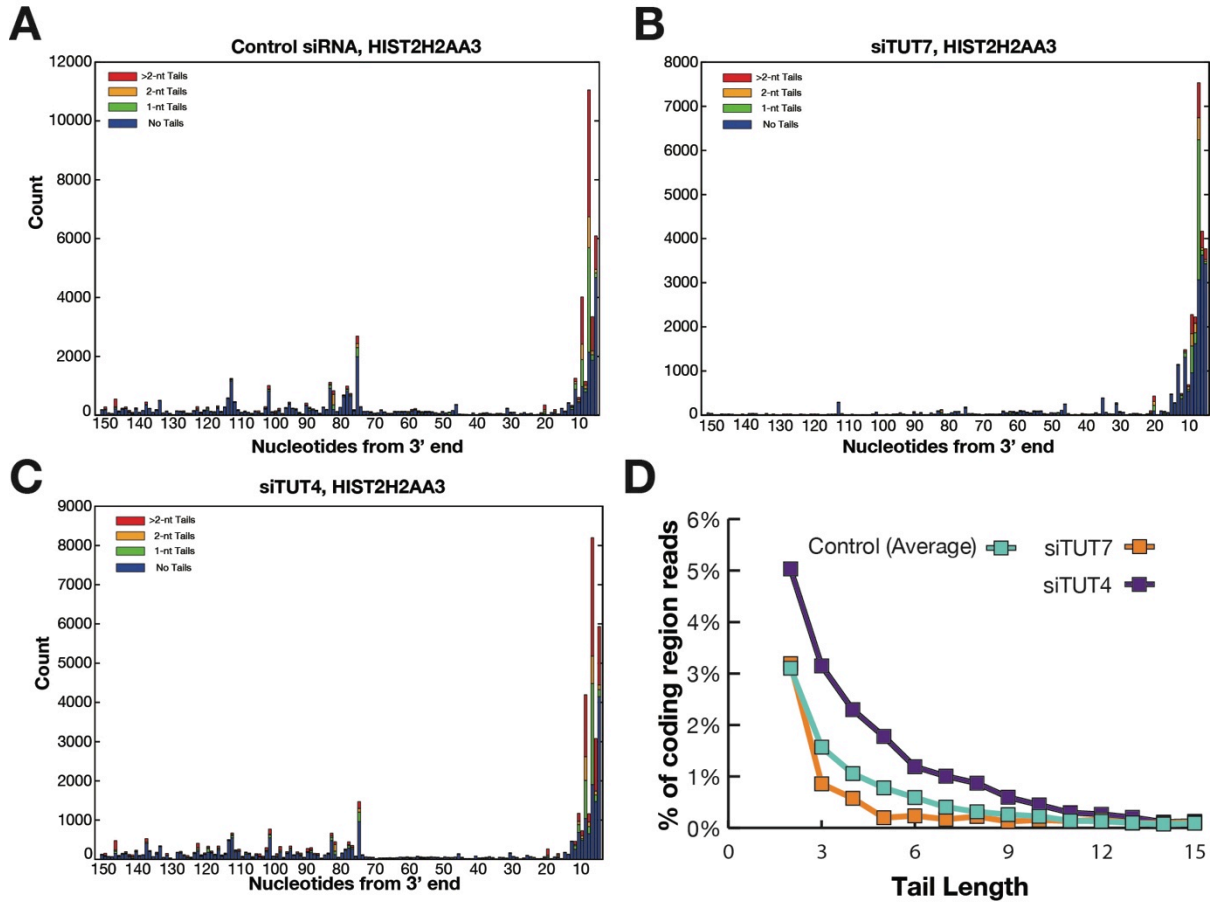
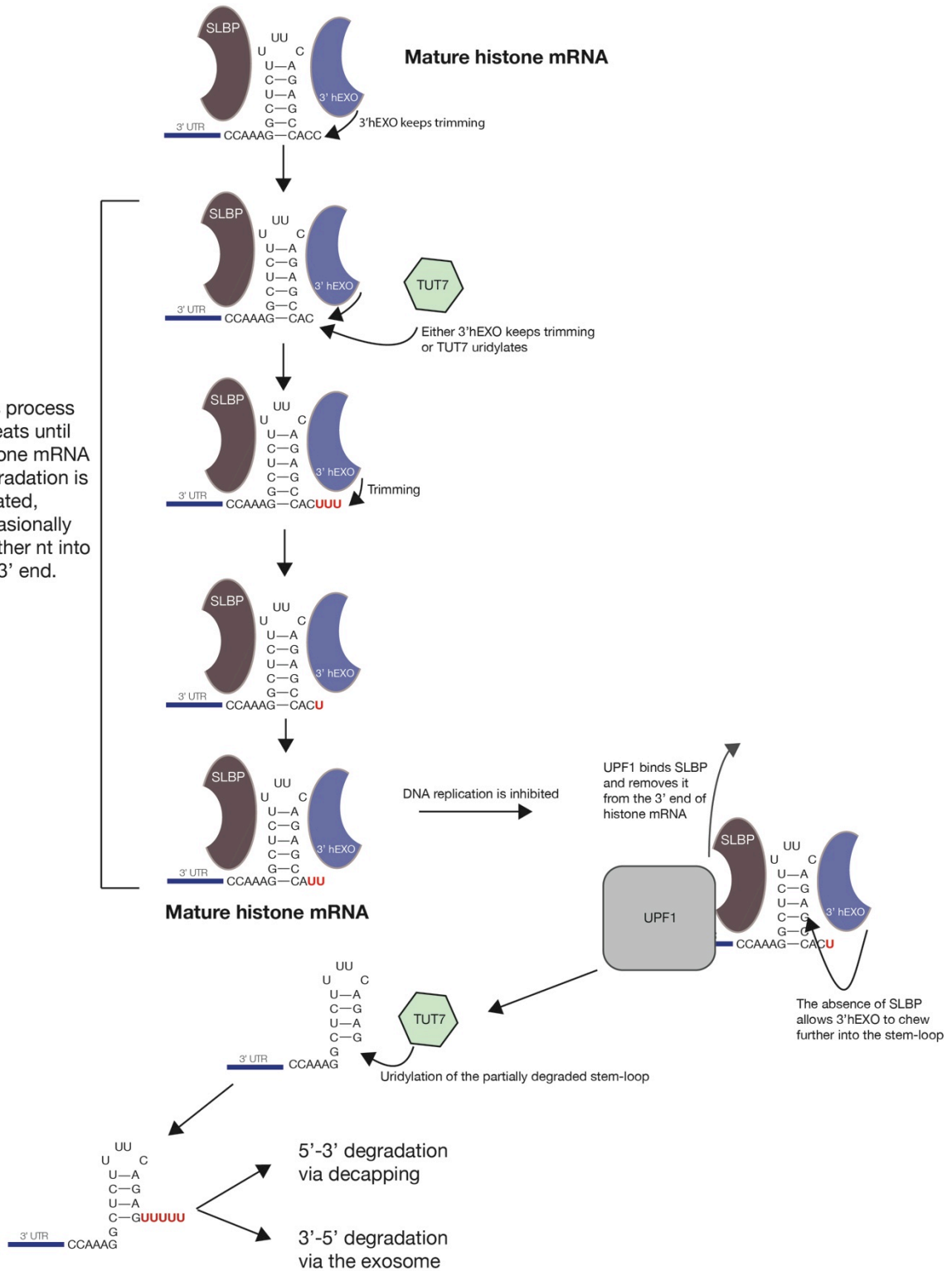


Figure 21. Model of histone mRNA degradation with uridylation carried out by TUT7 and mediated by 3' hEXO trimming

An update of the model shown in Figure 4, reflecting the EnD-Seq/AppEND data that TUT7 and 3'hEXO create a sort of feedback loop in uridylating the 3' end of histone mRNA both at a steady state, and that this steady-state is disrupted as degradation begins.

This process repeats until histone mRNA degradation is initiated, occasionally another nt into the 3' end.



CHAPTER 3

THE EFFECTS OF TUT1 AND TUT3 ON HISTONE mRNA METABOLISM

INTRODUCTION

In our lab's first look at the uridylation of histone mRNA, TUT1 and TUT3 (also known as mtPAP and PAPD5) were initially identified as the enzymes most likely to uridylate the message as a mark for degradation. This was based on the observation that both TUT1 and TUT3 knockdowns by siRNA partially stabilized the histone mRNA transcripts after HU treatment in a northern blot (Mullen and Marzluff 2008).

TUT1 had been identified as a primarily mitochondrial protein (Tomecki et al. 2004) and knockdown of the enzyme had large effects on both mitochondrial RNA stability and general cellular growth and activity (Nagaike 2005). Despite its localization to the mitochondria, its crystal structure and *in vitro* activity following purification of a recombinant TUT1 showed that TUT1 was capable of adding U-tails as well as A tails (Bai et al. 2011). Because of this and because TUT1 was reported to have a splice variant without the mitochondrial localization sequence (Fig. 22) (Bechtel et al. 2007) that would result in removal of the N-terminal mitochondrial localization signal, it was a protein worth studying despite its general classification as a mitochondrial enzyme.

TUT3 was also a bit of an unexpected result; its nucleotide sequence indicated similar with the yeast Trf proteins (Schmidt, West, and Norbury 2010b), which are involved in nuclear exosome targeting, and its earliest described functions were nuclear (Shcherbik et al.

2010). A structure/function analysis of mammalian TUT3 showed some differences to its yeast homolog; it is capable of activity without a cofactor and it has a non-traditional RNA-recognition motif (RRM) at its C-terminus (Rammelt et al. 2011). It's also potentially worth noting that TUT3's activity in the processing of snoRNAs, where it works in concert with PARN to adenylate to provide a substrate for trimming to form the mature 3' end of some snoRNAs (Berndt et al. 2012), is very similar to the TUT7/hEXO interplay mechanism described in Chapter 2 of this thesis.

