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QUANTITATIVE ANALYSIS OF THE HISTONE LOCUS BODY IN SUMO KNOCKOUT HUMAN CELLS

By

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ABSTRACT

Small ubiquitin-related modifiers (SUMOs) are proteins that can be reversibly conjugated to many other cellular proteins. Mammalian cells express up to five SUMO paralogs and our lab has recently generated paralog-specific knockout (KO) cells for SUMO1 and SUMO2 using CRISPR-Cas9. Analysis of these cells has exposed unique, paralog-specific phenotypes. In particular, SUMO1 and SUMO2 affected global gene expression patterns and PML nuclear body structure in unique ways. Using RNA-sequencing of poly(A)-selected mRNAs, we detected apparent lower levels of histone transcripts in SUMO1 KO cells but higher levels in SUMO2 KO cells, compared to wild type (WT) cells. Histone genes are not typically polyadenylated in healthy proliferative cells, but they can be polyadenylated in cases of cell differentiation, cancer, or 3' end processing errors. Our findings suggest that histone mRNA 3' ends may be misprocessed in SUMO KO cells. Because most core histone genes are both expressed and processed in a membrane-less organelle called the histone locus body (HLB), we assessed the localization of histone locus body factors NPAT and FLASH in WT, SUMO1 KO and SUMO2 KO cells. Using immunofluorescence microscopy, we observed similar colocalization of NPAT and FLASH in the HLBs of WT in SUMO KO cells, indicating no major defects in HLB assembly. We then used NPAT staining to further quantify the number and dimensions of HLBs in WT and SUMO KO cells. The mean HLB focus size was significantly larger in the knockout cells. Moreover, WT cells contained significantly more foci than both SUMO1 KO and SUMO2 KO cells. Based on our findings, we conclude that both SUMO1 and SUMO2 play a role in regulating histone mRNA processing through effects on the structure and function of HLBs.

PREFACE

Thesis readers:

Primary reader and advisor: Dr. Michael J. Matunis

Secondary reader: Dr. Anthony Leung

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Dedication:

This thesis is dedicated to my family for their steadfast support and encouraging me to find joy in life. It is also dedicated to all the brilliant women scientists past and present who inspire me to follow my curiosity about science.

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

\- Marie Curie

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INTRODUCTION

The diverse SUMO system

Small ubiquitin-related modifiers (SUMOs) are small (~100 amino acid; ~12 kDa) proteins that are covalently conjugated to other cellular proteins (Bohren et al., 2004; Johnson, 2004). SUMO conjugation is similar to that of its cousin ubiquitin (which shares the ubiquitin-like fold and 18% homology) in that it entails an enzymatic cascade of E1 activating enzyme, E2 conjugating enzyme, and E3 ligating enzyme. SUMO can be conjugated to substrate lysine residues within a canonical "ψKXE" motif, where ψ is a hydrophobic residue, K is the lysine to which SUMO is covalently conjugated, X is any amino acid, and E is glutamic acid (Rodriguez et al., 2001; Sampson et al., 2001). Also, like ubiquitination, sumoylation is made reversible by a family of SUMO-specific isopeptidases that cleave SUMO from the substrate protein (Kunz et al., 2018).

SUMO is conserved across eukaryotes. While some species, like *Saccharomyces cerevisiae* and *Drosophila melanogaster* express only one SUMO protein, others including vertebrates, plants, and some insects express multiple SUMO paralogs (Augustine et al., 2016; Citro and Chiocca, 2013; Estruch et al., 2016; Everett et al., 2013; Urena et al., 2016). Humans express four different SUMO paralogs: SUMO1, SUMO2, SUMO3, and SUMO4. SUMO1 and SUMO2 are ~45% identical, while SUMO2 and SUMO3 are ~96% identical. SUMO4 is ~87% homologous to SUMO2 . While SUMOs 1-3 are expressed ubiquitously across cell types, SUMO4 expression has primarily been detected in specific tissues (Bohren et al., 2004). A fifth human SUMO, SUMO5, has been identified and is most homologous to SUMO1 (Liang et al., 2016), but its expression in vivo has yet to be verified.

SUMO1 and SUMO2 are the most well-studied paralogs and, as their divergent homology suggests, differ in key molecular features. Only SUMO2 contains a sumoylation consensus lysine (K11) which can be sumoylated to form polySUMO chains (Tatham et al., 2001). These polySUMO2 chains function as protein scaffolds to facilitate assembly of large multi-protein complexes (Jansen and Vertegaal, 2021). Another functional role of polySUMO2 chains is in recognition by SUMO-targeted ubiquitin ligases, leading to substrate protein degradation (Erker et al., 2013; Tatham et al., 2008). In contrast, SUMO1 lacks a sumoylation consensus sequence and thus has less propensity to form chains. Other sequence differences between SUMO1 and SUMO2 also distinguish their protein interactions. Thus, some SUMO E3 ligases specifically act on either SUMO1 or SUMO2 and promote paralog-specific modification of substrates (Cappadocia and Lima, 2018). SUMO-specific isopeptidases also show preference or specificity for different paralogs (Mikolajczyk et al., 2007; Mukhopadhyay and Dasso, 2007). In addition to these distinct sumoylation dynamics, cellular proteins have preferential non-covalent binding affinities for either SUMO1 or SUMO2 (Hecker et al., 2006; Namanja et al., 2012; Zhu et al., 2008). These differences in the covalent and non-covalent interactions of SUMO1 and SUMO2 with different cellular proteins likely dictate distinct cellular functions.

