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Chromatin Regulation by the NuA4 Acetyltransferase Complex Is Mediated by Essential Interactions Between Enhancer of Polycomb (Epl1) and Esa1

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ABSTRACT Enzymes that modify and remodel chromatin act in broadly conserved macromolecular complexes. One key modification is the dynamic acetylation of histones and other chromatin proteins by opposing activities of acetyltransferase and deacetylase complexes. Among acetyltransferases, the NuA4 complex containing Tip60 or its *Saccharomyces cerevisiae* ortholog Esa1 is of particular significance because of its roles in crucial genomic processes including DNA damage repair and transcription. The catalytic subunit Esa1 is essential, as are five noncatalytic NuA4 subunits. We found that of the noncatalytic subunits, deletion of Enhancer of polycomb (Epl1), but not the others, can be bypassed by loss of a major deacetylase complex, a property shared by Esa1. Noncatalytic complex subunits can be critical for complex assembly, stability, genomic targeting, substrate specificity, and regulation. Understanding the essential role of Epl1 has been previously limited, a limitation now overcome by the discovery of its bypass suppression. Here, we present a comprehensive *in vivo* study of Epl1 using the powerful tool of suppression combined with transcriptional and mutational analyses. Our results highlight functional parallels between Epl1 and Esa1 and further illustrate that the structural role of Epl1 is important for promotion of Esa1 activity. This conclusion is strengthened by our dissection of Epl1 domains required *in vivo* for interaction with specific NuA4 subunits, histone acetylation, and chromatin targeting. These results provide new insights for the conserved, essential nature of Epl1 and its homologs, such as EPC1/2 in humans, which is frequently altered in cancers.

KEYWORDS NuA4; *EPL1*; *ESA1*; chromatin; acetylation

EUKARYOTIC genomes are packaged into chromatin, which is composed of nucleosome units containing DNA wrapped around a histone octamer (Kornberg and Lorch 1999). Chromatin is subject to multiple, diverse modes of post-translational regulation that have many established roles, including functions in recombination, DNA damage repair, and transcription (Kouzarides 2007). Acetylation is one such post-translational modification that regulates chromatin function, mediated by the opposing enzymatic activities of lysine acetyltransferases (KATs/HATs) and deacetylases (KDACs/HDACs) (Campos and Reinberg 2009). HATs often exist in large multimeric complexes, such as the deeply conserved NuA4 complex (Doyon *et al.* 2004).

In humans, the essential catalytic subunit of NuA4, KAT5/Tip60, along with additional essential subunits such as EPC1/2, are associated with several carcinomas (Avvakumov and Côté 2007; Lafon *et al.* 2007; Nakahata *et al.* 2009; Biankin *et al.* 2012; Huang *et al.* 2014), suggesting their importance for controlled cellular growth. Much of the basic understanding of NuA4 comes from studies performed in *Saccharomyces cerevisiae*. NuA4 in yeast includes six essential subunits: Esa1 (Tip60 ortholog), Epl1 (EPC1/2 ortholog), Tra1, Arp4, Act1, and Swc4, all of which are broadly conserved. NuA4 primarily acetylates histones H4 and H2A *in vivo* (Smith *et al.* 1998; Clarke *et al.* 1999) along with noncanonical histones, such as H2A.Z (Keogh *et al.* 2006), and >250 nonhistone substrates (Lin *et al.* 2009; Yi *et al.* 2012; Mitchell *et al.* 2013; Downey *et al.* 2015), including 91 essential proteins.

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There are two distinct smaller complexes containing NuA4 subunits: piccolo-NuA4, composed of Esa1, Epl1, Yng2, and Eaf6 (Boudreault *et al.* 2003; Mitchell *et al.* 2008; Rossetto *et al.* 2014), and the TINTIN triad of Eaf5/7/3 (Cheng and Côté 2014; Rossetto *et al.* 2014). Piccolo-NuA4 is thought to also exist alone (Ohba *et al.* 1999; Boudreault *et al.* 2003) and is sufficient for broad nucleosome acetylation *in vitro*, whereas the NuA4 holo-complex is required for more targeted NuA4 functions such as DNA damage repair and transcriptional activation (Figure 1A) (Bird *et al.* 2002; Boudreault *et al.* 2003; Selleck *et al.* 2005; Friis *et al.* 2009).

Because Esa1 is essential, much of our early understanding of it came from studying hypomorphic alleles, where Esa1 is only partially or conditionally functional (Clarke *et al.* 1999; Decker *et al.* 2008). Recently, the first bypass suppressor of Esa1 was identified, where *esa1*Δ is rescued by loss of the Rpd3L HDAC complex (Torres-Machorro and Pillus 2014). This bypass of Esa1 is promoted by establishing a relatively balanced cellular acetylation state. The discovery of this bypass allowed for the first studies in which cells were completely depleted of Esa1.

Among the six essential NuA4 subunits only Esa1 and Epl1 are found in the very active smaller piccolo complex (Galarneau *et al.* 2000). Epl1 was first reported as the yeast ortholog of *Drosophila melanogaster* Enhancer of Polycomb E(Pc), which can function as a suppressor of position-effect variegation and can increase the homeotic phenotype of Polycomb group mutations (Sinclair *et al.* 1998; Stankunas *et al.* 1998). Epl1 and E(Pc) are broadly conserved and are orthologous to the EPC1/2 paralogs in humans (Shimono *et al.* 2000; Doyon *et al.* 2004).

It is noteworthy that despite its conservation and discovery nearly two decades ago, Epl1 function has been only minimally characterized, primarily based on low-dosage variants, limited *in vitro* analyses, and most recently when its partial structure bound to nucleosomes was solved (Boudreault *et al.* 2003; Selleck *et al.* 2005; Chittuluru *et al.* 2011; Huang and Tan 2012; Xu *et al.* 2016). Phenotypes of *EPL1* depletion are quite similar to those of impaired *ESA1*. These include roles in cell-cycle progression through G2/M, H4 acetylation, DNA damage repair, telomeric silencing, and autophagy (Boudreault *et al.* 2003; Yi *et al.* 2012).

Epl1 bridges Esa1 and the Yng2 and Eaf6 subunits to the larger NuA4 complex (Boudreault *et al.* 2003; Mitchell *et al.* 2008; Rossetto *et al.* 2014). The C-terminus of Epl1 contacts the NuA4 holo-complex through Eaf1 (Auger *et al.* 2008), but only the N-terminus (the EPcA domain) is essential for viability (Boudreault *et al.* 2003), suggesting that integrity of piccolo-NuA4 is crucial.

Despite progress made in earlier studies, the essential function of Epl1 *in vivo* has remained unknown in *S. cerevisiae* and metazoans alike. Here, we report that Epl1 can be bypassed by the same loss of the Rpd3L deacetylase complex observed for Esa1 and present a comprehensive *in vivo* analysis of Epl1 made possible only by its bypass suppression. Although Epl1 has no known catalytic activity, we find striking phenotypic and transcriptional similarity between *esa1*Δ and *epl1*Δ mutant strains under bypass conditions, suggesting coordinated function and activity. Through mutational analysis of Epl1, we provide evidence that Epl1's essential function is directly linked to physical contact with Esa1, such that without the Epl1-Esa1 structural interaction, Esa1 is no longer fully active. These new findings thus help to illuminate the essential coordinated activity of a MYST-family acetyltransferase and its broadly conserved binding partner.

Materials and Methods

Yeast strains and plasmids

Strains, plasmids, and oligonucleotides are listed in Supplemental Material, Tables S1–S3 in File S1. *EPL1* and *ESA1* mutant strains were constructed initially with covering plasmids (pLP3189 or pLP796). *EPL1-13MYC-HISMX6* was derived from LPY21686 (QY237), and integrated at the endogenous *EPL1* locus in LPY79 by amplification of the *13MYC-HISMX6* tag with oLP2196 and oLP2172. *EPL1-13MYC-HISMX6* was similarly cloned into pLP74, as pLP3337, by amplifying *EPL1-13MYC-HISMX6* from LPY21686 with oLP2169 and oLP2180, digested with *Hin*DIII, and ligated into pLP74. Epl1 mutant plasmids were constructed using NEB Q5 site-directed mutagenesis on pLP3337. Mutants were tested for dominance by transforming into a wild-type (WT) strain. The mutations were then integrated at the *EPL1* locus in diploid WT W303 and dissected. Mutagenesis was verified by sequencing both prior to and after integration. Strains were backcrossed prior to use.

Growth assays

Plate-based assays were performed using fivefold serial dilutions on standard media as described (Chang and Pillus 2009). For temperature and DNA damage assays, cultures were grown at 24° in SC for 1–3 days and then plated with starting concentrations normalized to one A_{600} unit and imaged after 2–5 days. Methyl methanesulfonate (MMS) sensitivity was assayed at 0.0075% in SC. Hydroxyurea (HU) sensitivity was assayed at 0.05 M in SC. Camptothecin (CPT) sensitivity was assayed at 7 μg/ml in SC (DMSO as vehicle control) prepared with 100 mM phosphate buffer (pH 7.5). Cultures for 5-fluoroorotic acid (5-FOA) assays were grown for 2 days at 30° to reach saturation, normalized to starting dilutions of 5–7 A_{600} units, and imaged after 4–6 days after plating. 5-FOA assays performed in the W303 background were plated on 10% glucose; all other plate-based assays were performed with standard 2% glucose.