Even though TUT1 and TUT3 did not initially appear to be the most obvious enzymes to study, their effect on the stability of histone message along with all of the unknowns about the enzymes – the trouble annotating them that lead to uncertainty about the isoforms and localization, the interesting domain structures and activities with other RNA substrates – made them worth studying in the context of histone message degradation. My work focused on accomplishing several things with these enzymes. With TUT1, I focused on determining if its activity was limited to the mitochondria and therefore if it could have any function outside of the mitochondria. With TUT3, I wanted to learn about its interactions with proteins associated with histone mRNA metabolism, to further our knowledge about its subcellular localization, and to use our next-gen sequencing methods to ascertain how TUT3 knockdown affects the 3' end of histone mRNA, both at a steady state and during histone mRNA degradation.

MATERIALS AND METHODS

In vitro GST pulldown assays

TUT1, TUT3, and TUT3 truncations were all cloned into the pcDNA3 vector with a T7 promotor and expressed in rabbit reticulocyte using Promega's Quick Coupled Transcription/Translation (TNT) kit. This kit also allowed for ³⁵S methionine labeling during translation. GST-tagged SLBP and SLBP truncations were purified out of baculovirus as previously described (Erkman et al. 2005).

5 µg of the fusion protein (or GST alone, for the controls) was incubated with a 50 µL slurry of glutathione-sepharose beads (GE Healthcare) and TEN100 buffer (20 mM Tris pH 7.4, 0.1 mM EDTA, 100 mM NaCl) for one hour at 4°C, then the beads were pelleted. 10 µL of the reticulocyte was added to the beads, along with 14 µL GDB buffer (10% glycerol, 10 mM DTT, 0.05 mg/mL BSA), 10 µL 10X TEN100 buffer, and water to a final volume of 100 µL. This mix was incubated for 2 hours at 4°C, washed in TEN100 buffer, resuspended in SDS running buffer, boiled, and run on an SDS-PAGE gel. The resulting gel was dried and placed on a phosphor screen overnight for imaging.

siRNA knockdowns

siRNA knockdowns for TUT3 were done as described in Chapter 2, using Lipofectamine RNAi-MAX (Invitrogen) and a reverse transfection protocol. The sequence for the TUT3 siRNA is found in Table 1.

shRNA lentivirus knockdowns

Lentivirus was produced by co-transfecting the plasmid carrying the shRNA sequence (pLKO) with the plasmids for the lentiviral packaging (pVSVG and ΔNRF) in 293T cells

with Lipofectamine 2000 (Invitrogen).) A mix of 5 μ L Lipofectamine 2000 and 250 μ L OPTI-MEM (Gibco) was incubated at room temperature for seven minutes and combined with a mix of 1 μ g pLKO , 750 ng Δ NRF, and 250 ng pVSVG in 250 μ L OPTI-MEM. This second mixture was incubated at room temperature for 25 minutes, then added to a six-well plate with 293Ts growing at 80% confluency. After 24 hours, the media was aspirated and replaced at approximately half-volume (one mL of DMEM for a well in a six-well plate). After 24 more hours, this media was harvested and used for lentiviral infection (~100-250 μ L per well in a six-well plate for HeLa cells). Target cells were either selected with puromycin 24 hours later or harvested for analysis after 48-72 hours. The shRNA sequences used to target the TUT1 isoforms are collected in Table 3.

End-SEQ and AppEND

End-SEQ and AppEND on the TUT3 knockdown samples was carried out as described in Chapter 2 and (Slevin et al. 2014). Some further analysis was carried out on previously published Pm/Scl-100 knockdown samples.

RESULTS

TUT1 does not have a direct effect on histone mRNA degradation

Because TUT1 was originally identified as a mitochondrial protein (Tomecki et al. 2004), we focused on the the a splice-variant with an alternate N-terminal sequence omitting the mitochondrial location sequence (Figure 22A) (Bechtel et al. 2007). These two isoforms have distinctly different sizes, making it easy to express them and study them individually in HeLa cells (Figure 22B). I looked to study the two isoforms of TUT1 separately from each

other, to determine if the initial observation that TUT1 slows histone mRNA degradation (Mullen and Marzluff 2008) showed a specific effect on histone message or a more global one created by disrupting mitochondrial function.

After unsuccessful co-IP experiments, we used *in vitro* pulldown assays to determine if TUT1 interacts with SLBP, as SLBP is already associated with several components of the degradation complex. We expressed the two isoforms in rabbit reticulocyte lysates and did a GST-pulldown with the same baculovirus-expressed recombinant SLBP. Neither protein showed a significant interaction with SLBP in these experiments, as any potential interactions detected were well below the 5% input (Figure 23).

We then made isoform-specific shRNA lentiviruses to knock down the two isoforms of TUT1 individually, to determine the effects of each isoform on the stability of histone mRNA during degradation. After validating the effectiveness of the lentiviruses (Figure 24), we created knockdowns of the individual isoforms in HeLa cells and HU treated them for 15, 30, and 45 minutes, then took RNA samples for Northern blots (Figure 25). The results show that while the TUT1A knockdown seriously affects histone mRNA levels at all timepoints, including the zero timepoint, the TUT1B knockdowns have very little effect on message stability.

Because of TUT1A's role as a mitochondrial poly(A) polymerase important for regulating the levels of mitochondrial RNA, knockdown of TUT1 may have overall deleterious effects on cell growth. These results indicate that our lab's initial observation that TUT1 knockdown affects histone mRNA levels was likely caused by a global effect caused by TUT1 knockdown affecting the general growth and health of the cell. Since the TUT1B

specific knockdown did not affect histone mRNA degradation, we did not pursue TUT1 further after these experiments.

TUT3 interacts with SLBP

SLBP is a very unstructured protein (Tan et al. 2013) and it has known interactions with several other proteins involved in histone mRNA degradation, including 3'hEXO (Dominski et al. 2003), Lsm1 and Lsm4 (Mullen and Marzluff 2008; Lyons et al. 2013), and Upf1 (Kaygun and Marzluff 2005a). Because our initial hypothesis was that uridylation was a histone mRNA-specific mechanism to initiate uridylation, it made sense to look for an interaction between SLBP and one or more TUTases as a way of initiating uridylation.

For *in vitro* pulldown assays, I expressed TUT3 in rabbit reticulocyte using the pcDNA3 vector, and used recombinant GST-SLBP purified from baculovirus. After ruling out the TUT1 interaction (Figure 23), I then focused on TUT3 and TUT4. My initial pulldowns found a relatively strong interaction between SLBP and TUT3, but not between SLBP and TUT1 or TUT4 (Figure 26).

We then looked to characterize this interaction by using truncations of both TUT3 and SLBP. SLBP is a very well characterized protein, and different regions of the protein are associated with different steps in histone mRNA metabolism. Using various truncations of SLBP, we were able to map the binding between SLBP and TUT3 to C-terminal half of SLBP's RNA-binding domain (Fig. 27), which is separate from its binding sites for Upf1 (Stacie Meaux, unpublished) and Lsm4 (Lyons et al. 2013).

TUT3 has three major domains; the nucleotidyl transferase domain required for uridylation, its Poly(A) polymerase associated domain (PAPD), and a non-traditional RNA

recognition motif (RRM)(Rammelt et al. 2011). We made our truncations of TUT3 based on both existing splice variants in the gene databank and on these various domains, and were able to narrow the SLBP binding down to a 90 amino acid region that includes part of the PAPD, but neither of the other major domains (Fig. 28). The binding sites for TUT3 and SLBP are mapped in Figure 29.

We attempted to characterize this interaction in the cell, though this proved difficult. After co-immunoprecipitation was unsuccessful, we overexpressed both SLBP (using the pGLUE vector to put a tandem-affinity purification tag on the N-terminus) and TUT3 (using the pcDNA3 vector to create an N-terminal FLAG tag). We then pulled down SLBP using magnetic streptavidin beads and saw a very slight enrichment of TUT3 (on par with the 1% control) as opposed to our negative control cells, which were transfected with FLAG-TUT3 but not TAP-SLBP. HU treatment to stop DNA replication and induce histone mRNA degradation did not appear to affect this very slight interaction. Thus we could not conclusively demonstrate that TUT and SLBP interacted.

End-Seq and APPend show unexpected alterations to histone mRNA oligouridylation when TUT3 is knocked down.

Similar to the work done in the previous chapter, I also sought to determine the effect of siRNA knockdown of TUT3 using our next-gen sequencing methods, EnD-SEQ and AppEND.

Knocking TUT3 down has no effect on histone mRNA in normally growing HeLa cells. In looking at the effects of TUT3 knockdown on both HIST2H2AA3 and HIST1H2AG, I found no discernible change in the distribution of normal 3' ends, monouridylations, or diuridylations compared to the siRNA control (Fig. 30). While it's

possible that TUT3 has a slight effect on stable histone mRNA (say, similar to the very slight effect attributed to TUT4 in Chapter 2), it's clear that TUT3's effect on histone mRNA under these circumstances is either very slight or non-existent.

This changes substantially with an HU treatment. In HU treated samples, there is a large increase in degradation intermediates at the base pairs that we closely identified with the initial uridylation for degradation in Chapter 2 (7-10 nucleotides in from the 3' end) (Fig. 31A). An unusually large number of these intermediates are uridylated with long oligo(U) tails

An increase in uridylation is obviously the opposite of the effect expected by knocking down a TUTase, though it is worth noting that this pattern of uridylation and degradation intermediates matches the results from the knockdown of the exosome component Pm-Scl100 previously published by our lab (Slevin et al. 2014). In order to further explore the similarities between the two experiments, I re-analyzed the previous Pm-Scl100 results to match the current analysis methods for EnD-Seq/AppEND experiments seen in Chapter 2 (Fig. 31B).