Mounting evidence reveals unique and non-redundant functions for SUMO1 and SUMO2 in numerous cellular processes. Moreover, the SUMO paralogs have unique roles in various disease pathogeneses including neurodegeneration and cancers (Seeler and Dejean, 2017; Yau et al., 2020). Several approaches have been employed to better understand the distinct functions of SUMO1 and SUMO2. Gene expression

databases indicate SUMO2 is more abundantly expressed than SUMO1 in most cell types (Bouchard et al., 2021). Subcellular localization visualized by immunofluorescence microscopy of SUMO1 and SUMO2 also differ (Ayaydin and Dasso, 2004; Baczyk et al., 2018; Zhang et al., 2008). SUMO1 distinctly localizes at the nuclear envelope while SUMO1 and SUMO2 both localize in nuclear puncta identified to be PML nuclear bodies (de The et al., 2012; Muller et al., 1998). Despite these prominent, characteristic staining patterns, both SUMOs are ubiquitous throughout the nucleus and can also be detected in the cytoplasm. Another method to study the SUMO paralogs in cells is by exogenous expression of tagged SUMO constructs. These studies have revealed SUMO1 and SUMO2 exhibit distinct dynamics during cell cycle progression and cellular stress responses (Ayaydin and Dasso, 2004). However, exogenous overexpression may not model dynamics at endogenous SUMO levels. SUMO1 and SUMO2 genetic knockout mouse studies have revealed specific SUMO1 functions in adult adipogenesis (Mikkonen et al., 2013), while SUMO2 is essential for embryonic development (Wang et al., 2014). Recently, our lab characterized paralog-specific knockout cells generated by CRISPR-Cas9 in U2OS human osteosarcoma cells. Analysis of these cells demonstrated distinct roles for SUMO1 and SUMO2 in gene expression, cellular stress responses, cell morphology, and nuclear membrane-less organelle structure (Bouchard et al., 2021).

SUMO paralog-specific effects on histone transcripts

One intriguing phenotype of the SUMO1 and SUMO2 KO U2OS cells was the apparent opposite changes in expression of certain histone genes, as measured by

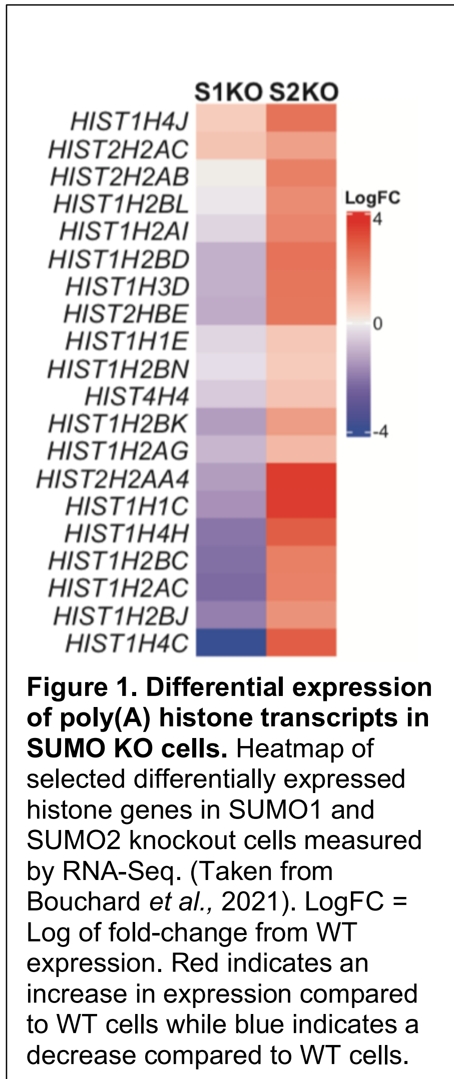


Figure 1. Differential expression of poly(A) histone transcripts in SUMO KO cells. Heatmap of selected differentially expressed histone genes in SUMO1 and SUMO2 knockout cells measured by RNA-Seq. (Taken from Bouchard *et al.,* 2021). LogFC = Log of fold-change from WT expression. Red indicates an increase in expression compared to WT cells while blue indicates a decrease compared to WT cells.

RNA-Seq (Figure 1). Compared to wild type cells, a similar subset of histone transcripts appeared to be less abundant in SUMO1 KO cells but more abundant in SUMO2 KO cells. Key to interpreting this finding, though, is that RNA-Seq was performed using poly(A)-selected transcripts, but histone genes are actually the only eukaryotic transcripts that are not typically polyadenylated (Marzluff and Koreski, 2017). With this in mind, it was curious that histone transcripts were detected at all. However, it has been observed that histone transcript polyadenylation occurs under certain physiological cellular conditions, as well as in cancer cells and in patients with type 1 interfonopathies (Ghule et al., 2009; Kari et al., 2013; Uggenti et al., 2020).

While not polyadenylated in normal proliferating cells, histone transcripts undergo co-transcriptional processing, involving cleavage of a histone downstream element (HDE), leaving a unique 3’ stem-loop structure as the terminal element of the processed mRNA (Marzluff and Koreski, 2017). Polyadenylation of a subset of histone gene mRNA transcripts does, however, occur under normal physiological conditions in terminally differentiated cells (Lyons et al., 2016). Furthermore, there is a baseline amount of polyadenylated histone mRNAs in wild type U2OS cells (Kari et al., 2013), explaining how SUMO1 knockout cells could have contained fewer mRNA transcripts than WT

cells. Interestingly, the subset of histone genes affected in SUMO KO cells correspond to the same transcripts that are polyadenylated in cancers and differentiated cells (Kari et al., 2013; Lyons et al., 2016).

However, our results indicating altered expression of detectable polyadenylated histone genes by RNA-Seq in SUMO1 and SUMO2 knockout cells may still be a result of altered regulation at either the transcriptional level or the processing level or a combination of both. Specifically, SUMO1 knockout cells may simply downregulate histone gene expression, or they may be more efficient at limiting polyadenylation than WT cells. In contrast, SUMO2 knockout cells may upregulate histone gene expression, leading to overall higher levels of misprocessed, polyadenylated transcripts compared to WT. Alternatively, SUMO2 may serve a role in histone 3' end processing, and in SUMO2 knockout cells, more aberrant processing and polyadenylation occurs. Additional experiments exploring histone mRNA levels and specific changes in 3' end processing will be needed to fully understand the molecular bases for the observed changes in SUMO KO cells.