Flow cytometry

Strains grown at 24° in SC for 1–2 days were diluted and grown to mid-log. One milliliter of exponentially growing cells (~3×10^7 cells) was fixed with cold 70% ethanol and prepared for flow cytometry, staining with propidium iodide (Chang *et al.* 2012). Thirty thousand cells were analyzed using a BD Accuri C6 Flow Cytometer.

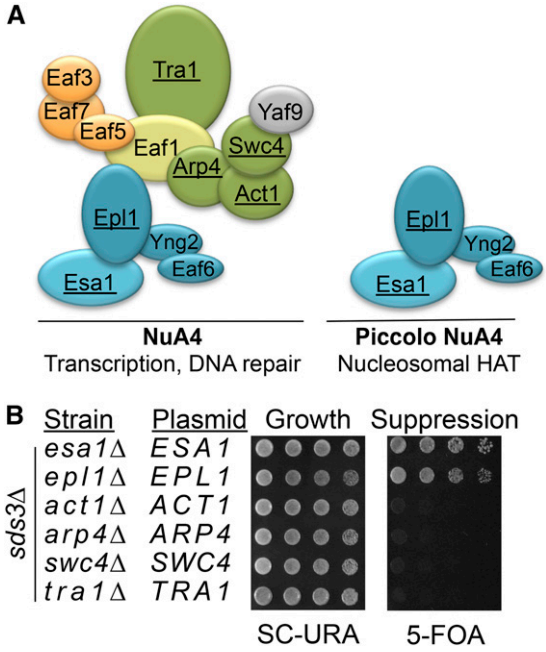

Figure 1 The requirement for two essential NuA4 subunits is bypassed by disassembly of Rpd3L. (A) The NuA4 histone acetyltransferase complex contains six essential subunits (underlined). The NuA4 holo-complex has targeted functions including roles in transcription and DNA damage repair, whereas the smaller piccolo-NuA4 complex is a broadly acting acetyltransferase complex. Piccolo-NuA4 contains the catalytic subunit Esa1, along with the essential subunit Epl1 and two nonessential subunits. NuA4 complex schematic is based on: Boudreault *et al.* (2003); Bittner *et al.* (2004); Doyon *et al.* (2004); Mitchell *et al.* (2008); Chittuluru *et al.* (2011); and Rossetto *et al.* (2014). (B) A screen of all essential NuA4 subunits for bypass potential. Double mutant analysis of *esa1*Δ *sds3*Δ (LPY20724), *epl1*Δ *sds3*Δ (LPY20609), *act1*Δ *sds3*Δ (LPY20974), *arp4*Δ *sds3*Δ (LPY20617), *swc4*Δ *sds3*Δ (LPY20611), and *tra1*Δ *sds3*Δ (LPY20443) revealed that only *epl1*Δ, like *esa1*Δ, could be bypassed by loss of *SDS3*, which encodes a central component of the Rpd3L deacetylase complex. Serial dilutions on the plasmid counterselective medium at 24° (and 30°, Figure S1 in File S1) illustrate that *epl1*Δ *sds3*Δ, like *esa1*Δ *sds3*Δ, survived without a plasmid-based copy of its corresponding essential gene.

Lysate preparation

Strains grown at 24° in SC were collected in mid-log for whole-cell extract preparations by bead-beating as described (Clarke *et al.* 1999). Fractionation was performed using spheroplasting, detergent-based lysis, and differential centrifugation (Liang and Stillman 1997) to yield whole-cell extract, and soluble and crude chromatin fractions. Lysates were briefly sonicated prior to immunoblot analysis.

Immunoprecipitations

Strains were grown for 1–2 days in 3 ml of SC at 24°, expanded to 10 ml, then diluted into 200 ml for growth and collected in mid-log phase. After pelleting and a phosphate-buffered saline (PBS) wash, cells were lysed by bead-beating in 1 μl of cold immunoprecipitation (IP) lysis buffer per A_{600} OD of cells (50 mM HEPES-KOH pH 7.5, 100 mM NaCl, 0.25% NP-40, 1 mM EDTA, 10% glycerol, and protease, phosphatase, and deacetylase inhibitors). The lysate was cleared then incubated with rotation for 3 hr with 5 μl of anti-Myc. IP mixtures were incubated for 50 min with 75 μl of Dynabeads Protein G (Thermo Fisher Scientific), prewashed with lysis buffer. Protein–antibody–bead conjugates were washed twice with lysis buffer and twice with wash buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA) prior to elution by boiling for 10 min in 40 μl of sample loading buffer (250 mM Tris-HCL pH 6.8, 10% SDS, 30% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue).

Immunoblots

To evaluate histones, proteins were separated using 15% SDS-PAGE, transferred to a 0.2-μm nitrocellulose membrane, and probed with: anti-H4K8Ac (1:2000, EMD Millipore, Darmstadt, Germany), anti-H4K5Ac (1:2000, Millipore), anti-H4K12Ac (1:2000, Active Motif), anti-H4 (1:2000, Active Motif), anti-H3K9/K14Ac (1:10,000, Upstate), and anti-H3 (1:2500, Abcam). Other proteins were separated on 7.5, 8, or 10% SDS-PAGE or for IP samples, 8–16% Novex Wedgewell Tris-Glycine gels (Thermo Fisher Scientific), transferred to a 0.2-μm nitrocellulose membrane and probed with: anti-Myc (1:2500 for detection of Epl1, 1:5000 for detection of all other Myc-tagged proteins) (Evan *et al.* 1985), anti-HA (1:1000, Covance), anti-Yng2 (1:1000, graciously provided by S. Tan), anti-Sir2 (1:10,000) (Garcia and Pillus 2002), anti-Pgk1 (1:20,000), and anti-β-tubulin (1:20,000) (Bond *et al.* 1986).

RNA-seq sample preparation and analysis

RNA was prepared in biological triplicate using hot-phenol extraction from mid-log cells grown in SC at 24°. RNA was DNase treated (Ambion). Quality was evaluated by gel electrophoresis and bioanalyzer (Agilent). Samples were depleted of rRNA (Ribo-zero Magnetic Gold Yeast, Epibio), and libraries were prepared (Tru-seq Stranded total RNA, Illumina). Twenty-four samples were sequenced with 50-bp single-reads on one lane of the HiSeq 2500 (Illumina), yielding a total of 287.52 million reads passing the quality filter.

Upon data generation, library adaptors were trimmed computationally with Cutadapt (Martin 2011), and reads were mapped to Repbase (Bao *et al.* 2015). Any reads mapping to Repbase were excluded from further analysis. The remaining reads were mapped to SacCer3 (Engel *et al.* 2014) with STAR (Dobin *et al.* 2013). Differential expression was assessed with DESeq2 (Love *et al.* 2014), and transcripts with a $\log_2$(fold change) ≥ 1 or ≤ -1 and *P*-adj ≤ 0.05 were called as differentially expressed. Further data analysis and visualization was completed using R computing software (R Development Core Team 2015) and the ggplot2 package (Wickham 2009).

qPCR validation

Select transcripts were validated using RT-qPCR. Briefly, cDNA was synthesized in biological triplicate from the RNA samples (TaqMan Reverse Transcriptase kit, Life Sciences)

and qPCR was performed using EvaGreen qPCR Master Mix (Lambda bio) on an MJ Research Opticon 2 to determine levels relative to the *SCR1* control. Significance was tested and assigned based on *P*-values calculated by a Student's *t*-test.

Data availability

Strains and plasmids are available upon request. Gene expression data have been deposited in the Gene Expression Omnibus with accession number GSE92774.

Results

Bypass and function of essential piccolo-NuA4 subunits

The finding that the essential requirement for Esa1 could be bypassed by loss of the Rpd3L deacetylase due to deletion of *SDS3* (Torres-Machorro and Pillus 2014) was significant because it marked the first condition where cellular viability was maintained without an essential NuA4 subunit. Similar to bypass suppression of *ESA1*, identification of other NuA4 bypass suppressors could facilitate *in vivo* analysis of these essential chromatin factors.

To test the extent to which disruption of Rpd3L by *sds3*Δ could bypass loss of genes encoding the essential NuA4 subunits (Esa1, Epl1, Act1, Arp4, Swc4, Tra1, underlined in Figure 1A), double mutants were constructed. Initially, each double mutant was recovered with a *URA3*-marked plasmid carrying the corresponding wild-type NuA4 gene. The strains were then challenged by plating on 5-FOA, which is toxic to cells expressing *URA3*. Growth on 5-FOA reveals mutant cells that can survive without the corresponding wild-type covering plasmid. Of the five new double mutants tested, only *epl1*Δ could be bypassed by *sds3*Δ; all remaining essential NuA4 subunits were still required for viability (Figure 1B and Figure S1 in File S1). The recovery of *epl1*Δ was of particular interest because of Epl1's limited *in vivo* characterization in any species and its close structural proximity to the catalytic Esa1 in piccolo-NuA4/NuA4.