These two experiments don't make perfect comparison points, as the Pm/Scl-100 experiment was one of our lab's earliest EnD-SEQ experiments and the number of reads is substantially lower than in my TUT3 knockdown experiments. Because of this, I separated out the tail length comparison so that the TUT3 knockdown would be compared to the control that was made in parallel with it (Fig. 31C) and the same would be true of the Pm/Scl-100 knockdown (Fig. 31C). Both experiments show an enrichment in the reads with tails of four or five nucleotides as compared to the control. As we showed in Chapter 2, these 4-5 nucleotide tails in this region of the mRNA seem to be precursors for degradation, and so

the fact that a TUTase knockdown would create a similar enrichment to these messages to an exosome knockdown is worth noting, even if the change in uridylation profile is unexpected.

Finally, I looked at the lengths of oligo(U) tails beyond the stem-loop and into the open reading frame, as Chapter 2 showed that neither TUT4 nor TUT7 were responsible for these uridylation. Figure 32 shows that TUT3 is not responsible for these tails either; in the control sample, approximately 19% of the samples that are degraded beyond the stem-loop are uridylated, while that number is 17% in the TUT3 knockdown. It is perhaps worth noting that a larger percentage of the reads from the TUT3 knockdown were found beyond the stem-loop. This is the opposite of what would be expected if TUT3 were somehow involved in exosome recruitment or targeting to histone mRNA, though it's difficult to draw conclusions based on these reads in particular as RNA degradation intermediates can pile up here and it is sometimes difficult to distinguish between genuine sequencing reads and chaff for any untailed read.

DISCUSSION

TUT3's interaction with SLBP indicates a role in histone mRNA metabolism

Despite our inability to pin down the function of TUT3 in histone message metabolism, its interaction with SLBP is notable, as it's an interaction not shared with other TUTases, including one (TUT4) that seems to be at least peripherally associated with histone message metabolism. It is worth mentioning, however, that this interaction does not necessarily tie TUT3 to any specific part of histone message metabolism, as SLBP is associated with the stem-loop for the entire life of the message, from transcription and processing to degradation. For example, unprocessed histone mRNAs in the nucleus that

result from failure to process histone mRNA are likely to be bound by SLBP and also are very unstable.

TUT3 is likely not involved in bulk histone mRNA degradation

Through our deep sequencing experiments done both here and in Chapter 2, we can infer that it is unlikely that TUT3 has a direct role in bulk histone mRNA decay. Our work in Chapter 2 with hEXO and TUT7 suggest roughly how degradation proceeds through the stem-loop: hEXO and TUT7 exist in steady-state, feeding back on each other to create the trimmed and mono-and-di-uridylation 3' end of histone mRNA. This exists until something disturbs the 3' end of histone mRNP at the end of S-phase (or when DNA replication is inhibited), allowing hEXO to chew further into the stem-loop than it does at the steady-state. Here, TUT7 uridylation in the stem-loop, seven to nine nucleotides in from the 3' end of the processed histone message. This uridylation catalyzes degradation of the histone message, both 3' to 5' and 5' to 3'.

Our TUT3 knockdown does not directly disturb this process. The 3' end remains almost completely unchanged at a steady-state, and rather than reducing uridylation of the degradation intermediates after HU treatment, we actually see an enrichment of them. The most likely explanation for this is TUT3's potential association with the exosome; TUT3 interacts with MTR4 and the zinc-knuckle protein ZCCHC6, both of which associate with the human nuclear exosome (Lubas et al. 2011).

Two exosome components, Rps41 and Pml/Scl100, have been associated with histone mRNA degradation (Mullen and Marzluff 2008; Slevin et al. 2014). It was previously thought that this exosome function was mainly cytoplasmic; the exosome functions in bulk RNA degradation (J S Anderson and Parker 1998) and our lab has sequenced partially

degraded histone mRNAs that appear to have been degraded from the 3' end. It is worth noting, though, that both proteins are part of the nuclear exosome complex that functions in various types of mRNA surveillance as well as the cytoplasmic complex associated with message degradation (Sloan et al. 2012). Given TUT3's many nuclear-specific roles, it's possible that it works with the nuclear exosome in surveillance of histone message in an unexpected and yet unexplored pathway.

TUT3's alteration of the histone mRNA degradation pattern resembles the exosome and may provide clues to its function in histone mRNA metabolism

Although more work needs to be done to verify TUT3's localization to the histone locus body, thinking of it in conjunction with the HLB may help provide clues to its ultimate function. We do know that knockdown of SLBP results in the retention of unprocessed message in the nucleus (Sullivan et al. 2009). Some mechanism must exist to degrade these messages under normal circumstances; it's possible that TUT3's function is to facilitate the exosome in rapidly degrading these aberrant transcripts under normal circumstances to prevent them from accumulating.

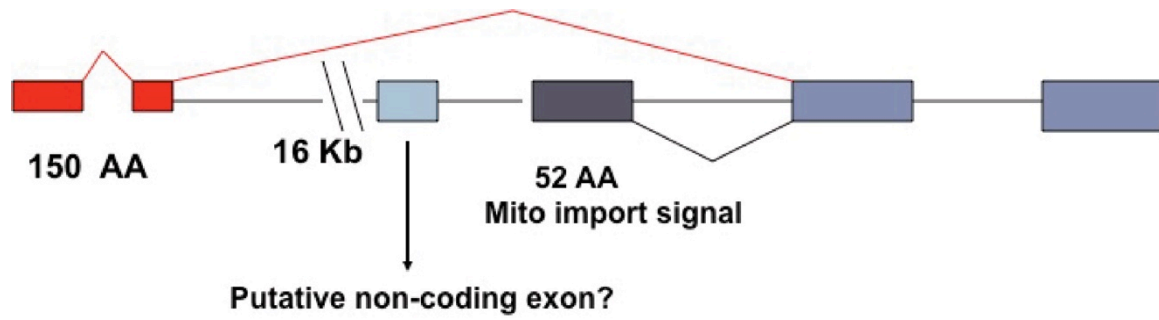
It is currently unclear exactly how this might work; the human TRAMP complex, of which TUT3 is a part, has been shown to be localized to the nucleolus, while general RNA surveillance is carried out by the Nuclear Exosome Targeting complex, or NEXT. Still, these complexes are relatively understudied in humans, as compared to their yeast counterparts, and so there is room for uncertainty in their role in histone mRNA surveillance. In *S. cerevisiae*, two Trf proteins, Trf4 and Trf5, do have a role in regulating histone mRNA metabolism (Reis and Campbell 2006); when the proteins are knocked out, the core histone genes are overexpressed. This suggests an interaction between the Trf4 and Trf5 proteins and

proper regulation of histone mRNA in *S. cerevisiae*. Yeast histone genes are polyadenylated and do not have stem-loops, so it's hard to draw 1:1 comparisons between the regulation of yeast histone genes and human histone genes, but the role of TUT3/Trf4 in yeast histone mRNA metabolism may provide some insight into how the protein functions in humans.

Figure 22. Splice variants of TUT1

A. The most common variant of TUT1 has a mitochondrial location sequence, but genome annotation indicates an alternative splicing variant with a unique N-terminus that removes the mitochondrial location sequence and replaces it with two exons that do not have specific localization sequences. B. The mitochondrial (TUT1A) and non-mitochondrial (TUT1B) isoforms of TUT1 were expressed in HeLa cells using the pcDNA3-FLAG vector (* is a non-specific band used as a loading control, and the bottom band in the top panel is the endogenous enzyme). The over-expression of the two isoforms was detected by our lab's antibody to TUT1.

A



B

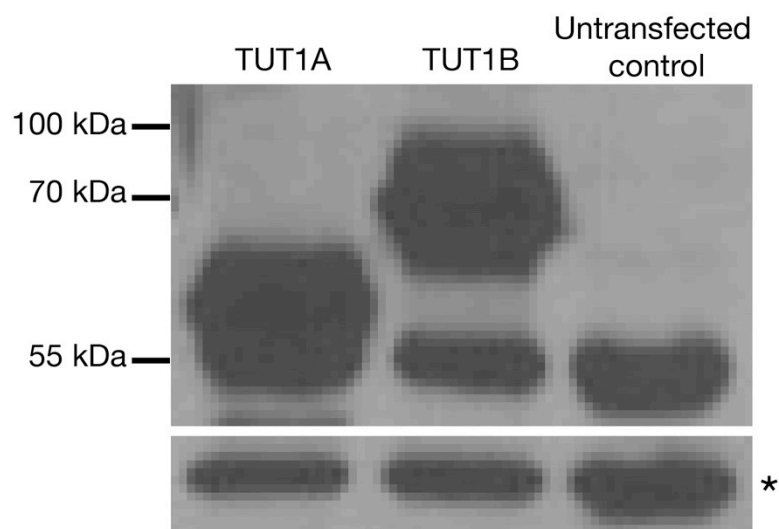


Figure 23. *In vitro* pulldown assays for TUT1 and SLBP

The two isoforms of TUT1 (TUT1A left panel, TUT1B right panel) were expressed via TnT reaction in rabbit reticulocyte, and recombinant GST-SLBP expressed in *Baculovirus* was used for the experiment.