Possible roles for SUMO1 and SUMO2 in histone mRNA biogenesis

In proliferating cells, the histone genes that code for the canonical core histones required for organizing newly synthesized DNA are expressed at high levels specifically during S phase, and are therefore referred to as replication-dependent histone genes (Marzluff and Koreski, 2017). Cell cycle coordination is mediated by cyclin E/Cdk2, which phosphorylates a factor required for histone gene expression called NPAT (Nuclear protein at the ataxia- telangiectasia locus) (Zhao et al., 2000). While sumoylation functions in cell cycle progression (Mukhopadhyay and Dasso, 2017), our

lab found that the SUMO1 KO and SUMO2 KO cells do not significantly differ in time spent in any of the cell cycle stages (G0/G1, S, G2/M), as measured by flow cytometry (Bouchard et al., 2021). We therefore do not suspect that differences observed in histone gene expression are due to differences in time spent in S phase.

Histone gene transcription and processing occur in a membrane-less organelle called the histone locus body (HLB) which forms around the two major clusters of replication-dependent histone genes (Duronio and Marzluff, 2017; Marzluff and Koreski, 2017). The assembly and size of HLBs is affected by alterations in histone mRNA processing, as demonstrated by expression of misprocessed histone transcript mutants containing an uncleavable HDE or a functional poly(A) tail (Shevtsov and Dundr, 2011). Based on these observations, we hypothesize that histone mRNA 3' end processing defects in SUMO KO cells will correlate with detectable changes in HLB number and size.

In the Shevtsov & Dundr experiments (Shevtsov and Dundr, 2011), HLB biogenesis was measured by the association of mutant histone transcripts (visualized by RNA FISH) with the HLB protein NPAT visualized by immunofluorescence microscopy. NPAT is both required for HLB formation as well as histone gene expression (Duronio and Marzluff, 2017; Zhao et al., 2000). NPAT is a large protein, consisting of 1,427 amino acids, and contains four predicted sumoylation consensus sequences, suggesting possible regulation by sumoylation. To test our hypothesis that HLB number and size may be affected in SUMO KO cells, we therefore analyzed NPAT localization and quantified HLB size and number in WT, SUMO1 KO and SUMO2 KO cells, as summarized in the results section of this thesis.

There are several more factors required for histone mRNA production; when misregulated, processing errors and polyadenylation occur (Duronio and Marzluff, 2017; Tatomer et al., 2016). These include Flice-associated huge protein (FLASH), which both promotes transcription and is required for processing, and U7 small nuclear ribonucleoprotein complex (U7 snRNP). The U7 snRNP subunit Lsm11 binds to the N terminus of FLASH, which is localized to the HLB, and in turn, the histone cleavage complex (HCC) is recruited to the FLASH-Lsm11 interface (Kolev and Steitz, 2005; Sabath et al., 2013; Yang et al., 2011). HCC contains endonuclease CPSF-73 and symplekin, which are both required for the 3' end processing of histone mRNAs (Kolev and Steitz, 2005). Intriguingly, both CPSF-73 and symplekin are also required for mRNA cleavage and polyadenylation of non-histone mRNAs and their activities are regulated by SUMO2 modification (Vethantham et al., 2007). Based on this knowledge, we hypothesize that possible defects in histone 3' end processing in SUMO2 KO cells may also be related in part to defects in CPSF-73 and symplekin function.

Disruption of the HCC association with FLASH and U7 snRNP causes defects in 3' end processing that leads to aberrant polyadenylation of histone mRNAs. For example, in *Drosophila* the mislocalization of FLASH away from the HLB slows 3' end processing and as a consequence transcripts accumulate in the histone locus body where they are polyadenylated (Tatomer et al., 2016). The structural function of FLASH in HLBs to coordinate interactions between multiple complexes is reminiscent of another structural protein, PML, in the membrane-less organelles PML nuclear bodies. PML acts as a structural protein by self-associating and binding resident proteins, functions that are dependent on its covalent sumoylation and non-covalent interactions with SUMO

(Matunis et al., 2006). PML is sumoylated at multiple residues, contains multiple SUMO interaction motifs, and even acts as an E3 SUMO ligase (Guo et al., 2014; Lallemand-Breitenbach et al., 2001). FLASH is a huge protein with 1,692 amino acids containing seventeen predicted sumoylation sites, one of which has been confirmed experimentally (Alm-Kristiansen et al., 2009), and in addition, four tandem SUMO interaction motifs (Sun and Hunter, 2012). Sumoylation of FLASH has been proposed to affect both its stability (Vennemann and Hofmann, 2013) and its transcriptional activities (Alm-Kristiansen et al., 2009). We further hypothesize that FLASH and sumoylation may affect HLBs in a manner similar to sumoylaiton and PML in PML nuclear bodies.

Thesis research overview

Through studies presented in this thesis, we examined how apparent shifts in levels of polyadenylated histone mRNAs detected in SUMO1 KO and SUMO2 KO cells by RNA-Seq are related to possible changes in HLB number and morphology. We analyzed HLBs in U2OS WT, SUMO1 KO, and SUMO2 KO cells in three ways: (1) assessment of co-localization of HLB proteins NPAT and FLASH, (2) assessment of the size of NPAT-labeled HLBs, and (3) assessment of the number of NPAT-labeled HLBs.

We first examined NPAT and FLASH co-localization in U2OS WT, SUMO1 KO, and SUMO2 KO cell nuclei by immunofluorescence microscopy. We hypothesized that mislocalization of either NPAT or FLASH would negatively affect HLB structure and lead to decreased histone gene expression and 3’ processing efficiency. We found that NPAT and FLASH co-localized to HLB foci in both SUMO1 KO and SUMO2 KO cells to a similar extent as observed in WT cells. We therefore next used NPAT staining as a presumptive marker for HLBs in all three cell lines to test the hypothesis that HLB size

and number would be altered in SUMO KO cells. To compare HLB size between cell lines, we quantitatively measured the areas and perimeters of NPAT foci from immunofluorescence microscopy images. Consistent with our hypothesis, we found that HLBs are significantly larger in SUMO KO cells compared to WT cells. Finally, we compared the number of NPAT-labeled foci in WT, SUMO1 KO, and SUMO2 KO cells. Again, consistent with our hypothesis, we found that SUMO KO cells had significantly fewer detectable HLBs compared to WT cells. We thus conclude that SUMO1 and SUMO2 both play roles in regulating histone mRNA biogenesis in part through effects on HLB structure and function.