Previous *in vitro* and *in vivo* studies of Epl1 were reported using two hypomorphic alleles and repressible expression. Epl1 was shown to have roles similar to Esa1, such as in histone H4 acetylation, DNA damage repair, and cell-cycle progression (Boudreault *et al.* 2003). To evaluate potential distinctions between Epl1 and Esa1 function *in vivo*, we examined phenotypes of the bypass strains. The *esa1*Δ *epl1*Δ *sds3*Δ triple mutant was viable (Figure 2A) and thus included in the phenotypic analysis.

The NuA4 bypass strains were surveyed for growth across a range of temperatures: all showed extreme sensitivity to high temperatures, and general growth defects at lower temperatures (Figure 2A). The *epl1*Δ *sds3*Δ and *esa1*Δ *epl1*Δ *sds3*Δ mutants were sensitive to DNA-damaging agents (Figure 2B) as shown previously for *esa1*Δ *sds3*Δ (Torres-Machorro and Pillus 2014). These strains were also sensitive to the vehicle control for CPT, DMSO, which has been shown to broadly decrease cellular proliferation (Kakolyri *et al.* 2016). This sensitivity mirrors that which has been identified for mutants of other chromatin regulators (Gaytán *et al.* 2013; Sadowska-Bartosz *et al.* 2013). As illustrated by H4K8 and H4K12 acetylation, *EPL1* bypass strains had low levels of histone H4 acetylation relative to WT and *sds3*Δ. By contrast, H3 acetylation remained unaffected (Figure 2C). Finally, loss of *EPL1* resulted in a similar defect in cell-cycle progression as loss of *ESA1*, characterized by a G2/M delay (Figure 2D).

Thus, loss of *EPL1*, despite not encoding acetyltransferase activity, had similar phenotypic and functional consequences as loss of *ESA1*. The observation that no distinct phenotypes were found when both *ESA1* and *EPL1* were lost, as compared to when only a single subunit was bypassed, further emphasized a high degree of functional overlap.

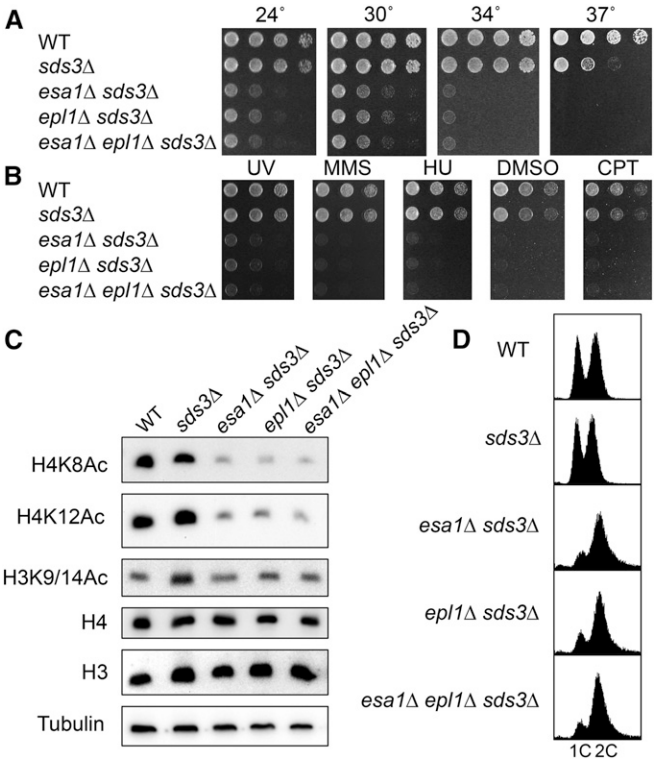

Figure 2 Bypass of *EPL1* is phenotypically akin to *esa1*Δ *sds3*Δ. (A) The *epl1*Δ *sds3*Δ (LPY21299) bypass strain shared growth defect and temperature-sensitivity phenotypes of *esa1*Δ *sds3*Δ (LPY21631), relative to both WT (LPY79) and *sds3*Δ (LPY20877). Likewise, in the triple mutant, loss of both *EPL1* and *ESA1* (LPY21751) had similar growth defects to loss of either essential subunit alone. (B) Bypass strains were surveyed at 24° for DNA damage, revealing sensitivity to all agents tested, relative to growth control (A), and the DMSO-vehicle control for CPT. (C) Histone H4 acetylation is significantly reduced upon loss of *ESA1* and/or *EPL1* relative to WT and *sds3*Δ. Two acetylation isoforms were probed as representatives for acetylation. Histone H3 acetylation remained unchanged relative to WT upon Esa1 or Epl1 mutation, highlighting the effect on histone H4 acetylation as a NuA4 target rather than the H3–H4 tetramer. (D) Cell cycle profiles demonstrated that loss of Esa1 and Epl1 resulted in a G2/M delay. All experiments were completed in three or more independent assays. Representative results from each are shown here.

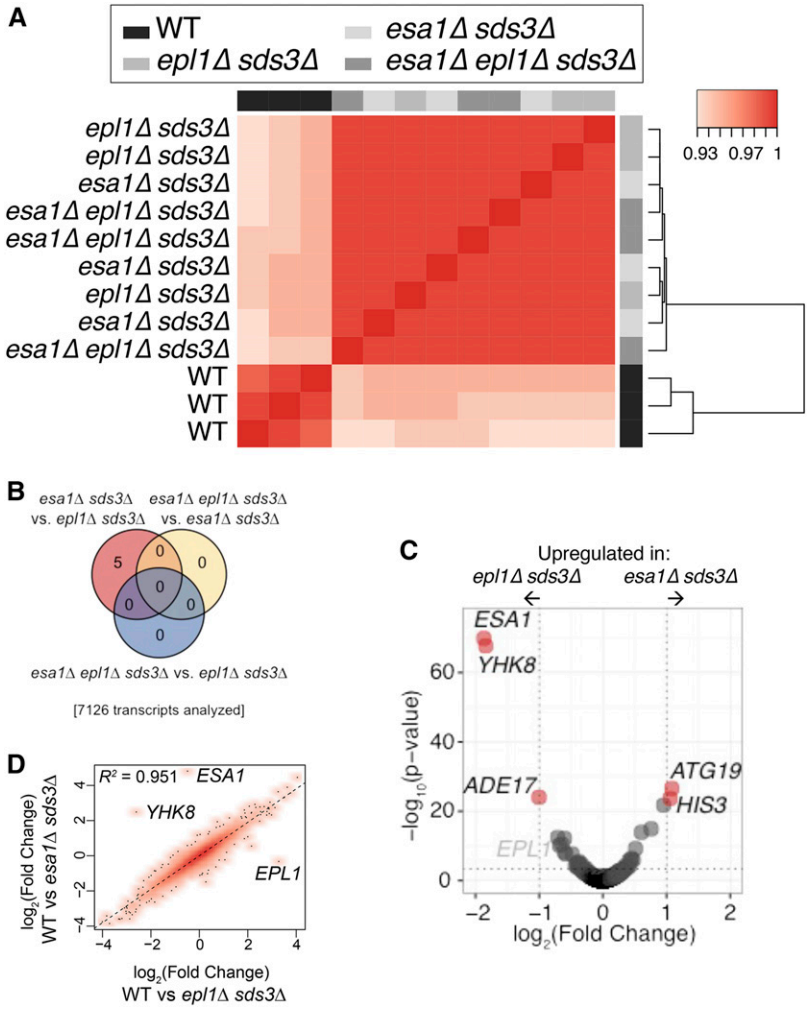

Figure 3 *ESA1* and *EPL1* bypass strains have nearly identical gene expression profiles. (A) Transcriptome analysis of bypass strains (LPY21299, LPY21631, and LPY21751) demonstrated a significantly high degree of similarity. Pairwise correlation analysis by Spearman's correlation coefficient was performed among strains and biological replicates shown by hierarchical clustering and a correlation heatmap. The three bypass strains clustered with near-perfect correlation coefficients. The biological replicates had analogous degrees of similarity, yet were clearly distinct from WT (LPY79) and *sds3*Δ (data not shown). (B) Differential expression analysis depicted by Venn diagram, highlights the similarity between *ESA1* and *EPL1* mutants. Analysis of 7126 transcripts that passed quality-control filters demonstrated that only five were differentially expressed above/below the threshold of $\log_2$(fold change) ± 1, respectively, and a false discovery rate (FDR) adjusted *P*-value $\leq$ 0.05. (C) Volcano plot illustrating the fold change and significance of transcripts in analysis of differential expression between *esa1*Δ *sds3*Δ and *epl1*Δ *sds3*Δ. A negative fold change indicates down-regulation in *esa1*Δ *sds3*Δ relative to *epl1*Δ *sds3*Δ. Transcripts meeting the significance threshold are in red with gene name indicated. *EPL1* (gray) was not differentially expressed above threshold. (D) Fold change between WT and *epl1*Δ *sds3*Δ and between WT and *esa1*Δ *sds3*Δ is plotted in a smooth scatter plot, with color intensity corresponding to density of individual points. Linear regression analysis is indicated by R^2, with the major outlying transcripts labeled. All differential expression analysis is of three biological replicates for each strain: WT (LPY79), *esa1*Δ *sds3*Δ (LPY21631), *epl1*Δ *sds3*Δ (LPY21299), and *esa1*Δ *epl1*Δ *sds3*Δ (LPY21751).