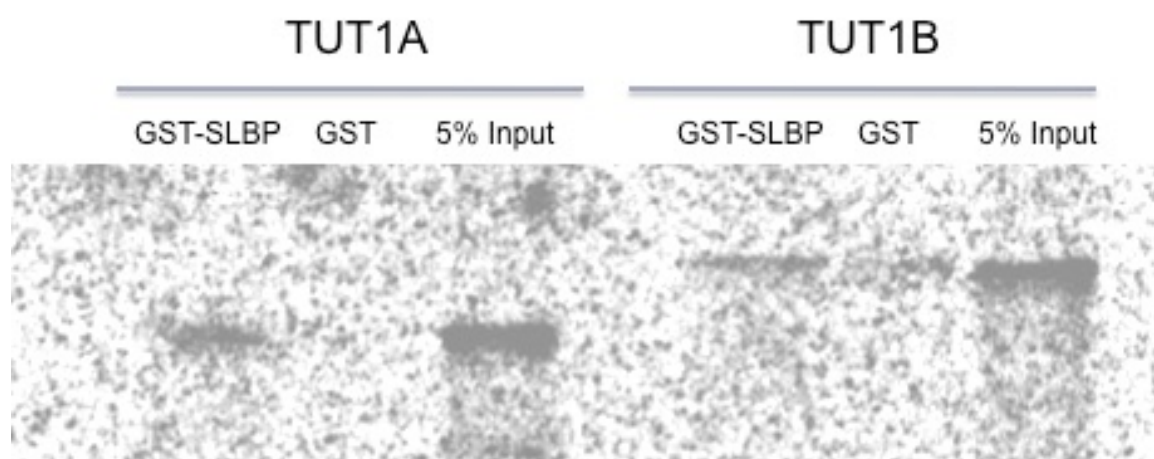


Figure 24. Western blots for TUT1 specific isoform knockdowns

Cells expressing TUT1A (left) and TUT1B (right) were treated with shRNA against either TUT1A and TUT1B, and the knockdowns were checked via Western blot.

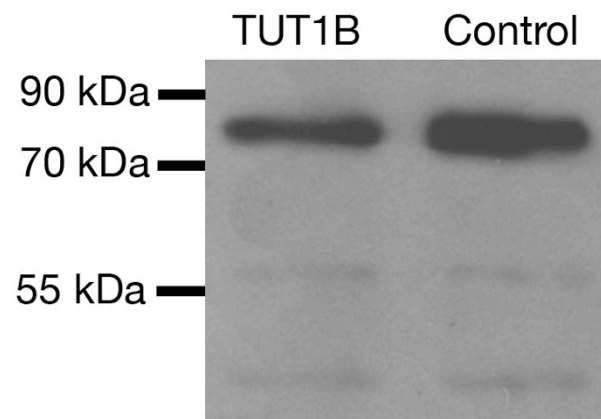
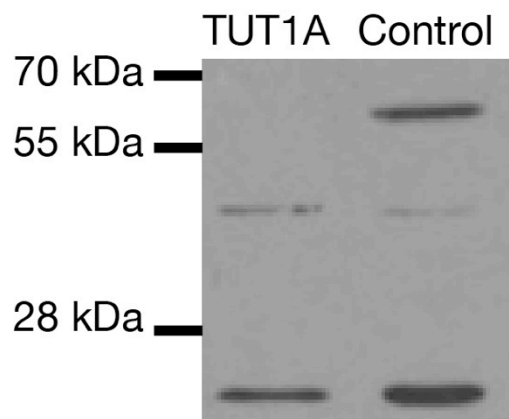


Figure 25. Northern blot for histone mRNA stability after TUT1 isoform-specific knockdowns

HeLa cells were treated with lentivirus specific for TUT1A and TUT1B, then HU treated.

RNA samples were taken and a Northern blot was done to measure how the knockdowns affected histone mRNA stability.

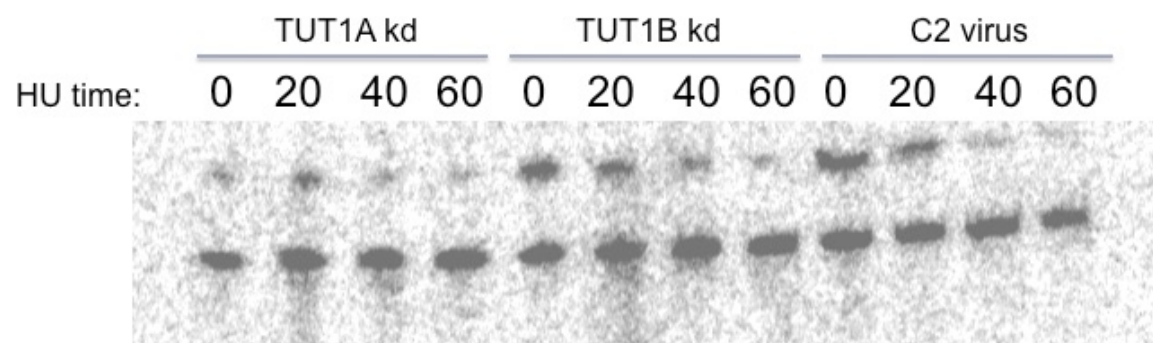


Figure 26. GST pulldowns with *in vitro* translated TUT3 and TUT4 and recombinant SLBP

TUT3 and TUT4 were *in vitro* translated and incubated with recombinantly expressed GST-SLBP immobilized on glutathione beads to look for interactions between the proteins.

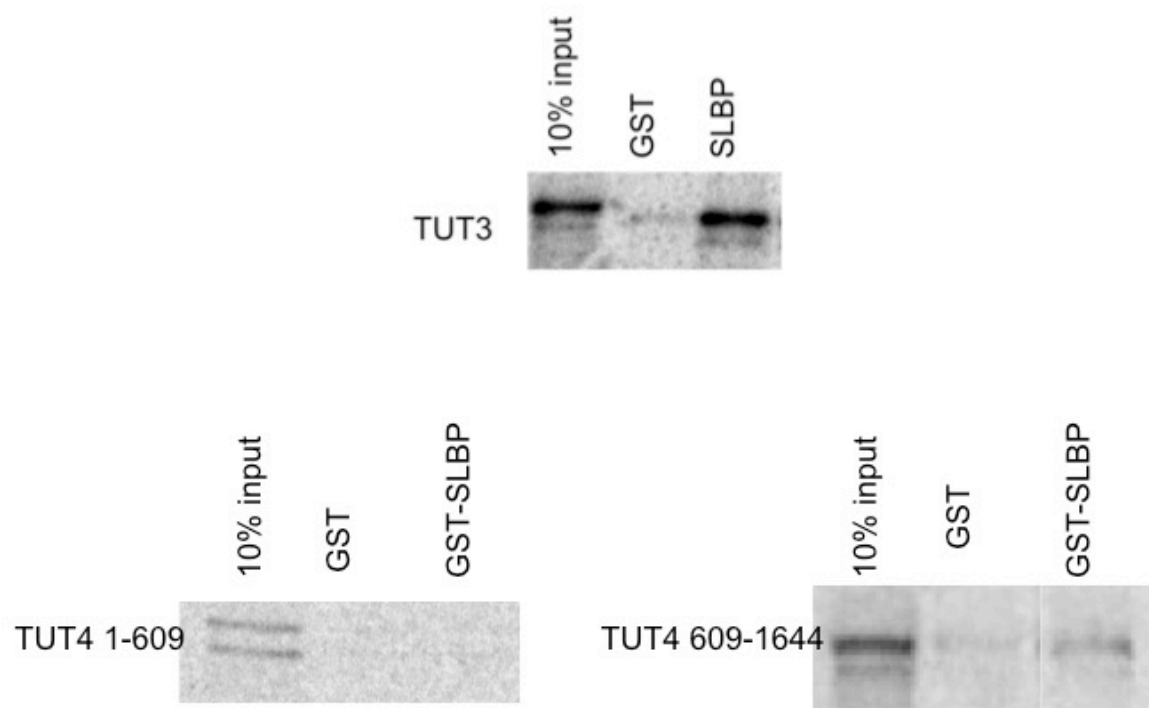
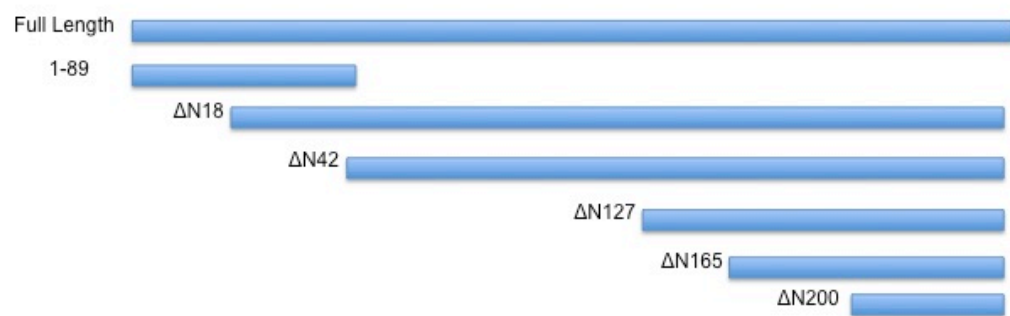
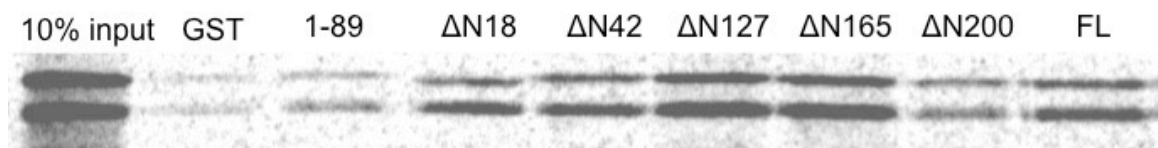


Figure 27. TUT3 interacts with SLBP somewhere between SLBP's amino acids 165 and 200

The pulldown experiments were repeated with various truncations of GST-SLBP to find the region of SLBP that interacts with TUT3.