RESULTS

Previous RNA-Sequencing experiments by our lab revealed changes in levels of polyadenylated histone transcripts in SUMO1 KO and SUMO2 KO cells (Bouchard et al., 2021). Compared to WT cells, SUMO1 KO cells exhibited a decrease in polyadenylated histone transcripts, while SUMO2 KO cells exhibited an increase in polyadenylated histone transcripts (Figure 1). Because histone gene expression and 3' mRNA processing occur in the HLB, we hypothesized that these changes in histone mRNAs will be associated with changes in HLB morphology.

NPAT and FLASH colocalize in WT, SUMO1 KO, and SUMO2 KO cells

Our first approach to assess HLB morphology in SUMO1 KO and SUMO2 KO cells was to visualize localization of key HLB proteins by immunofluorescence microscopy. We assessed the localization patterns of NPAT, which is required for HLB formation and histone gene expression, as well as FLASH, which is required for histone transcript processing (Bongiorno-Borbone et al., 2008; Marzluff and Koreski, 2017).

As expected, anti-NPAT and anti-FLASH antibodies consistently co-localized in discrete nuclear foci in WT cells (Figure 2). It is established from previous studies that NPAT and FLASH both co-localize to the HLB (Bongiorno-Borbone et al., 2008; Duronio and Marzluff, 2017). We therefore concluded that the nuclear foci containing both NPAT and FLASH are likely HLBs. Similar colocalization patterns of NPAT and FLASH were observed in both SUMO1 KO and SUMO2 KO cells (Figure 2). Because NPAT and FLASH colocalization in SUMO1 KO and SUMO2 KO cells did not appear to differ from

that in WT cells, we conclude that SUMO1 and SUMO2 do not regulate NPAT or FLASH localization to the HLB.

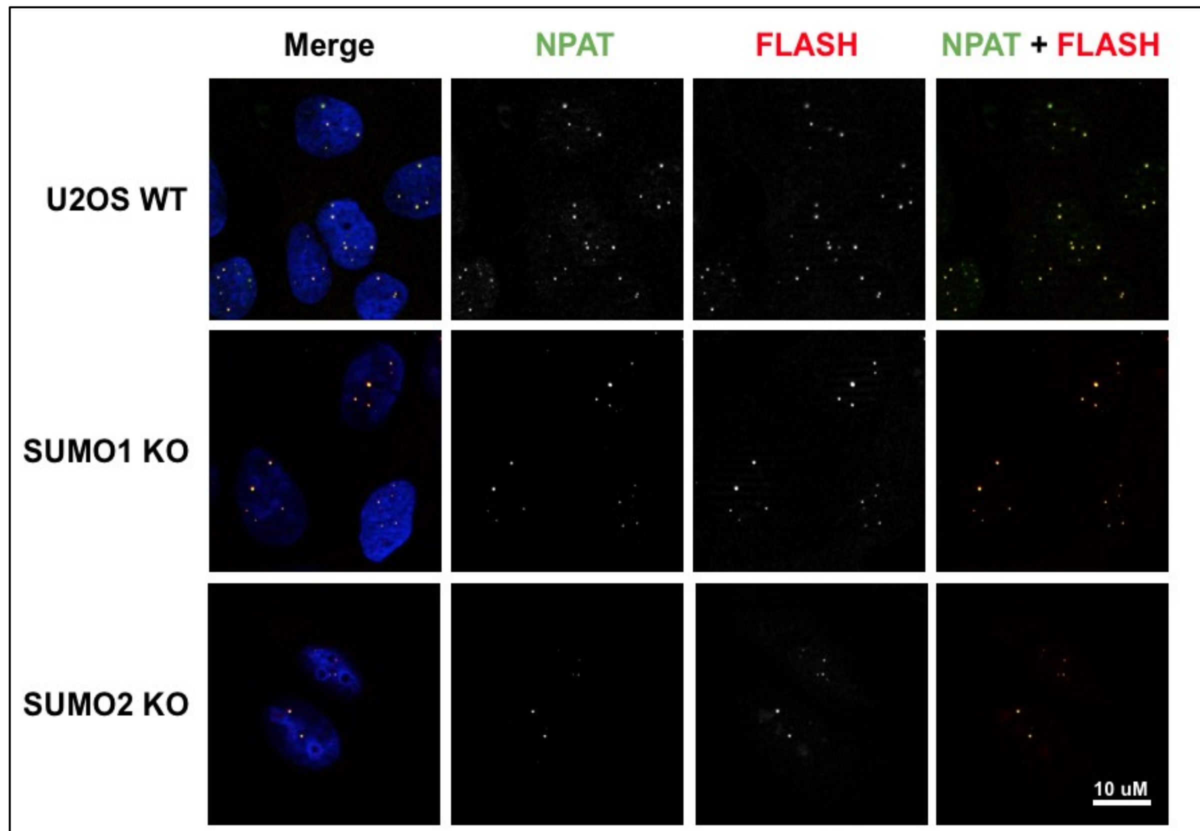
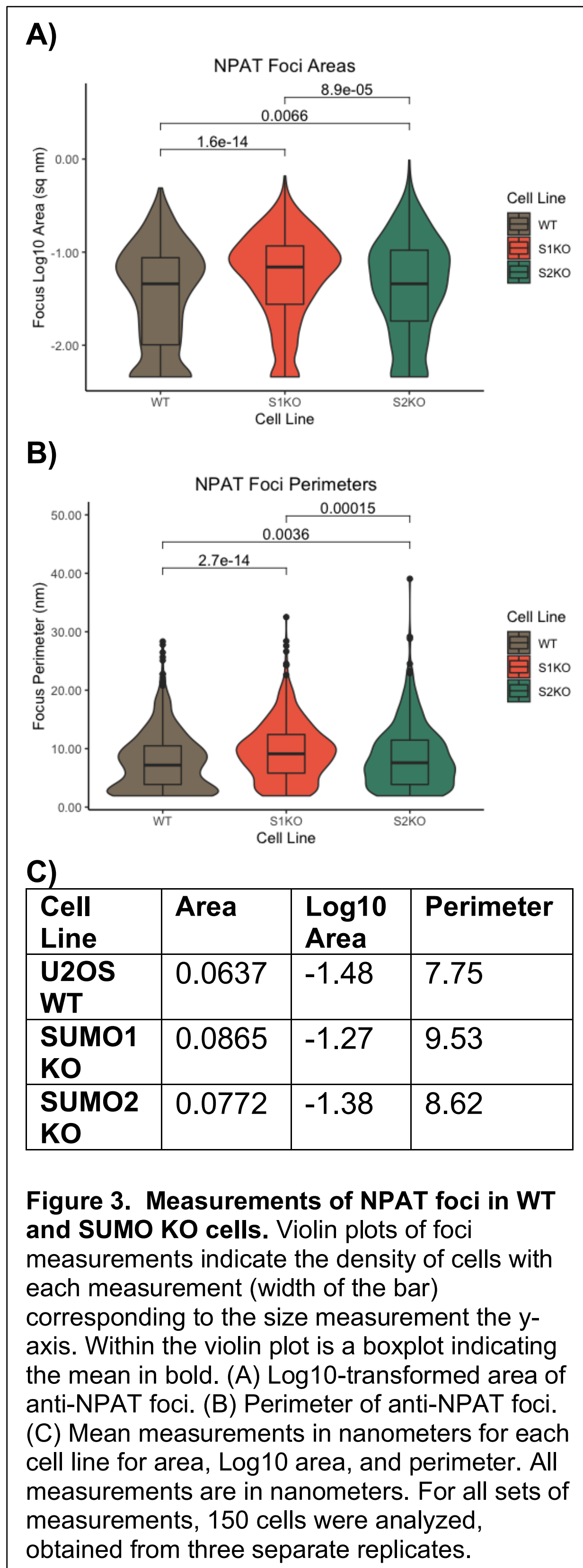