ESA1 and EPL1 bypass strains have nearly identical gene expression profiles

NuA4, and Esa1 specifically, contribute to the transcriptional regulation of ribosomal protein genes and many other targets genome-wide (Reid *et al.* 2000; Durant and Pugh 2006; Uprety *et al.* 2015). *ESA1* and its metazoan counterparts have roles in heterochromatin regulation, gene expression, and DNA damage repair (Clarke *et al.* 2006). Mutation or transcriptional repression of *EPL1* leads to similar phenotypes as those of *ESA1* mutants (Sinclair *et al.* 1998; Boudreault *et al.* 2003).

We asked if loss of *EPL1* mirrored loss of *ESA1* during bypass at the level of transcription. We performed RNA-sequencing and found that *epl1*Δ *sds3*Δ, *esa1*Δ *sds3*Δ, and *esa1*Δ *epl1*Δ *sds3*Δ had extremely similar transcriptomes. In fact, hierarchical clustering analysis illustrates that the similarity between these mutants is nearly equivalent to that of biological replicates, such that the different mutants cluster in the same group as, and interspersed within, the replicates of each mutant (Figure 3A). It should be noted that this intermixed clustering of mutants and replicates is not due to high variability between biological replicates, as the given correlation coefficients are >0.99. Rather, the clustering highlights the striking similarity between the three NuA4 bypass mutants.

Expression analysis of 7126 transcripts in the *ESA1* and *EPL1* bypass strains revealed that just over 1000 transcripts are differentially expressed between WT and *esa1*Δ *sds3*Δ and a similar number between WT and *epl1*Δ *sds3*Δ. However, only five transcripts were differentially expressed between *ESA1* and *EPL1* bypass strains (Figure 3B). Notably, these five transcripts were only differentially expressed between *esa1*Δ *sds3*Δ and *epl1*Δ *sds3*Δ; there were no transcripts differentially expressed between the triple *esa1*Δ *epl1*Δ *sds3*Δ and either *esa1*Δ *sds3*Δ or *epl1*Δ *sds3*Δ. Further analysis of these five differentially expressed transcripts by volcano plot (Figure 3C) illustrates that two of the differentially expressed transcripts, *ESA1* and *HIS3*, were expected due to the genetic background of the strains: these strains are auxotrophic for histidine and contain a *his3-11* mutation, affecting the expression of *HIS3*. However, in the *esa1*Δ *sds3*Δ strain, *ESA1* is replaced with *HIS3*, thereby restoring *HIS3* transcription and explaining the observed differential expression. Although *ADE17* was not differentially expressed at statistical significance by RT-qPCR, expression

trended toward its down-regulation in *esa1*Δ *sds3*Δ as compared to *epl1*Δ *sds3*Δ. The *ATG19* and *YHK8* differential expression was validated by RT-qPCR (Figure S2 in File S1), and in fact, *YHK8*, a largely uncharacterized open reading frame (ORF), is greater than sixfold up-regulated in *epl1*Δ *sds3*Δ as compared to *esa1*Δ *sds3*Δ by RT-qPCR. However, all three of these transcripts are only differentially expressed by one- to twofold by RNA-sequencing, and there is no functional theme underlying and unifying their differential expression, nor are the corresponding genes directly bound by Esa1 (Robert *et al.* 2004).

An analysis examining the differential expression of transcripts between WT and *esa1*Δ *sds3*Δ plotted against the differential expression of transcripts between WT and *epl1*Δ *sds3*Δ was also telling. Plotting the $\log_2$ (fold change) of all transcripts relative to WT in *esa1*Δ *sds3*Δ *vs.* that in *epl1*Δ *sds3*Δ illustrated a high correlation between differential expression in *esa1*Δ *sds3*Δ and in *epl1*Δ *sds3*Δ, both relative to WT (Figure 3D). As such, transcripts that differ between WT and *esa1*Δ *sds3*Δ also differ, and to a similar magnitude, between WT and *epl1*Δ *sds3*Δ. Thus, the transcriptional profiles of Epl1 and Esa1 bypass conditions are virtually identical, despite their distinct noncatalytic and catalytic roles in NuA4.

Epl1 promotes the chromatin association of Esa1

Both phenotypic and transcriptional analyses of *epl1*Δ and *esa1*Δ emphasize their similarity, despite the overt difference of Esa1's catalytic activity. To further probe distinctions between the roles of Epl1 and Esa1, and to determine the nature of *EPL1*'s essential function, we considered the *in vitro* characterization of Epl1, which reported that it associates with the nucleosome core particle to promote Esa1's enzymatic activity (Chittuluru *et al.* 2011). We could ask for the first time if Epl1 drives Esa1's chromatin association *in vivo*, and if Esa1 would remain chromatin associated in the absence of Epl1.

To test the role of Epl1 in targeting Esa1 to chromatin, subcellular fractionation (Liang and Stillman 1997) and immunoblotting were performed (Figure 4). Controls included probes for the chromatin-associated protein Sir2 and the glycolytic enzyme Pgk1, a predominantly cytoplasmic protein. In WT, *sds3*Δ, and *epl1*Δ *sds3*Δ strains, Sir2 was primarily localized to the chromatin (C) fraction, whereas Pgk1 was more enriched in the soluble (S) fraction (Figure 4, A and B). In contrast, whereas Esa1 is largely localized to the chromatin fraction in WT and *sds3*Δ, it becomes shifted to the soluble fraction upon loss of *EPL1* and depleted from chromatin. Notably, this shift in association is specific for Esa1, as Sir2 remains chromatin associated.

NuA4 contains subunits that have chromatin activity independent of NuA4, including several that contain their own chromatin targeting domains. We sought to determine if the newly defined role for Epl1 in promoting chromatin association of Esa1 *in vivo* was extended to other NuA4 subunits, and therefore, if its loss might have more widespread consequences. To test this possibility, we selected Swc4 for its

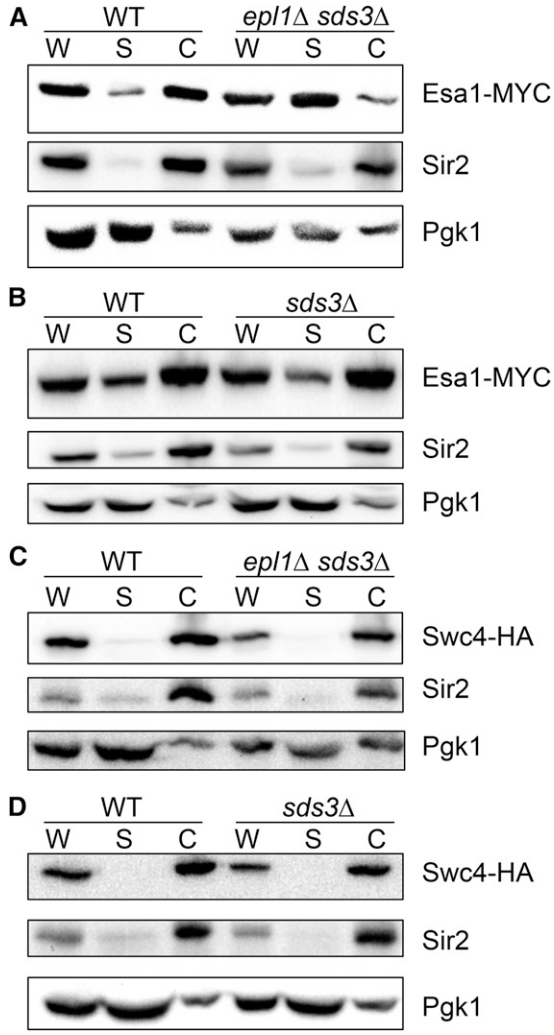

Figure 4 Epl1 is required for stable chromatin association of Esa1. (A) Subcellular fractionation assays reveal that in the absence of Epl1, a fraction of Esa1 is released from chromatin. Cells were collected and lysed for whole-cell extracts (W). Additional fractionation was performed to yield soluble (S) and crude chromatin (C) fractions. In WT cells (LPY21568), the majority of Esa1 is associated with the chromatin fraction, much like Sir2. However, in *epl1*Δ *sds3*Δ (LPY21596), Esa1 is shifted to the soluble fraction, analogous to the Pgk1 control. A brief chemical cross-link prior to lysis and fractionation was performed in parallel (Figure S3 in File S1). (B) The *sds3*Δ single mutant (LPY21579) alone does not alter Esa1 chromatin association, as illustrated by subcellular fractionation followed by immunoblotting for Esa1, and the Sir2 and Pgk1 controls. (C) Swc4 remains chromatin associated upon loss of *EPL1*. Subcellular fractionation demonstrates that Swc4, another essential NuA4 subunit, remains chromatin associated in *epl1*Δ *sds3*Δ (LPY21942), consistent with WT (LPY22201) and much like the Sir2 control. (D) The *sds3*Δ single mutant (LPY22202) alone also does not affect Swc4 chromatin association.