SLBP truncations

Figure 28. SLBP interacts with TUT3 somewhere between TUT3's amino acids 459 and 549

Truncations of TUT3 were expressed through the *in vitro* transcription and translation system, then used in pulldown assays with full-length SLBP to try and determine the region of TUT3 that interacts with SLBP.

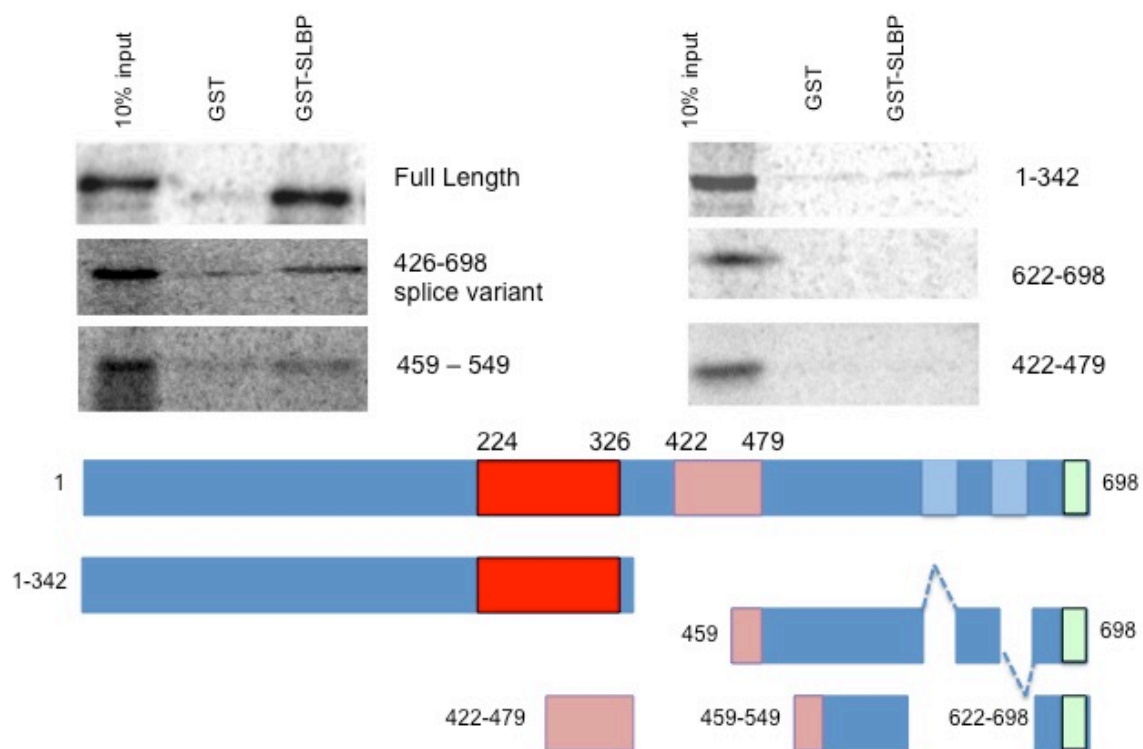


Figure 29. Mapping the interaction between TUT3 and SLBP

Using the data from the pulldowns, we can see that the region of SLBP that TUT3 interacts with is in SLBP's RNA binding domain, but notably does not overlap with SLBP's Lsm4 binding site. The region of TUT3 that SLBP interacts with is part of TUT3's PAP-associated domain.

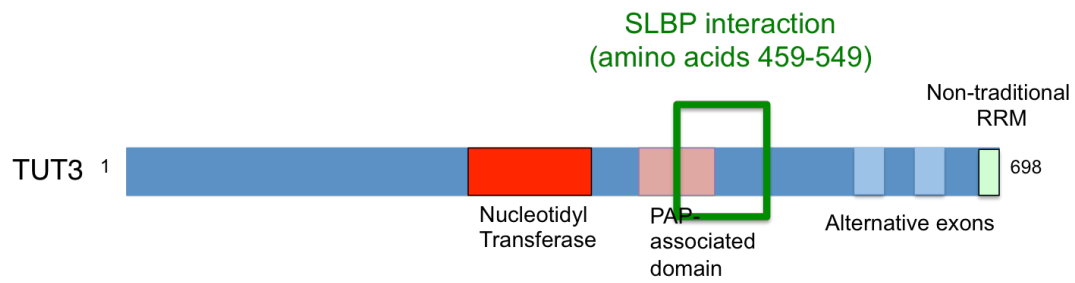
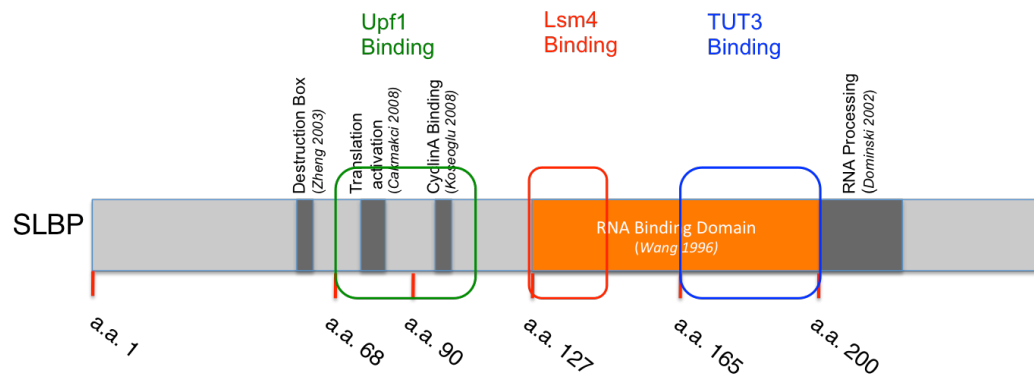


Figure 30. EnD-Seq results for TUT3 siRNA knockdown without HU treatment

A. Western blot showing TUT3 knockdown, with a non-specific band as loading control marked with the asterisk and TUT3 indicated by the arrow. B. Stacked bar plots for the TUT3 knockdown at the 3' end in HIST2H2AA3. C. Stacked bar plots for the TUT3 knockdown at the 3' end in HIST1H2AG.

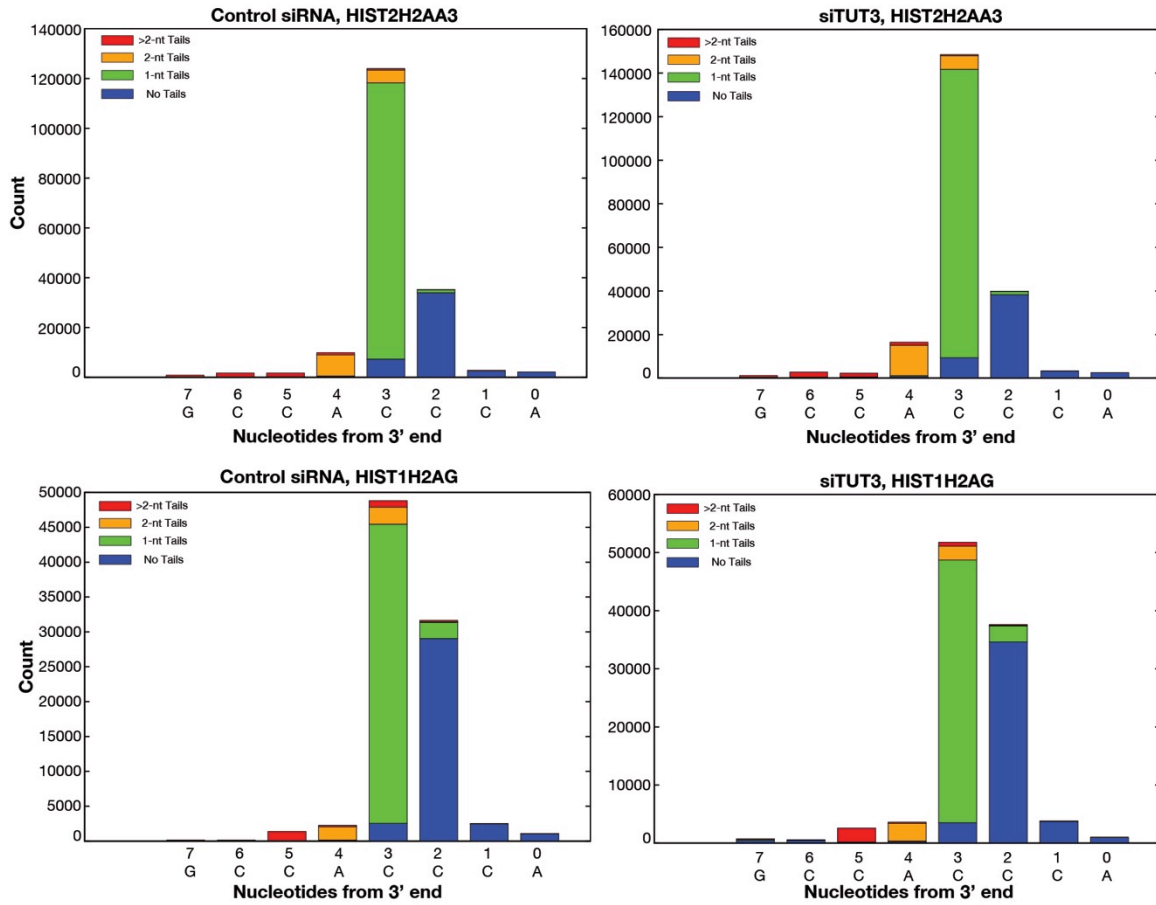
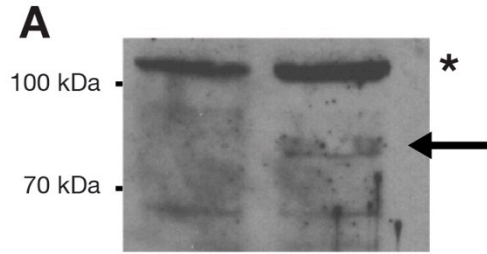


Figure 31. TUT3 knockdown results in accumulation of messages uridylated in the stem-loop in HU-treated samples

As in Chapter 2, the TUT3 knockdown samples were treated with HU and harvested when approximately 50% of the message remained. A. Stacked bar graph for nucleotides 5-22 (the stemloop) in the TUT3 knockdown (right panel) and siRNA (left). B. Stacked bar graph for nucleotides 5-22 (the stemloop) in the PM/Scl-100 knockdown (right panel) and siRNA (left) – data originally published in (Slevin et al. 2014) and reanalyzed here. C. Number of reads with a particular tail length expressed as a percentage of total reads in the 5-22 region in the siTUT3 experiment. D. Number of reads with a particular tail length expressed as a percentage of total reads in the 5-22 region in the siPM/Scl-100 experiment.