Figure 2. Immunofluorescence microscopy of NPAT and FLASH in SUMO KO cells. Cells were co-labeled with anti-NPAT (green) and anti-FLASH (red) antibodies and stained with DAPI (blue). First panel in each row is a merge of all three signals, second panel is only the anti-NPAT signal, third panel is only the anti-FLASH signal, and the fourth panel is a merge of only the anti-NPAT and anti-FLASH signals.

SUMO1 KO and SUMO2 KO cells contain larger NPAT-labeled HLBs

NPAT and FLASH are routinely used as markers for the HLB (Duronio and Marzluff, 2017). A convenient conclusion from observing similar NPAT and FLASH colocalization patterns across WT and KO cell lines is that the antibodies could serve as putative markers for the HLB in these cells.



Cell Line	Area	Log10 Area	Perimeter
U2OS WT	0.0637	-1.48	7.75
SUMO1 KO	0.0865	-1.27	9.53
SUMO2 KO	0.0772	-1.38	8.62

Figure 3. Measurements of NPAT foci in WT and SUMO KO cells. Violin plots of foci measurements indicate the density of cells with each measurement (width of the bar) corresponding to the size measurement the y-axis. Within the violin plot is a boxplot indicating the mean in bold. (A) Log10-transformed area of anti-NPAT foci. (B) Perimeter of anti-NPAT foci. (C) Mean measurements in nanometers for each cell line for area, Log10 area, and perimeter. All measurements are in nanometers. For all sets of measurements, 150 cells were analyzed, obtained from three separate replicates.

While examining NPAT and FLASH localization, we observed apparent differences in sizes of the labeled nuclear foci in the SUMO KO cells. In particular, SUMO1 KO cells appeared to contain larger foci than WT cells. We hypothesized that lower amounts of polyadenylated histone mRNAs (Figure 1) might be due to more efficient processing in larger HLBs in SUMO1 KO cells.

Using immunofluorescence localization of NPAT, we investigated the size of nuclear NPAT-labeled foci of 150 cells of each cell line from three replicate immunofluorescence experiments (50 cells per replicate). To objectively and quantitatively examine potential differences in foci size, anti-NPAT immunofluorescence microscopy images were analyzed using the ImageJ plugin BioVoxxel's Speckle Inspector

(Brocher, 2015). Obtained area and perimeter measurements of foci were then compared between cell lines (Figure 3).

Consistent with our previous observations by microscopy, focus size quantitation revealed SUMO1 KO nuclear NPAT foci mean area and mean perimeter were significantly greater than that of WT (Figure 3). In addition, SUMO2 KO cell NPAT nuclear foci were also significantly larger than that of WT, but the difference was greater in SUMO1 KO cells (Figure 3). For example, mean focus perimeter was 23% greater than WT in SUMO1 KO cells, whereas mean focus perimeter was 11% greater than WT in SUMO2 KO cells.

Analysis of violin plots of size measurements revealed a larger proportion of small sized foci in WT cells compared to both KO cell lines (the base of the violin plot is wider than in other cell lines (Figure 3). How this relates to our hypotheses about how SUMO1 and SUMO2 regulate NPAT localization to HLBs will be explored in the discussion section below.