essential nature, dual-role in NuA4 and the Swc4 chromatin-remodeling complex, and its SANT domain (Krogan *et al.* 2004). We found that Swc4 remained chromatin associated in the absence of *EPL1* (Figure 4C), demonstrating that loss of Epl1 did not broadly affect all NuA4 subunits. Like Esa1 localization, Swc4 is unaffected by *sds3*Δ alone, and

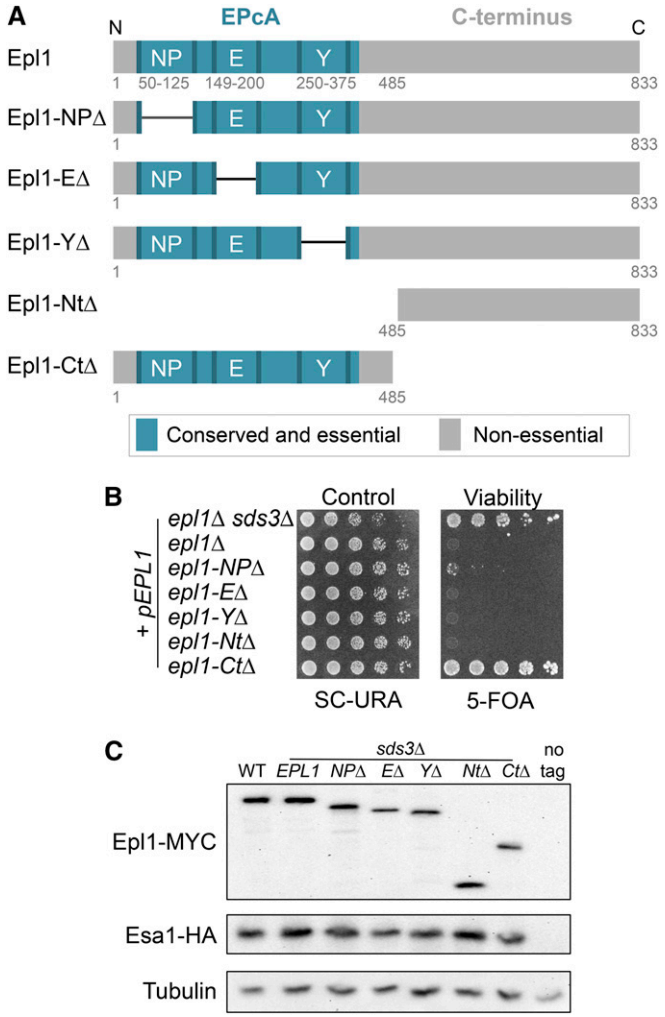

Figure 5 Defining functional regions of Epl1 *in vivo*. (A) Epl1 contains a conserved and essential EPcA domain, and a more variable and nonessential C-terminus. EPcA contains three subdomains that were previously classified by *in vitro* assays (Boudreault *et al.* 2003; Selleck *et al.* 2005; Chittuluru *et al.* 2011) and validated in recent structural studies (Xu *et al.* 2016). NP (nucleosome core particle) interacts with the nucleosome core particle, E (Esa1) makes physical contact with Esa1, and Y (Yng2) makes contact with the nonessential piccolo-NuA4 subunits Yng2 and Eaf6. Although the nomenclature for these domains follows that set by previous studies, it should be noted that the residues in the defined domains are not identical to past studies, varying by one or two amino acids. The C-terminus does not contain any conserved domains; however, *in vitro* it has a structural role in tethering the piccolo-NuA4 subunits to the NuA4 holo-complex by interacting with Eaf1. (B) Evaluation of dominance and viability of the Epl1 mutants. Serial dilution assays reveal that at 24° the mutants are not dominant (Control). In the *epl1*Δ mutant, only the *EPL1-NP* (LPY22120) construct supports viability of *epl1*Δ, although cells have a significant reduction in fitness. Epl1 mutants for each of the other putative subunit-interaction domains fail to support viability, demonstrating an essential *in vivo* function for each (LPY22012, LPY22001, LPY22084). Confirming previous results, *epl1-Ct*Δ (LPY22010) is viable and robust. The *epl1*Δ *sds3*Δ (LPY21071) and *epl1*Δ (LPY20759) strains are plated as viable and inviable controls, respectively. (C) The *EPL1* mutations do not have gross effects on protein levels of either Epl1 or Esa1 in whole cell lysates prepared from exponentially growing cells. A representative blot for one of at least three independently prepared lysates is shown. Eight strains were assayed: *EPL1-13MYC ESA1-3HA* (LPY22231), *EPL1-13MYC sds3*Δ ESA1-3HA (LPY22232), *epl1-NP*Δ*-13MYC sds3*Δ *ESA1-3HA* (LPY22213), *epl1-E*Δ*-13MYC sds3*Δ *ESA1-3HA* (LPY22208), *epl1-Y*Δ*-13MYC sds3*Δ *ESA1-3HA* (LPY22226), *epl1-Nt*Δ*-13MYC sds3*Δ *ESA1-3HA* (LPY22209), *epl1-Ct*Δ*-13MYC sds3*Δ *ESA1-3HA* (LPY22211), and the WT no-tag control (LPY79).

localization patterns in *sds3*Δ mirror WT (Figure 4D). Thus, Epl1 is important specifically for the association of Esa1 with chromatin, and its loss does not generally disrupt chromatin association of two other chromatin proteins with distinct functions in transcription and remodeling.

Defining the critical regions of Epl1 in vivo

Due to its essential nature and a limited number of hypomorphic alleles (Boudreault *et al.* 2003), much of Epl1's characterization has been performed *in vitro*. Accordingly, we wanted to determine if Epl1's chromatin-association function was essential, and concurrently, which regions were most critical for promoting Epl1's essential role. Several prior studies defined regions of Epl1 essential for viability and *in vitro* activity, such as the conserved EPcA N-terminal domain (Boudreault *et al.* 2003; Selleck *et al.* 2005; Chittuluru *et al.* 2011; Huang and Tan 2012). This is in contrast to the more variable, nonessential C-terminus. We used mutational analysis to construct four distinct Epl1 mutants that targeted the EPcA domain, and one mutant targeting the C-terminus (Figure 5A).

Given that, to our knowledge, this represents the most comprehensive *in vivo* structure–function mutational analysis of Epl1 to date, we next moved to assess the essential nature of each of the subdomains. The EPcA domain was shown earlier to be essential, to interact with the nucleosome core particle *in vitro*, to contribute to substrate specificity, and together with Yng2, to position Esa1 to acetylate nucleosomes (Boudreault *et al.* 2003; Selleck *et al.* 2005; Chittuluru *et al.* 2011; Huang and Tan 2012; Lalonde *et al.* 2013). To date, no *in vivo* assessment has been reported for the requirement for all subdomains within EPcA. We found that among the mutants in the essential N-terminus, only *epl1-NP*Δ is viable (Figure 5B). However, its growth was not as robust as the *epl1-Ct*Δ strain. Therefore, although important, the NP subdomain of Epl1 is not essential.

As many of the Epl1 mutations were not viable in an otherwise WT-background (*epl1-E*Δ, *epl1-Y*Δ, *epl1-Nt*Δ), and those that were viable were not robust (*epl1-NP*Δ, *epl1-Ct*Δ), we capitalized on the resource of bypass suppression, using the *sds3*Δ background to further study the functional consequences of the *EPL1* mutations *in vivo*. To begin, we found that the mutations did not significantly disrupt either Epl1 or Esa1 protein levels (Figure 5C). This suggests that there are no gross changes in protein stability, although effects due to changes in protein conformation remain possible.

We next evaluated the phenotypic consequences of the *EPL1* domain mutants in the *sds3*Δ background, such that the only Epl1 that is expressed is the mutant version, integrated

at the genomic locus. In these bypass conditions, mutants of the Epl1 subunit interaction domains (*epl1-E*Δ and *epl1-Y*Δ) are sensitive to high temperature (Figure 6A), and DNA damage (Figure 6B). Accordingly, complete loss of *EPL1* or loss of the entire essential EPcA domain (*epl1-Nt*Δ) is phenotypically similar to loss of either of the subunit interaction domains (*epl1-E*Δ and *epl1-Y*Δ) alone. In contrast and consistent with *epl1-NP*Δ sufficiency for viability, this mutant has the most robust growth in bypass conditions when challenged with higher temperatures and DNA-damaging agents. These results suggest that the residues of Epl1 that interact with other subunits *in vitro* (Epl1-E and Epl1-Y) are most critical for both viability and function *in vivo*, and that in bypass conditions, loss of either of these regions is as detrimental to cellular fitness as loss of the entire gene. In contrast, the domain previously defined as critical for nucleosome targeting (Epl1-NP) *in vitro*, although important, is less critical during bypass suppression.