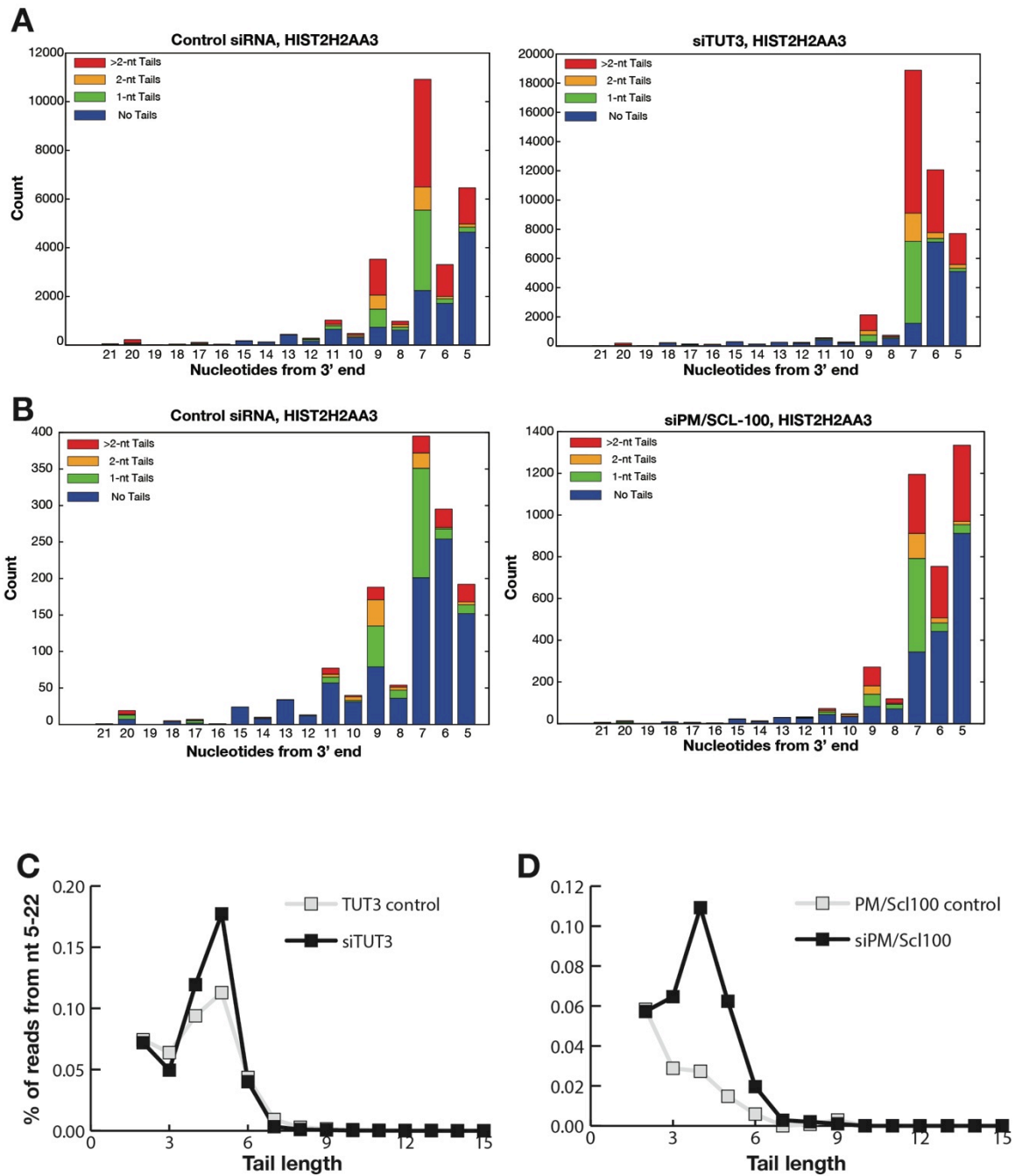
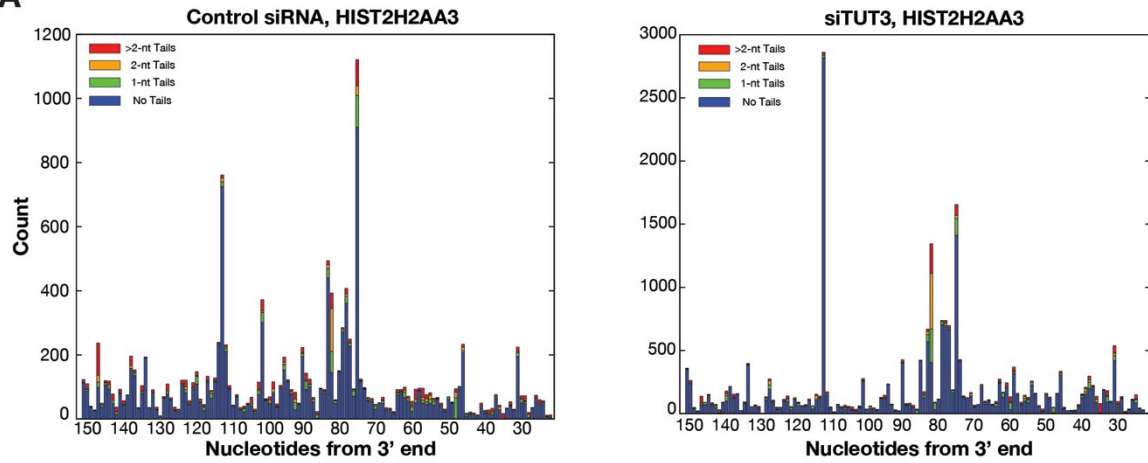


Figure 32. TUT3 knockdown has little effect on uridylation in the open reading frame

A. Stacked bar graph showing uridylation patterns in the open reading frame of HIST2H2AA3 for control experiment (left) and siTUT3 experiment (right). B. Table quantifying the percentage of these reads that are uridylated, and the number of these reads as the percentage of the whole sequencing experiment.

A



B

	% tailed	% of total
siTUT3	17.4%	15.2%
Control	19.1%	6.2%

CHAPTER 4

SUMMARY AND CONCLUSIONS

INTRODUCTION

When we first discovered oligouridylation on histone mRNAs, we thought it was a degradation-dependent modification of the 3' end that simulated the oligo(A) stump left after deadenylation on bulk mRNAs (Mullen and Marzluff 2008). This would allow Lsm1-7 to bind to the histone mRNA and potentially initiate the same pathway of degradation that occurs on polyadenylated mRNAs. Our deeper look at the 3' ends of histone message using our next-gen sequencing methods revealed that oligouridylation is much more dynamic and prevalent in histone mRNA metabolism, associated with multiple steps in histone mRNA degradation as well as occurring on molecules that are not undergoing degradation (Slevin et al. 2014; Welch et al. 2015).

Previous work by our lab implicated a wide variety of factors involved in a number of different types of mRNA degradation in histone mRNA degradation; decapping enzymes, the exosome, and the Lsm proteins required for normal mRNA degradation are all implicated in different steps in histone mRNA degradation (Mullen and Marzluff 2008; Lyons et al. 2013). It also requires the main NMD protein Upf1 and No-Go Decay proteins Dom34 and Hbs1 (Kaygun and Marzluff 2005a; Slevin et al. 2014). Uridylation of the oligoA tail remaining after deadenylation of poly(A) mRNAs has also been implicated in mRNA decay in

mammalian cells (Lim et al. 2014), suggesting that uridylation has a very broad role in mRNA decay.

UPF1 MAY BE CRITICAL FOR THE FIRST STEP OF HISTONE mRNA DEGRADATION

To this point, the role of Upf1 in histone mRNA degradation has been unclear. In a recent collaboration, though, our lab showed that Upf1 binds to the 3' UTR 21 nucleotides from the cleavage site (Brooks et al. 2015). My results show an increase in uridylation at this point, especially in the TUT4/TUT7 double knockdown

It is unclear exactly how all of these parts fit together; one explanation is that each disparate piece of machinery is involved in a specialized step in degradation of histone mRNA. Since histone mRNA is both unique at the 3' end and tightly cell-cycle regulated, it would make sense that it has a very unique and specific mechanism of mRNA decay.

Another explanation would be that histone mRNA might be degraded by several mechanisms at once, with the two ends of the same molecule being degraded simultaneously by different pathways, or different molecules being degraded by different pathways. Thus some might be degraded by a mechanism similar to bulk mRNA decay, others by a mechanism similar to No Go Decay, and others more by a mechanism similar to NMD. This would make some sense from a logistical perspective; the cell must degrade a lot of histone mRNA and it must do it quickly at the end of S-phase. Having multiple pathways available to degrade the message would certainly be one way to approach that problem.