SUMO1 knockout and SUMO2 knockout nuclei contain fewer counted NPAT foci

We next investigated the hypothesis that SUMO1 and SUMO2 regulate HLB formation by counting the number of NPAT foci per nucleus in WT, SUMO1 KO, and SUMO2 KO cells. In human diploid cells, there are 2-4 HLBs depending on cell cycle stage. U2OS cells are reported to be hypertriploid and contain 6-8 HLBs (Ghule et al., 2009). We hypothesized that if SUMO1 or SUMO2 regulate HLB formation or stability, we would observe fewer NPAT-labeled foci in SUMO1 KO and SUMO2 KO cells. For instance, SUMO1 and SUMO2 promote PML nuclear body formation and stability and,

as predicted, SUMO1 KO and SUMO2 KO cells contain fewer PML nuclear bodies (Bouchard et al., 2021).

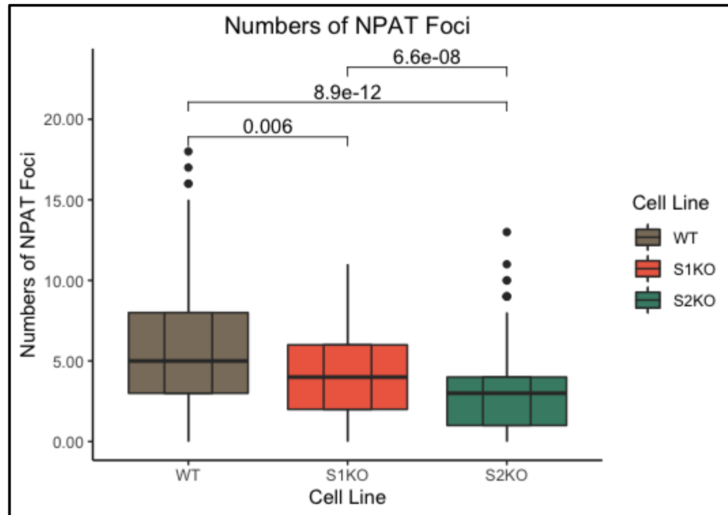


Figure 4. Number of NPAT foci counted in each nucleus by cell line. Boxplot comparing mean number of foci counted in WT, SUMO1 KO, and SUMO2 KO cells. Bold lines indicate means, which are as follows: WT = 5.95, S1KO = 4.49, S2KO = 3.07 foci/nucleus. 3 independent replicates, 50 cells per replicate = n = 150 n = 150 nuclei/cell line.

To test this hypothesis, we quantified the number of NPAT foci per nucleus using the ImageJ plugin BioVoxxel's Speckle Inspector function (Brocher, 2015). The same cells analyzed in Figure 3 were used for this analysis (150 cells/ cell line; 3 independent replicates of 50 cells).

Both SUMO1 KO and SUMO2 KO cells contained fewer foci than WT (Figure 4). WT cells contained a mean of 5.95 foci per nucleus, consistent with the literature. In contrast, SUMO1 KO cells contained a mean of 4.49 foci per nucleus and SUMO2 KO contained a mean 3.07 foci per nucleus. Interestingly, the effects of SUMO1 or SUMO2 loss paralleled what was observed for PML nuclear bodies in these cells; SUMO2 contains fewer counted foci than those in SUMO1 KO (Bouchard et al., 2021). These data suggest that SUMO2 may play a distinct role from that of SUMO1 in regulating nuclear body formation and structure.

Together, these experiments indicate that SUMO1 and SUMO2 affect HLB size and number. Loss of SUMO1 had a greater effect on increasing HLB size than loss of SUMO2, while loss of SUMO2 had a greater effect on decreasing HLBs counted in

nuclei. These distinct phenotypes suggest that SUMO1 and SUMO2 regulate HLB stability in unique ways.

DISCUSSION

In this study, we found that HLB morphology is altered in SUMO1 KO and SUMO2 KO cells compared to WT U2OS cells. Specifically, SUMO KO cells contained fewer, but larger NPAT-labeled HLBs. Because HLB proteins NPAT and FLASH co-localized in both WT and SUMO KO cells (presumably to the HLB; this would need to be formally verified with additional FISH analysis), we conclude that SUMO1 and SUMO2 do not regulate their targeting and localization to the HLB. Instead, we conclude that SUMO1 and SUMO2 regulate the dynamics of protein targeting and association with HLBs and possibly the assembly and structure of the HLB itself.

Curiously, the number of NPAT foci varied between cell lines, but all cell lines are expected to contain the same number of histone loci, which are defined by the tandem arrays of core histone genes on chromosomes 1 and 6 (Duronio and Marzluff, 2017). U2OS cells have previously been determined hypertriploid by FISH analysis and contain 6-8 HLBs (Ghule et al., 2009). Because WT cells contained more foci than the SUMO KO cells (Figure 4), and a greater number of small foci (Figure 3), WT cells may have NPAT foci that assemble independently of the HLB. This would suggest that SUMO affects NPAT self-assembly or the localization and stability of its association with HLBs. In future studies, we plan to explore this further by evaluating the dynamic associations of NPAT and other HLB-associated factors with HLBs using fluorescence recovery after photobleaching experiments in live cells.

Different phenotypes in SUMO1 and SUMO2 KO cells

While both SUMO1 KO and SUMO2 KO cells contained fewer and larger HLBs than WT, the degree to which the HLBs were changed differed between the cell lines. Specifically, SUMO1 KO cells contained a greater number of large HLBs compared to SUMO2 KO cells, and SUMO2 KO cells contained fewer HLBs than in SUMO1 KO cells. We therefore conclude that SUMO1 and SUMO2 regulate HLB morphology in unique ways.

The finding that SUMO1 and SUMO2 regulate the HLB in unique ways is consistent with our previous finding that apparent levels of polyadenylated histone transcripts were differentially altered in SUMO1 KO and SUMO2 KO cells. Specifically, RNA-Seq analysis revealed that SUMO1 KO cells contained reduced levels of polyadenylated histone mRNA transcripts compared to WT cells, while SUMO2 KO cells contained higher levels compared to WT cells (Figure 1). Considering our finding that SUMO1 KO cells contained larger NPAT-labeled HLBs than WT, larger HLBs therefore appear to correlate with more efficient histone mRNA 3' end processing. Larger foci and more efficient processing would be consistent with more efficient and stable recruitment of processing factors to HLBs in the absence of SUMO1, and therefore suggest a negative role for SUMO1 in regulating these processes in WT cells.