Because nucleosomal H4 acetylation by Esa1 in the piccolo-NuA4 complex is one of its defining features (Boudreault *et al.* 2003), we evaluated H4 acetylation as a proxy for NuA4 catalytic activity in the *EPL1* mutants. For the lysines probed, we observed that mutants of all three EPcA subdomains (*epl1-NP*Δ, *epl1-E*Δ, *epl1-Y*Δ) were defective for H4 acetylation in the bypass state (Figure 6C). Accordingly, loss of the entire EPcA domain (*epl1-Nt*Δ) leads to similarly low levels of H4 acetylation. This is a striking distinction from the growth assays where *epl1-NP*Δ was more robust than *epl1-E*Δ or *epl1-Y*Δ, thus pointing to the idea that substrates in addition to H4 may be critical for full biological function.

One of the key findings from the initial Epl1 bypass analysis was that Epl1 promotes the stable chromatin association of Esa1 (Figure 4A). Because our mutational studies revealed that the most critical Epl1 residues (Epl1-E and Epl1-Y) were required for growth at high temperature, response to DNA damage, and histone H4 acetylation, we initially hypothesized that these same residues might be important for promoting chromatin association. We performed fractionation assays as above, in this case with each of the Epl1 mutants in the *sds3*Δ bypass background (Figure 7A). We found that each of the mutants in the essential EPcA domain retained chromatin association and, likewise, Esa1 remained chromatin associated in each of these mutants.

Previous *in vitro* studies suggested a key role for the Epl1-NP region of the protein in nucleosomal binding (Chitturuluru *et al.* 2011; Xu *et al.* 2016). However, the observed (Figure 7A) *epl1-NP*Δ mutant protein associated with chromatin *in vivo*. In contrast, a small amount of the Epl1-CtΔ protein shifted to the soluble pool (S), with a similar shift observed for Esa1 in this background. Sir2 remained chromatin bound regardless of *EPL1* mutations. The shift to the soluble pool in *epl1-Ct*Δ *sds3*Δ for both Epl1 and Esa1 supports the idea that the C-terminus acts to stabilize both Esa1 and Epl1 in chromatin, thus defining a new role for this most divergent region of Epl1 and its orthologs.

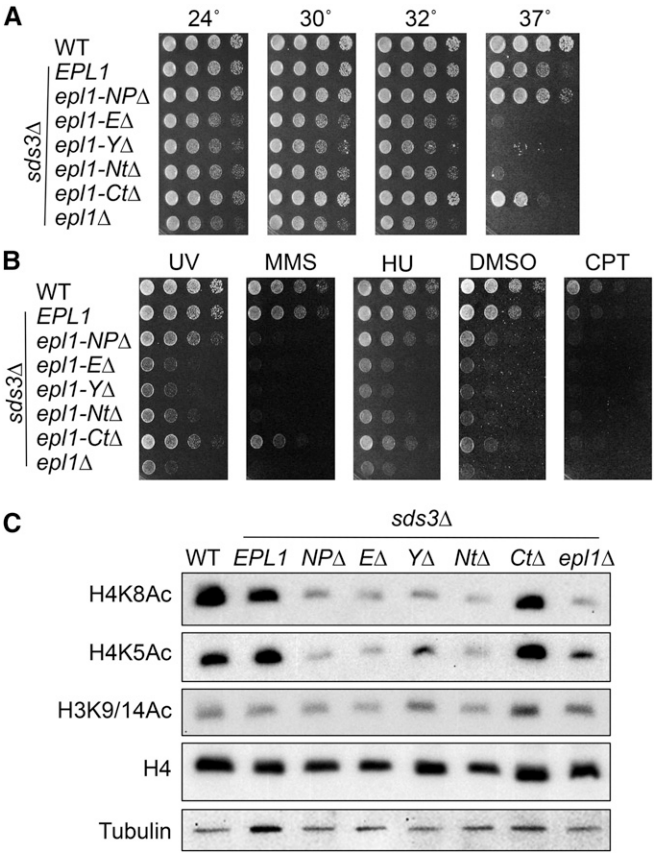

Figure 6 Subunit interaction domains are critical for Epl1 function *in vivo*. (A) Under *sds3*Δ bypass conditions, *EPL1* mutants were surveyed for growth on SC medium: *epl1-NP*Δ (LPY22111), *epl1-E*Δ (LPY22017), *epl1-Y*Δ (LPY22185), *epl1-Nt*Δ (LPY22091), *epl1-Ct*Δ (LPY22033). Growth at increasing temperatures is shown in comparison to WT (LPY22004) and *sds3*Δ (LPY22006). (B) Sensitivity to a spectrum of DNA-damaging agents at 24°. The growth control 24° SC plate is shown in (A), and DMSO is included as the vehicle control for CPT. (C) Histone H4 acetylation is low among mutants in the essential EPcA domain of *EPL1* relative to WT, whereas H4 acetylation is at WT levels in the *epl1-Ct*Δ mutant.

Physical association between Esa1 and Epl1 is required for activity

From earlier *in vitro* studies, Epl1 was divided into two domains: the EPcA domain that physically interacts with the Esa1, Yng2, and Eaf6 piccolo-NuA4 subunits and the C-terminus that tethers Epl1 and the piccolo subunits to the NuA4 holo-complex through Eaf1 (Boudreault *et al.* 2003; Auger *et al.* 2008; Rossetto *et al.* 2014). These regions had not yet been evaluated *in vivo*, so we sought to determine which are essential for the interaction with Esa1, and simultaneously which are required for interaction with Yng2 and, by extension, Eaf6.

We immunoprecipitated Epl1 in WT, in each of the three EPcA subdomain mutants, and in the C-terminal deletion mutant, and then immunoblotted for Esa1 and Yng2. We found that only Epl1-NPΔ and Epl1-CtΔ retained interaction with both Esa1 and Yng2 (Figure 7B). This connection of

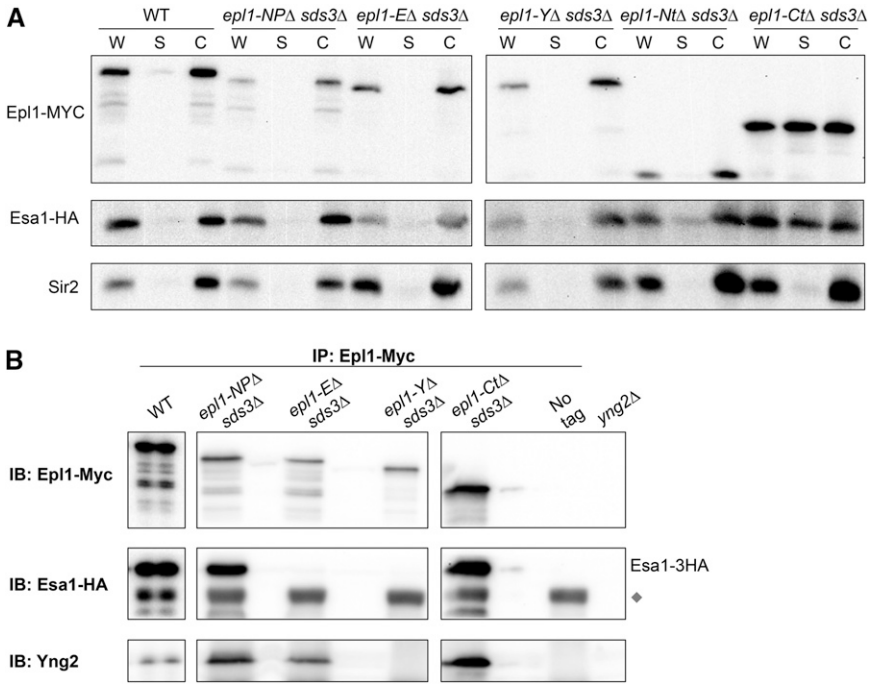

Figure 7 Viability of *epl1* mutants is linked to stable Epl1–Esa1 interaction, not chromatin association. (A) Both Epl1 and Esa1 remain chromatin associated like WT (LPY22231) in all mutants of the essential EPcA domain [*epl1-NPΔ-13MYC sds3Δ ESA1-3HA* (LPY22213), *epl1-EΔ-13MYC sds3Δ ESA1-3HA* (LPY22208), *epl1-YΔ-13MYC sds3Δ ESA1-3HA* (LPY22226), *epl1-NtΔ-13MYC sds3Δ ESA1-3HA* (LPY22209)]. However, upon loss of the C-terminus of Epl1 [*epl1-CtΔ-13MYC sds3Δ ESA1-3HA* (LPY22211)], both Epl1 and Esa1 are shifted to occupy both soluble and chromatin-bound pools. The observed shift of Epl1 association in *esa1Δ sds3Δ* here also controls for a possibility of the *MYC*-tag causing unintended association. (B) The physical interaction of Esa1–Epl1 is disrupted in the *epl1-EΔ* and *epl1-YΔ* mutants, but not in the *epl1-NPΔ* and *epl1-CtΔ* mutants. Immunoblots following immunoprecipitation illustrate the loss of the physical interaction in the two essential domain mutants as seen in WT, *epl1-NPΔ*, and in *epl1-CtΔ*, and lack of nonspecific binding at the relevant molecular weights in the no-tag control (LPY79). Additionally, physical interaction with Yng2 is lost only in *epl1-YΔ*. ◆ marks cross-reactivity with IgG-heavy chain of the antibody. Whole-cell lysate was prepared for *yng2Δ* (LPY22421) in no-tag control background and is included as a negative control for the Yng2 antibody.