The evidence we do have points towards the first model, at least for the initial steps in degradation, although it is far from certain. We know that Upf1 is the only NMD factor involved in histone mRNA decay (Kaygun and Marzluff 2005a), which suggests that the

histone mRNA decay pathway differs from NMD in significant ways. This means that NMD itself is not a mechanism by which histone mRNA is degraded. Still, there remains much to be uncovered about the role of uridylation, the various factors involved in degradation, and the cell's preference for degradation mechanisms (ie 5'-3' vs 3'-5') when it comes to histone mRNA.

The role of oligouridylation and the non-canonical poly(A) polymerases is similarly muddled. The meaning of oligouridylation in miRNAs is context dependent; under some circumstances TUT7 can add an oligo(U) mark that protects a piece of RNA or promotes process and other different circumstances the same enzyme can leave a similar mark that tags the RNA for destruction (Kim et al. 2015). TUT3, meanwhile, has been implicated in activity all over the cell; oligoadenylating snoRNAs to promote trimming and processing (Berndt et al. 2012), polyadenylating rRNA to mark for degradation (Shcherbik et al. 2010), and oligoadenylating miRNAs in the cytoplasm (Burns et al. 2011).

The goal of this thesis is to provide some clarity to both of these subjects by providing a greater understanding of the role of oligouridylation in histone mRNA metabolism and degradation. I set out to determine both the enzyme responsible for histone mRNA oligouridylation, as well as the contribution of these oligo(U) marks to the degradation of the histone message.

TUT7 IS THE PRIMARY TUTASE AT THE 3' END OF HISTONE mRNA

My knockdown and sequencing experiments show three very clear effects that implicate TUT7 in the oligouridylation of histone mRNA. The first is that the knockdown of TUT7 resulted in a reduction of bulk uridylation of histone mRNA as compared to the control sample. The second is that we saw a shift inwards of the position of the mono-and-di-

uridylation at the 3' end of steady-state histone message that 3'hExo activity trimmed the mRNA further when TUT7 was reduced. When the TUT7 knockdown data is analyzed with the hEXO knockdown experiment in mind, it indicates that the absence of TUT7 resulted in a slower uridylation response by the cell. This is also supported by the TUT7/hEXO double knockdown. Finally, we saw a reduction in the uridylation of the key intermediates in the stem loop of histone mRNA associated with degradation in the knockdown cells. These three pieces of data taken together implicate TUT7 in the uridylation of histone mRNA at the 3' end.

Thus two distinct classes of uridylation were affected by the TUT7 knockdown, the uridylation at the 3' end of the mRNA to maintain the proper mRNA length, and the uridylation of the initial degradation intermediate formed by 3'hexo digestion into the stem. There is also uridylation of degradation intermediates that have a 3' end in the open reading frame or in the 3' UTR before the stem-loop. In general, about 10% of these intermediates are uridylated and TUT7 knockdown did not affect these intermediates (in fact, nothing we did affected these intermediates much, though this is a topic for later discussion). These intermediates (uridylated and not uridylated) were greatly diminished in the TUT7 knockdown, suggesting that they are formed slowly and then rapidly removed in by the exosome, in a step independent of TUT7.

In addition to finding that TUT7 affected both the uridylation of the 3' ends of the steady-state and degrading histone mRNAs, we also found that neither TUT3 nor TUT4 had much of an effect on either. A TUT4 knockdown by itself had very little effect on the uridylation profile of histone mRNA. However when the two enzymes were codepleted, the

effect was more severe than knockdown of TUT7 alone, consistent with the possibility that TUT4 could partially compensate for TUT7 when TUT7 was knocked down.

I did not run deep sequencing experiments on TUT1, TUT2, TUT5, or the U6 TUTase. Because TUT1 is unlikely to be involved in histone message metabolism and knockdown of the U6 TUTase has been shown to be lethal in HeLa cells, they were not considered for deep sequencing in this thesis and are likely poor candidates to pursue in the future. Both TUT2 and TUT5 are interesting, however, as TUT2 has been implicated in some miRNA processing functions (Heo et al. 2012) and TUT5 is a structural homolog to TUT3, that has been very sparsely studied to this point.

TUT7 AND 3'hEXO FUNCTION TOGETHER TO PROMOTE URIDYLATION AND DEGRADATION

One of the most interesting and unexpected discoveries in these experiments studying the oligouridylation of histone mRNA is that the mono-and-di-uridylation at the 3' end of the message and the oligouridylation that encode for the earliest steps of degradation in the stem-loop have entirely different functions, yet are produced by the same enzyme.

The mono-and-di-uridylation at the 3' end of histone mRNA exist at a high level (when looking at HIST2H2AA3, they are more prevalent than non-uridylated messages) in growing cells. They can be perturbed by both TUT7 knockdown and 3'hEXO knockdowns, with the TUT7 knockdown shifting the balance from monouridylation towards diuridylation and shorter messages and the 3'hEXO knockdowns resulting in messages with longer uridylation and U-tails closer to the 3' end of the message. Importantly, once degradation is initiated by hydroxyurea treatment, these mRNA isoforms exist in the roughly the same ratios as in the growing cells, indicating that they are *not* preferentially degraded once the

cell begins to destroy histone message. This implies that they are not potential degradation intermediates, nor have they been “primed” for degradation. Instead, they are treated the same as unuridylated, mature histone mRNA.

My results also indicate that TUT7 is responsible for the uridylation that initiates degradation (this turns out to be a bit of a difficult event to define, as it appears that there is a general amount of exonucleolytic degradation by 3'hEXO into the stemloop under normal conditions as well as the larger actions of the exosome and 5'-3' degradation machinery to more completely degrade the message – as such, when I refer to “initiating degradation” I’m referring to initiating those latter steps, and not the degradation done by 3'hEXO), after inhibition of DNA replication. When TUT7 is knocked down, this uridylation is reduced, although there is still exonucleolytic degradation into the stem. Uridylation at this location in the stemloop after HU treatment is also reduced when 3'hEXO is knocked down, likely because hEXO is required for partial degradation that opens the message up into the stem-loop. Presumably, hEXO degrades into the stem-loop, then falls off of the message, allowing for TUT7 to add a longer tail that marks the remainder of the mRNA for degradation.

Taken together, the two results show us that length and function of an oligo(U) tail on histone mRNA is context-dependent. While the cell is growing and the SLBP/3'hEXO complex is bound to the stem-loop, TUT7 oligouridylates the message, while hEXO trims it back to the appropriate length, three nucleotides beyond the stem. These uridylations function to protect the message; by restoring the length of the mRNA, giving 3'hEXO a substrate to trim and possibly preventing the exonuclease from prematurely degrading into the stem-loop. This maintains the stem-loop/SLBP complex necessary for translation and other aspects of histone mRNA life in the cytoplasm. Once degradation is initiated, the

stability of the 3' mRNP is reduced and hEXO is able to chew into the stem until it forces itself to fall off of the message. Without hEXO present, the tails added by TUT7 are much longer than the one-to-two nucleotide tails that we see at the 3' end, and these tails seem to be precursors to the full degradation of the message.

TUT7 AND 3'hEXO COOPERATE TO ACHIEVE PROPER URIDYLATION OF HISTONE mRNA

Our data suggest that not only do TUT7 and hEXO combine to create this context-dependent uridylation, but that there is some of feedback between the two proteins that impact the function of the other. When hEXO is knocked down, the uridylation of HU-treated histone mRNA is reduced in the important region 7-12 nucleotides from the cleavage site as a percentage of total reads in the region. This is an unexpected result; in the 3'hEXO knockout mice, these intermediates don't exist because hEXO is not present to degrade further into the stem-loop (Hoefig et al. 2012). In the knockdown (as opposed to knockout) cells, there's less degradation by 3'hEXO into the stem, but I expected to find the messages that were degraded that far back would be uridylated normally. The fact that they are not is an indication that there may be some sort of interaction or regulation between the two enzymes that further their contribution to histone mRNA metabolism.

Similarly, in the 3'hExo knockdown cells the uridylations at the 3' end of the mRNA often extend longer than 1 or 2 nts, suggesting that TUT7 may normally add slightly longer tails which are then trimmed back by 3'hExo to maintain the proper length at the 3' end of histone mRNA. Given these pieces of data, one could imagine TUT7 and 3'Hexo might function together to both digest and uridylate their targets.

IS THERE A ROLE FOR TUT3 IN HISTONE mRNA METABOLISM ?

While the role of TUT7 was relatively clear from our sequencing, the role of TUT3 was much less apparent. Instead of a shift of the pattern of uridylation or a global decrease in uridylated message, we saw an increase of the degradation intermediates that were chewed back into the stem-loop and uridylated, similar to what we previously reported in the Pm/Scl-100 knockdown (Slevin et al. 2014). The connection to the exosome was not entirely unexpected, since TUT3 is a human Trf4 analogue and Trf4 is part of the TRAMP complex that targets messages to the exosome in yeast.

Nothing in our results ties TUT3's function to the cytoplasm, though. We discovered and analyzed an interaction between the enzyme and SLBP, but SLBP's involvement and function in histone mRNA metabolism is not limited by cellular localization. The exosome component most studied by our lab and the one that has knockdown sequencing results that resemble the TUT3 knockdown sequencing experiment, Pm-Scl100, is part of both the nuclear and cytoplasmic exosome.

This raises the possibility that the reason we're not seeing a change in oligouridylation or oligoadenylation (as you might expect with TUT3, given its currently documented activities (Rammelt et al. 2011)) is because our deep sequencing approach is targeting the wrong region of histone message. If TUT3 is indeed involved in some sort of nuclear surveillance, it would stand to reason that it affects unprocessed targets. Our current approach places histone primers ~150 nucleotides upstream of the 3' cleavage point and is optimized to amplify cDNA fragments of approximately this length. In each experiment, however, we see a statistically insignificant number of unprocessed reads. While it is difficult to draw conclusions from these reads, it may be possible to move our primer closer to the

stop codon or cleavage point in the hopes of amplifying these unprocessed messages. Since SLBP knockdown results in an increase in unprocessed message retained in the nucleus, it may also be possible to couple this with a TUT3 knockdown to gain a better understanding of how this works.