It is also possible that in the absence of SUMO1, more SUMO2 modification occurs where SUMO1 would normally be conjugated. If this is the case, we would conclude that SUMO2 positively regulates HLB function by promoting larger and presumably more stable HLBs, and more efficient mRNA processing. However, if SUMO2 promotes HLB stability and function, we would have expected a decrease in

the size of HLBs in SUMO2 KO cells, which is in contrast to our findings. Nonetheless, the finding that normal histone mRNA 3' end processing is apparently reduced in SUMO2 KO cells supports a positive role for SUMO2 in HLB function. These discrepancies raise possible questions and concerns about our methodologies used to quantify HLB number and size, as discussed below.

To further distinguish the specific roles for SUMO1 and SUMO2 in histone mRNA biogenesis and HLB morphology, future studies could take advantage of genetic rescue experiments. For example, in SUMO1 KO cells, either a conjugation-defective mutant SUMO1 or SUMO1 with a mutated SIM (SUMO interaction motif) binding domain could be introduced to the SUMO1 KO cells. The resulting phenotypes could reveal if SUMO1 regulates these processes by covalent conjugation to substrates or by non-covalent interactions with SIM-containing proteins. Similarly, reintroduction of a mutant SUMO2 that cannot form chains through the consensus site lysine in the N-terminus (K11R mutation) into SUMO2 KO could help distinguish if SUMO2 regulates these processes through polymeric chain formation, as we hypothesized. To determine if paralog compensation occurs in KO cells, we would predict that the phenotype would be accentuated with exogenous expression of the other paralog. For example, would SUMO1 KO cells with extra SUMO2 contain even larger NPAT-labeled HLBs? Would they also express relatively fewer polyadenylated histone mRNAs than SUMO1 KO cells without the added SUMO2? It should be noted that we have preliminary data indicating SUMO2 KO rescued with WT SUMO2 or with SUMO1 fails to rescue HLB morphology to WT level phenotypes. Interestingly, PML nuclear body number was not fully rescued in SUMO2KO rescue cells either (Bouchard et al., 2021).

SUMO regulation of histone gene transcription and processing

It still remains to be formally demonstrated that changes in histone mRNAs detected in SUMO1 and SUMO2 KO cells are due to 3' end mis-processing and polyadenylation. Both SUMO paralogs regulate transcription factors (Cubenas-Potts and Matunis, 2013) and changes in mRNA expression levels could also contribute to our RNA-Seq results. Further RNA-Seq analysis using ribominus-selected mRNAs, which avoids poly(A) purification, is currently underway to help further distinguish between changes in histone mRNA expressing and processing.

The primary regulators of histone gene transcription are NPAT and FLASH (Duronio and Marzluff, 2017; Marzluff and Koreski, 2017), and as we observed, both proteins properly localize to the HLB (Figure 2). We did not formally test the functionality of either protein in the KO cells, however. FLASH is a huge protein with 1,692 amino acids containing 17 predicted sumoylation sites, one of which has been confirmed experimentally, and in addition, 4 tandem SUMO interaction motifs (Alm-Kristiansen et al., 2009; Sun and Hunter, 2012). Sumoylation of FLASH has been proposed to affect both its stability (Vennemann and Hofmann, 2013) and its transcriptional activities (Alm-Kristiansen et al., 2011; Alm-Kristiansen et al., 2009). The functions of the tandem SUMO interaction motifs, however, have not been explored. It is nonetheless intriguing to speculate that both covalent sumoylation and non-covalent SUMO binding may allow FLASH to affect the recruitment and assembly of other factors with HLBs, in a manner analogous to PML and its role in PML nuclear body assembly and function (de The et al., 2012; Matunis et al., 2006). Given the currently known connections between SUMO1 and SUMO2 regulation of FLASH activity and stability, further studies are

needed to fully investigate how these contribute to HLB function and histone mRNA biogenesis.

Levels of polyadenylated histone transcripts are associated with alterations in 3' end processing that have been observed in terminally differentiated cells (Lyons et al., 2016), some cancers (Ghule et al., 2009; Kari et al., 2013), and other disease conditions (Uggenti et al., 2020). Thus, cellular mechanisms exist to shift the 3' end processing of histone mRNAs from cleavage and stem loop formation to cleavage and polyadenylation under specific conditions. Mechanisms regulating this shift, however, are not known. Histone transcripts contain a 3' HDE that is cleaved by endonuclease CPSF73, which is also required for non-histone mRNA cleavage and polyadenylation (Marzluff and Koreski, 2017). Similarly, symplekin is required for 3' end cleavage and processing of histone mRNAs as well as cleavage and polyadenylation of non-histone mRNAS. Intriguingly, both of these factors are modified by SUMO2, and sumoylation is required for efficient cleavage and polyadenylation of non-histone mRNAs in vitro and in cells (Vethantham et al., 2007). Thus, it can be hypothesized that the sumoylation status of these factors may play a role in regulating their assembly into specific complexes uniquely involved in histone and non-histone mRNA processing. Defects in sumoylation could thus alter the assembly or functions of CPSF73 and symplekin in ways the affect 3' end processing. In this regard, it will be important to evaluate possible alterations in the 3' end processing of non-histone mRNAS as part of future studies.

Quantification of NPAT foci and considerations for future studies

We used ImageJ (Rueden et al., 2017) to quantify HLB foci, an approach that allowed for analysis of a large number of cells in a mostly unbiased manner.

Nonetheless, our approach did require user-defined intensity thresholds that influenced the final results. Data were analyzed using a number of different thresholds, with slightly varying outcomes. However, although outcomes varied, trends between cell lines were consistent and the results that are reported here were deemed to be best representative of the collected data sets.