Epl1-NPΔ to the NuA4 holo-complex offers an explanation for its ability to survive under nonbypass conditions, and upon DNA damage and high-temperature stress. Consistent with recent structural analysis (Xu *et al.* 2016), we found that Epl1-YΔ lost physical interaction with Yng2 *in vivo*. Interestingly, we found that Epl1-E and Epl1-Y, the two most critical domains of Epl1 defined phenotypically, were both essential for robust physical interaction with Esa1. Our results, in tandem with earlier *in vitro* studies illustrating that HAT activity of Esa1 is directly augmented by Epl1 (Boudreault *et al.* 2003), support the idea that Epl1 is a critical NuA4 subunit due to a role as an Esa1-cofactor. Thus, Epl1 is a central regulator that is as crucial for NuA4 complex function as the Esa1 enzyme itself.

Discussion

Defining the function of a noncatalytic component of a macromolecular complex can be a challenge, particularly when that component is essential for viability. Such has been the case for Enhancer of polycomb, originally identified as a suppressor of position-effect variegation in *Drosophila* (Sinclair *et al.* 1998). Shortly after its genetic discovery, E(Pc) was cloned and found to be both deeply conserved from yeast (Epl1) to humans (EPC1/2) (Stankunas *et al.* 1998; Doyon *et al.* 2004) and to be essential for chromatin-directed functions. Specifically, Epl1 was identified as a critical subunit of the conserved MYST-family histone acetyltransferase NuA4 complex (Galarneau *et al.* 2000) and appears to be dedicated to NuA4/piccolo-NuA4.

Progress made toward understanding the functions of Epl1 include identification of the essential EPcA domain of Epl1, characterization of essential *in vitro* functions, and most recently structural analysis of Epl1 as part of nucleosome-bound NuA4 core complex (Boudreault *et al.* 2003; Selleck *et al.* 2005; Chittuluru *et al.* 2011; Huang and Tan 2012; Xu *et al.* 2016). Despite this progress, analysis of Epl1 *in vivo* has been relatively modest. The discovery reported here, that the requirement for *EPL1* could be bypassed by deletion of a component of a histone deacetylase complex, provided a unique advantage for performing *in vivo* studies in *epl1Δ* strains.

We found that the essential requirement for Epl1 and Esa1 could be bypassed by loss of the Rpd3L deacetylase, but not for the other four essential subunits in NuA4 (Figure 1B). Earlier studies suggested that among the essential subunits, only Epl1 and Esa1 appear to be dedicated to NuA4/piccolo-NuA4, whereas the others participate in additional chromatin-modifying complexes or cellular structures. These include Tra1, an ATM-family cofactor, which serves as a recruitment module in SAGA and SLIK/SALSA complexes (Grant *et al.* 1998) and Swc4, Arp4, and Act1, which are components of the SWR1 chromatin-remodeling complex (Krogan *et al.* 2004; Mizuguchi *et al.* 2004) and serve other cellular roles. Specifically, Arp4 and Act1 are also found in the INO80 ATP-dependent chromatin-remodeling complex (Shen *et al.* 2000) and Act1 is an essential cytoskeletal protein (Shortle

et al. 1982). Given this context, the bypass suppression of Epl1, but not the other essential subunits, underscores its exclusive importance as a NuA4 subunit.

We have demonstrated that Epl1 is important for promoting the stable chromatin association of Esa1 through the nonessential C-terminus of Epl1. This was counter to expectations because Esa1 nucleosomal association was reported previously to occur via the N-terminal EPcA subdomain (Epl1-NP), essential for H4 but not H2A acetylation (Boudreault *et al.* 2003; Selleck *et al.* 2005; Chittuluru *et al.* 2011; Huang and Tan 2012; Lalonde *et al.* 2013; Xu *et al.* 2016). An important distinction is that in contrast to *in vitro* experiments utilizing recombinant piccolo-NuA4 components, *epl1-NP*Δ *sds3*Δ retains an assembled NuA4 holo-complex. Thus, it is possible that whereas an isolated Epl1 requires Epl1-NP for nucleosomal association, in *epl1-NP*Δ *sds3*Δ, other chromatin-interacting subunits in NuA4 that are still attached to Epl1 may efficiently target Epl1 (and Esa1) to chromatin.

We found that without the Epl1 C-terminus (*epl1-Ct*Δ), H4 acetylation remained at WT levels. Additionally, in Epl1 mutants with disrupted Epl1-Esa1 physical interactions, Esa1 remained chromatin-targeted. Whereas these findings may at first appear to be at odds, there are several key considerations. It is possible that, by default, Esa1 is associated with chromatin. When loss of the C-terminus dissociates Epl1 from chromatin, it may bring along Esa1. This possibility is supported by the fact that in *epl1-E*Δ *sds3*Δ and *epl1-Y*Δ *sds3*Δ, Esa1 remains chromatin associated (Figure 7A) despite not physically interacting with Epl1 (Figure 7B). Even without Epl1, Esa1 may transiently associate with chromatin (Figure S3 in File S1), allowing the dynamic and rapid process of acetylation to occur. It is also possible that the small amount of chromatin association that remains is sufficient for Esa1 catalytic activity, especially in the bypass state, where Rpd3L does not actively deacetylate histone H4. The NuA4 holo-complex may also be required for stable chromatin association, such that the interaction of Esa1 is facilitated by other subunits, where loss of the C-terminus of Epl1 specifically represents dissociation of piccolo-NuA4. Further studies involving the nonessential NuA4 Eaf1 subunit and the dynamics between Epl1 and Eaf1 *in vivo* may provide insight into these possibilities.

Our results support a model in which Epl1 is physically required for promoting Esa1 enzymatic activity as a part of the piccolo-NuA4 and/or the NuA4 holo-complexes, much like Ada2 and Ada3, which act in a catalytic core to potentiate the activity of the Gcn5 acetyltransferase (Balasubramanian *et al.* 2002). In WT or *sds3*Δ cells, Epl1 acts as an anchor for piccolo-NuA4 subunits, including Esa1, and also tethers these subunits to the NuA4 holo-complex (Figure 8A). With both NuA4 and piccolo-NuA4 intact, as they are in WT and in *sds3*Δ, there are normal levels of acetylation. However, upon loss of the nonessential C-terminus (*epl1-Ct*Δ *sds3*Δ), piccolo-NuA4 becomes untethered, and the NuA4 holo-complex is disrupted, leaving only the broad nucleosomal HAT function of piccolo-NuA4 (Figure 8B). This is sufficient for global, less targeted acetylation of histone H4 but perhaps not for acetylation of nonhistone substrates that contribute to fitness. In *epl1-NP*Δ *sds3*Δ, all piccolo-NuA4 subunits, including Esa1 and Epl1, still physically interact, with *epl1-NP*Δ still permitting both NuA4 and piccolo-NuA4 integrity; however, this mutation causes a reduction in acetylation relative to WT and *sds3*Δ (Figure 8C), perhaps due to inefficient nontargeted chromatin binding.

Despite reduced acetylation levels in *epl1-NP*Δ, we hypothesize that the presence and targeted activities of the NuA4 holo-complex may be sufficient to promote cellular viability and in bypass conditions, response to cellular stresses such as DNA damage. In fact, we found that disruption of the NuA4 holo-complex by *eaf1*Δ results in lethality of *epl1-NP*Δ in nonbypass conditions (Figure S4 in File S1), highlighting the importance of the NuA4 holo-complex for growth in the *epl1-NP*Δ mutant background. Further, we found that if Esa1 was simply disassociated from Epl1, such that it was no longer a component of NuA4 or piccolo-NuA4, as was the case in both *epl1-E*Δ *sds3*Δ and *epl1-Y*Δ *sds3*Δ, cellular growth was severely compromised, with low levels of acetylation, and death at elevated temperatures or with DNA damage (Figure 8, D and E). These findings complement the recently published structural insights for piccolo-NuA4 (Xu *et al.* 2016), which illustrate that residues within those deleted in *epl1-E*Δ and *epl1-Y*Δ were most critical for contacting both Esa1 and the nucleosome.