THE MECHANISM OF DEGRADATION FOR INTERMEDIATES IN THE OPEN READING FRAME REMAINS UNKNOWN

In every experiment we did, the uridylation of degradation intermediates in the open reading frame remained unperturbed; remaining at about 10% of these molecules. There are a number of questions about these intermediates that are not immediately easy to answer. Our working model for these intermediates has always been that they have been partially degraded by the exosome, which then stalled out when it contacted a ribosome. Once the ribosome is removed, the messages are re-uridylated and then fully degraded. If the assumption is that these messages are treated like normal mRNAs at this point, other published work indicates that the uridylation should come from either TUT7 or TUT4, but our results thus far do not support this possibility, although we cannot rule out that the TUT4 knockdown was not sufficient to reduce the activity of that enzyme enough to have an effect on these RNAs.

It is worth reconsidering this model some in light of the information that Dom 34 and Hbs1 are involved in histone message decay (Slevin et al. 2014). In yeast, the model of No-Go Decay involves Dom 34 and Hbs1 dissociating the ribosome, followed by an endonucleolytic cleavage and degradation of the resulting messages. It seems possible that the same thing is happening in histone message; that our reads in the open reading frame are not a result of partial degradation, but rather cleavage after the dissociation of the ribosome.

It's not clear why these messages would be differently uridylated under either circumstance; all evidence we have points to these sorts of messages being degraded normally. They no longer have a stem-loop, which eliminates the histone mRNP, and so they should be treated like normal untailed messages, which would be uridylated by TUT7 and TUT4. It is possible that if they are treated like normal messages, that a more complete knockdown is needed to fully detect a change in uridylation levels.

SORTING OUT RELATIVE CONTRIBUTIONS OF 5'-3' AND 3'-5' DEGRADATION

One thing that EnD-Seq/AppEND is currently unable to sort out is the question of 3'-5' degradation vs. 5'-3' degradation. Because our primers target the 3' end of the histone message specifically, there's no way to know what the 5' end of the message looks like. An mRNA which appears intact on the 3' end might be undergoing degradation from the 5' end. Finally the amounts of different intermediates present will depend on the relative rates of 5' to 3' and 3' to 5' degradation. Two earlier published studies from the lab have given us some insight into this, but the question looms large. In our lab's first look at uridylation in 2008, treating the RNA from HeLa cells with a decapping enzyme before circular PCR to look at both the 3' and 5' ends of actively degrading RNA showed that some mRNA molecules can be simultaneously degraded in both directions (Mullen and Marzluff 2008). In our second paper on uridylation we showed that messages that were immunoprecipitated with a 7-methylguanosine antibody are uridylated in a pattern similar to normal histone mRNAs, indicating that these molecules had undergone partial 3' to 5' degradation but still contained a cap.

Still, the nature of our sequencing method makes it impossible to determine if the tails we see in the stem-loop are precursors for Lsm1-7 binding and decapping, or to prime the exosome for degradation. Knockdown of exosome components has a greater effect on degradation than knockdown of XRN1, the 5' to 3' exonuclease (Mullen and Marzluff 2008), consistent with 3' to 5' degradation being an important pathway. Also, in the 3'hExo knockdown, uridylated RNAs accumulate after inhibition of DNA replication and aren't subject to decapping. One clue may be found in the length of the oligo(U) tails. The SLBP/Lsm complex is not capable of binding to a tail made of five uridines, but the binding is much stronger with 10 nucleotide tails (Lyons et al. 2013). In our sequencing results, we find that the tails on HIST2H2AA3 peak at five nucleotides and decline precipitously after this. It's possible that the reason for this is that any tail longer than five nucleotides is being bound by the Lsm complex, quickly degraded, thus it cannot be detected by sequencing. Similarly, the other uridylated intermediates further into the 3' UTR and ORF do not have tails that approach that length, so these shorter tails may simply function to prime for the exosome.

This is all speculation, of course. The only way to truly answer this question would be to design a sequencing method capable of sequencing the entire histone mRNA. Because histone genes tend to be of relatively smaller size (HIST2H2AA3 is about 400 nucleotides), both in the coding sequence and in the UTR, this is possible. We've done some very preliminary work with the TeloPrime kit from Lexogen that utilizes a specific 5' ligation for capped RNAs and had some success amplifying a full histone gene, from the 5' cap all the way to the 3' end. This would give us a much more reliable way to ensure that we are only sequencing capped intermediates than the cap IP that we've previously done, and by doing

that we may be able to separate some of the different pathways of degradation out from each other.

It may also be worthwhile to do a larger study in *Drosophila*, as flies have a large number of oligoadenylations in addition to oligouridylations. It's possible that the oligoadenylations might represent RNA marked for a different mechanism than the oligouridylation that appears as all oligouridylations in human cells.

FINAL CONCLUSIONS

The work in this thesis accomplishes several goals. It gives a new, more detailed model for the oligouridylation of histone mRNA, both as it applies to the message in its steady state and as it's being degraded. It also points to some future directions for both the study of the oligouridylation of histone mRNA and the study of the degradation of the message.

Our knockdown and sequencing studies of TUT7 and 3'hEXO in Chapter 2 establish TUT7 as the main enzyme that uridylates histone mRNA, both at a steady state and during degradation. They also indicate that the utility of the uridylation is determined by context; at steady state, 3'hEXO is part of the histone mRNP with SLBP, and TUT7 uridylates to restore the length of the message to prevent hEXO from trimming too far into the stem and destroying the equilibrium of the histone mRNP. Once degradation begins, that equilibrium is disturbed by some unknown factor, and hEXO trims further into the stem. Once hEXO trims into the stem, existing structure data tells us that it likely falls off of the stem. TUT7 continues to uridylate, but without hEXO to trim, the tail is considerably longer. This longer tail initiates the full degradation of the message, both via the exosome and the 5'-3' decapping pathway in a balance that is as yet undetermined.

In Chapter 3, we focus on the other TUTases. TUT1 is likely not involved in histone mRNA metabolism, as we initially speculated, but TUT3 seems very likely to have some role in the life of histone message, even if it is not directly involved in degradation. We show an *in vitro* interaction between SLBP and TUT3 and a knockdown and sequencing experiment that resembles a pattern previously published from an exosome component. More work needs to be done to firmly establish TUT3's localization, especially as it relates to histone mRNA.

This leaves several avenues for future studies. There is still work to be done to fully understand the roles of the exosome, the balance between it and the 5'-3' degradation pathway, the true role of the uridylation that begins degradation, how the degradation intermediates in the UTR (beyond the stem-loop) and the ORF are formed, the role of Upf1, and the role of the no-go decay proteins Dom34 and Hbs1. EnD-Seq and AppEND are very powerful tools that can help achieve this, with a combination of knockdowns, CRISPR knockouts, and model organism studies in *Drosophila*.

The question of TUT3 also remains; the enzyme has ties to the nuclear exosome and the exosome is tied to histone message degradation, but there's no real known role for the nuclear exosome during histone mRNA metabolism at this point. This obviously does not mean that a role cannot exist, but there is a lot of work left to be done to connect these dots.

Table 1. siRNA sequences used to target 3'hEXO and TUTases of interest

All siRNAs are targeted to the open reading frame of the genes in question (except the C2 control siRNA) and were generated by Dharmacon with either dTdT or UU overhangs.

siRNA	Sequence	Source
C2	5'-GGUCCGGCUCCCCAAAUG-3'	(EJ Wagner and Garcia-Blanco 2002)
TUT3	5'-GGACGACACUCAAUUAUU-3'	(Mullen and Marzluff 2008)
TUT4	5'-CAGCAAAAGCAGUGAAUA-3'	This paper
TUT7	5'-GAAAAGAGGCACAAGAAAA-3'	(Mullen and Marzluff 2008)
3'hEXO	5'-UUACGAAUGGCUGUAUUA-3'	(Mullen and Marzluff 2008)

Table 2. Important linker and primer sequences for EnD-Seq/AppEND

All sequences here originally reported in (Slevin et al. 2014). The off-set sequence for the P7 second round primer is a variable Illumina indexing sequence and changes from sample to sample.

Preadenylated Linker	5' Ap-CTGTAGGCACCATCAATCTCACTCCG-NH ₂ -3'
RT Primer (linker complement)	5'-CGGAGTGAGATTGATGGTGCCTACAG-3'
Round 1 primers	
V1.5-N5-RT Primer	5' GGTCAGAGTTCTACAGTCCGACGATC-NNNN-CGGAGTGAGATTGATGGTGCCTACAG-3'
Reverse N5 Consensus H2A	5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-CTGGCGGGCAACGCGGC-3'
Reverse N5 Consensus H2B	5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-GGTCCACCCGACACCGGCATCT-3'
Round 2 primers	
P5-V1.5	5' AATGATACGGCGACCACCGAGATCTACAC-CGACAGGTTTCAGAGTTCTACAGTCCGAC-3'
P7-Reverse-Index-Reverse	5' CAAGCAGAAGACGGCATACGAGAT-CGTGAT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'

Table 3. shRNA sequences used to target TUT1 isoforms

These sequences were flanked with hairpin sequences for cloning into the pLKO vector for use in lentiviruses, as the UNC lentivirus database did not include TUT1 isoform specific lentiviruses.

shRNA	Sequence	Source
TUT1A	5'GGCCAAAGACCUUAGGAGAGA-3'	This paper
TUT1B	5'-AGGACAAAGAGGAGAUGAAGA-3'	This paper

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