Our analysis also relied on analysis of NPAT foci, which may not all necessarily represent HLBs. While NPAT is generally considered a good marker for HLBs, a more rigorous approach to measuring the size of *de facto* HLBs would include FISH detection of histone loci, as has been described (Ghule et al., 2009).

Additionally, our analysis involved collection of two-dimensional images and HLBs out of the focal plane of these images were not captured. Thus, analysis of reconstructed three-dimensional images may further improve the quality of data collection. This technique would also allow us to measure differences in nuclear volume or shape, which may be relevant given that SUMO2 KO cells are known to have a unique, fibroblast-like cell morphology as opposed to the epithelial morphology observed of U2OS WT and SUMO1 KO cells. If the fibroblast-like morphology of SUMO2 KO cells results in a flatter-shaped nucleus, we might expect that more NPAT foci in SUMO2 KO nuclei were in focus for the current analysis of foci per cell. Therefore, compared to SUMO2 KO cells, WT and SUMO1 KO cell NPAT foci may be undercounted.

Additionally, directly tagging NPAT with GFP using genetic engineering may also improve and simplify analysis. GFP-tagged NPAT would also allow for the study of HLB

dynamics in live cells, and these studies could reveal how SUMO1 and SUMO2 affect NPAT localization associations with HLBs.

SUMO2 and HLB structure and PML nuclear bodies

If SUMO1 KO cells exhibit larger HLBs due to an increase in SUMO2 conjugation in place of SUMO1, and SUMO2 KO cells contain more polyadenylated histone mRNAs due to defects in their normal 3' end processing, we would conclude that SUMO2 promotes HLB structure, function and histone mRNA 3' end cleavage and stem loop formation. A number of mechanisms may explain this positive role, including the intriguing idea that SUMO2 modification of HLB-associated factors may promote phase separation. SUMO2 has been shown to facilitate phase separation and formation of other membrane-less organelles, in particular, PML nuclear bodies (Banani et al., 2016). Consistent with this role, we found that PML nuclear body size and number were affected in SUMO2 KO cells (Bouchard et al., 2021). Specifically, the number of PML nuclear bodies decreased compared to WT cells, while the size (perimeter) of the nuclear bodies was greater than WT cells, which parallels SUMO2 KO phenotypes for NPAT foci found in this study. Taken together, these findings suggest that SUMO2 may have roles in affecting HLB structure and function similar to its roles in PML nuclear bodies.

PML nuclear bodies are a hub of SUMO modification, and SUMO2 is visualized as bright foci co-localizing with PML in the nucleus by immunofluorescence microscopy (de The et al., 2012; Muller et al., 1998). Because we hypothesize that SUMO2 promotes HLB assembly and stability similar to effects on PML nuclear bodies, we can

further predict that SUMO2 co-localizes with NPAT at HLBs. However, preliminary immunofluorescence microscopy experiments did not indicate obvious co-localization of SUMO2 at NPAT foci similar to co-localization with PML. This, however, does not rule out the possibility that SUMO2 regulates factors at relatively low levels within HLBs (the SUMO2 signal is abundant throughout the nucleus and makes co-localization to foci difficult if not above the nucleoplasmic signal).

In conclusion, based on our preliminary findings, further studies to determine the precise mechanisms by which SUMOs regulate histone mRNA biogenesis and the function of HLBs is clearly warranted. Numerous HLB-associated factors and factors involved in mRNA cleavage and 3' end processing are known SUMO substrates, which provide immediate avenues and hypotheses to follow. In addition, our findings suggest that roles for SUMO in PML nuclear bodies may be extended to the HLB. Future experiments to explore these and other ideas will provide valuable insights into histone mRNA biogenesis and general functions and properties of sumoylation.

METHODS

Cell Lines and Cell Culture Conditions

U2OS WT, SUMO1 KO, and SUMO2 KO cells were grown at 37°C, 5% CO_2 in DMEM (Gibco, catalog number: 11965-092) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologics, catalog number: S11550).

Immunofluorescence Microscopy

Cells were seeded in a 6-well dish and grown overnight, washed twice with phosphate buffered saline (PBS), then fixed in 3.5% paraformaldehyde in PBS for 7 minutes, followed by permeabilization in 0.05% Triton-X-100 in phosphate buffered saline for 20 minutes, and finally washed twice in PBS. Cells were then incubated with anti-FLASH (Sigma, catalog number: LS-C81573, 1:500 dilution) and/or anti-NPAT (SantaCruz, catalog number: SC-136007, 1:100 dilution) antibodies for 1 hour. Cells were then washed three times, followed by incubation with Alexa fluorescent secondary antibodies (anti-rabbit-594 at 1:400 dilution and anti-mouse-488 at 1:400 dilution) for 40 minutes. After a final 3 washes, coverslips were then mounted using Fluoroshield Mounting Medium with DAPI (Abcam, catalog number: ab104139). Microscopy images were taken using an upright Zeiss Observer Z1 fluorescence microscope with an Apotome VH optical section grid. Representative images of each cell line were taken at the same exposures using a 63x objective.

Quantitative Immunofluorescence Analysis

Samples were prepared as above with anti-NPAT antibody and DAPI. Images were acquired with the 63x objective. 16-bit grayscale images were exported from the AxioVision Release 4.8 software. Images were opened in FIJI (Schindelin et al., 2012), where nuclei (DAPI) and foci (NPAT) signal thresholds were optimized for each of the three replicate experiments. The resulting images were then used for the Speckle Inspector function of the Biovoxxel plug-in (Brocher, 2015). Settings for the Speckle Inspector included using 3000 pixels for minimum

primary object size (to exclude micronuclei) and checking “Exclude objects on edges.” Speckle Inspector then measured the number of foci per nucleus, foci areas, and foci perimeters. Non-parametric Wilcoxin test was used to calculate p-values and graphs were generated using ggplot2 in R.

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