Overall, our results support the concept that Epl1 is required to function in tandem with Esa1, tethering Esa1 to other subunits for full and robust function. This concept is supported by *in vitro* experiments demonstrating negligible HAT activity of Esa1 in the absence of the Epl1 and Yng2 piccolo-NuA4 subunits (Boudreault *et al.* 2003). We have shown that if Epl1 is not present (*epl1*Δ *sds3*Δ), or unable to physically interact with Esa1 (*epl1-E*Δ *sds3*Δ and *epl1-Y*Δ *sds3*Δ), Esa1 becomes ineffectual.

The majority of the studies reported here have been performed where the requirement for Epl1 is conditionally bypassed by *sds3*Δ, serving to balance cellular acetylation, as in our studies of Esa1 (Torres-Machorro and Pillus 2014). Historically, bypass suppression has promoted fundamental understanding of multiple and diverse pathways. This includes, for example, studies of cell-cycle checkpoints where suppression of *mec1*Δ and *rad53*Δ lethality is bypassed by concurrent loss of *SML1* (Zhao *et al.* 1998), and more recent studies of transcription regulation, where the requirement for the COMPASS methyltransferase subunit Swd2 is bypassed by *set1*Δ (Soares and Buratowski 2012). Likewise, bypass suppression served as a powerful tool here that has allowed comprehensive functional assessment of *EPL1 in vivo*. However, the concurrent loss of a major deacetylase should be kept in mind in the interpretation of data. It is only in this context that the relative comparisons between the mutants *in vivo* can be made with the earlier biochemical analyses to provide a deeper, valuable, and more holistic understanding

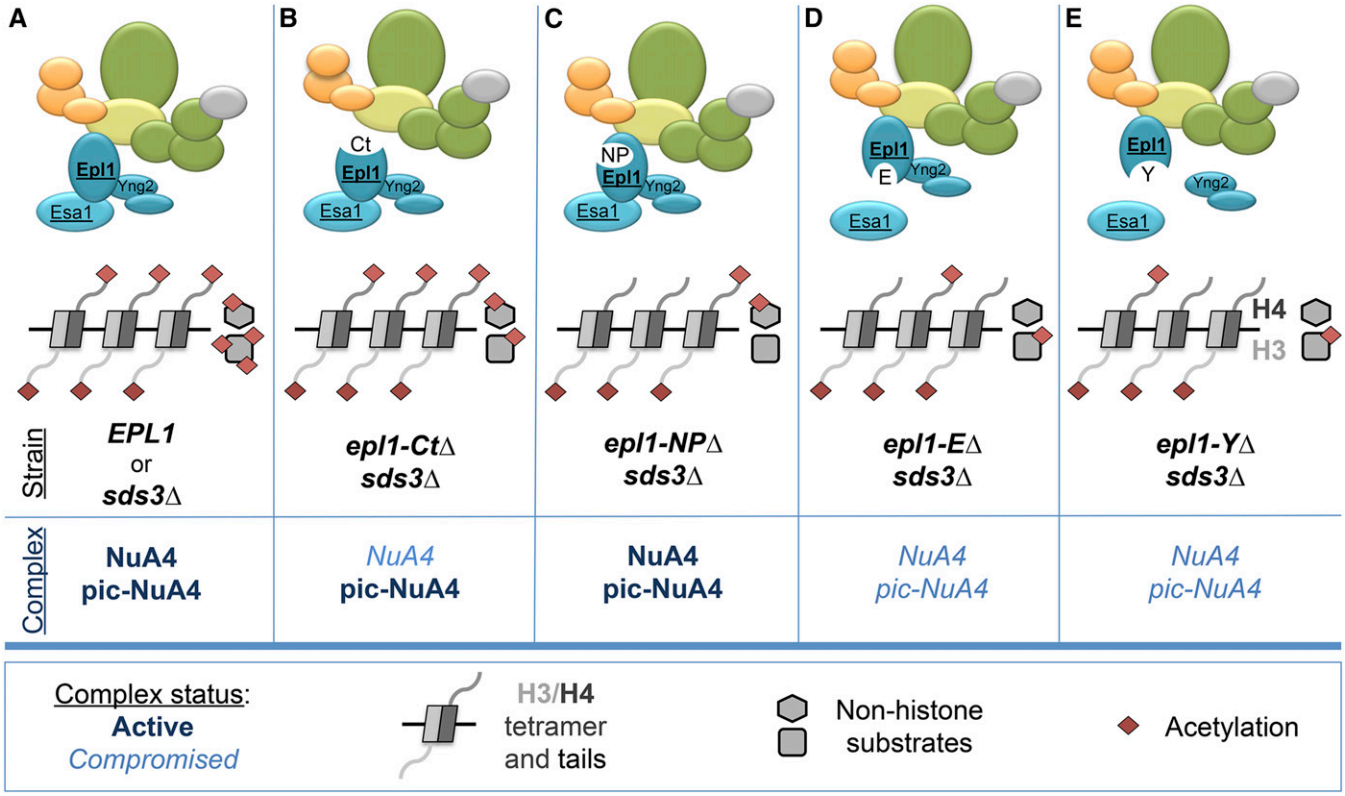

Figure 8 Model: Epl1 is a core NuA4 regulator in tandem with Esa1. (A) Epl1 is a central component of NuA4 and piccolo-NuA4 (abbreviated pic-NuA4, shown here only as a part of NuA4). In WT and *sds3*Δ, both complexes are intact and active, resulting in normal levels of acetylation, both of histone H4, as shown in the tails of the H3–H4 tetramer, and of nonhistone substrates, with two representative substrates illustrated, out of over 250 reported in proteomic studies. (B) Loss of the Epl1 C-terminus results in loss of the NuA4 holo-complex, but largely uncompromised pic-NuA4 function, and slightly reduced acetylation levels of nonhistone substrates only in the *sds3*Δ background. (C) *epl1-NP*Δ keeps NuA4 intact, and the essential components of pic-NuA4 remain tethered, promoting robust fitness in the bypass state; however, low acetylation levels are present. The assembly of the NuA4 holo-complex is critical here, such that upon loss of *EAF1*, *epl1-NP*Δ is no longer viable in nonbypass conditions (Figure S4 in File S1). (D) Loss of the subunit-interaction domains [*epl1-E* here and *epl1-Y* in (E)] results in Esa1 no longer structurally bound to Epl1, causing both NuA4 and pic-NuA4 to be compromised. This results in low acetylation and overwhelming loss of cellular fitness similar to *epl1*Δ *sds3*Δ. (E) By comparison, *epl1-Y*Δ *sds3*Δ results in the similar loss of physical contact with Esa1, but also loss of physical interaction with Yng2, and therefore by extension, Eaf6. This mutant has the same severe fitness-deficits as *epl1-E*Δ *sds3*Δ and *epl1*Δ *sds3*Δ, underscoring the primary importance of the Esa1–Epl1 interaction. Although not illustrated in the model, *epl1*Δ *sds3*Δ would be similar to *epl1-E*Δ *sds3*Δ and *epl1-Y*Δ *sds3*Δ, with low levels of acetylation; however in the complete absence of Epl1, all piccolo-NuA4 subunits would be disassociated from the NuA4 holo-complex. Note that in our experiments, analysis of H4 acetylation is a proxy for NuA4/piccolo-NuA4 activity. Proteomic studies define many additional substrates for NuA4 activity, some of which are likely to contribute to processes affected upon loss of Epl1 or Esa1 functions. Of note, both Yng2 and Epl1 were identified as substrates of Esa1, where acetylation has already been demonstrated to have a significant functional impact (Yi *et al.* 2012; Mitchell *et al.* 2013; Downey *et al.* 2015). Therefore, we believe that nonhistone substrates, though not specifically analyzed here, are a critical part of the observed phenotypes and model presented.

of an essential protein-modifying activity. Studies in *Drosophila* and humans alike illustrate that Epl1 orthologs play key roles in development and cancer, akin to Epl1's essential role in yeast. In addition to established roles in DNA damage (Figure 2B) (Boudreault *et al.* 2003), human EPC1 potentially has critical roles in DNA damage repair both within and independently of NuA4 (Attwooll *et al.* 2005; Wang *et al.* 2016). Failures in DNA damage repair are associated with genomic instability, which is a major driving force in cancer. The observation that mutational profiles of cancer patients reflect frequent alterations in EPC1/2 highlights the importance of these proteins in human biology and disease. Analysis of genomic cancer data illustrates, for example, that EPC1 is frequently amplified in neuroendocrine prostate cancer, but deleted in prostate adenocarcinoma (Cerami *et al.* 2012; Gao *et al.* 2013). Additionally, independent analysis demonstrates that EPC1 and EPC2 are often mutated across the gene body in many cancer subtypes, including in the critical domains studied here (Forbes *et al.* 2014). These frequent yet diverse alterations underscore the importance of understanding the critical functions of specific residues and domains of Epl1, along with the consequences of complete deletion of this essential gene. Our results provide new insights into both aspects of altered function and will be instrumental in deepening the understanding of Epl1 orthologs in development and disease.

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