Protection from Disulfide Stress by Inhibition of Pap1 Nuclear Export in *Schizosaccharomyces pombe*

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ABSTRACT Appropriate subcellular localization of regulatory factors is critical for cellular function. Pap1, a nucleocytoplasmic shuttling transcription factor of *Schizosaccharomyces pombe*, is redox regulated for localization and antistress function. In this study, we find that overproduction of a peptide conjugate containing the nuclear export signal of Oxs1, a conserved eukaryotic protein that, along with Pap1, regulates certain diamide responsive genes, can retain Pap1 in the nucleus before stress by competing for nuclear export. The nuclear retention of Pap1 upregulates several drug resistance genes to prime the cells for higher tolerance to disulfide stress. Overproduction of Oxs1 also upregulates these same genes, not by competing for export but by binding directly to the drug resistance gene promoters for Pap1-mediated activation. Of medical relevance is that this may suggest a gene therapy approach of using nuclear export signal conjugates to suppress the nuclear export of biomolecules.

KEYWORDS nuclear export signal; subcellular localization; drug-resistant genes; Oxs1; Pap1; disease therapy

EUKARYOTIC cells contain a highly coordinated membrane system dividing a cell into structural and functional compartments, allowing proteins to function properly in a sequestered context. Stringent regulation of subcellular localization is crucial, as dysregulation results in aberrant localization and consequent loss of function or deleterious gain of function. For example, cytoplasmic mislocalization induces the inactivation of the tumor suppressor p53 and the oncogenic activity of the apoptosis inhibitor survivin in various types of cancer (Moll *et al.* 1992; Knauer *et al.* 2007; Sun *et al.* 2016).

In the fission yeast *Schizosaccharomyces pombe*, Pap1, a homolog of mammalian cJun and *Saccharomyces cerevisiae* Yap1, is a redox-regulated transcription factor for oxidative stress, heavy metal detoxification, and multidrug resistance (Turner and Tjian 1989; Toda *et al.* 1991; Toone *et al.* 1998; Toone and Jones 1999; Vivancos *et al.* 2005). Pap1 is also highly regulated in its subcellular localization through a potential nuclear localization signal for importin-α Imp1 (Umeda *et al.* 2005) and a nuclear export signal (NES) for exportin Crm1 (Toone *et al.* 1998; Kudo *et al.* 1999). It shuttles between the nucleus and the cytoplasm, but is cytoplasmic prior to stress as nuclear export through GTP hydrolysis predominates over its import (Castillo *et al.* 2002; Vivancos *et al.* 2004). With Ran-GTP primarily in the nucleus (Gorlich 1998; Kalab *et al.* 2002), binding to Ran-GTP in the nucleus destabilizes Imp1-Pap1 but stabilizes the Crm1-Pap1 complex (Figure 1A). Upon export to the cytoplasm, Ran-GTP is hydrolyzed to Ran-GDP (Gorlich 1998).

Oxidative stress in *S. pombe* is best described in the H_2O_2 response. When exposed to low H_2O_2 concentrations (<0.2 mM), Tpx1, a direct sensor of H_2O_2, oxidizes the proteasome-associated thioredoxin-like protein Txl1 as well as Pap1 (Brown *et al.* 2013). Oxidation of Pap1 forms at least one intramolecular disulfide bond between the Pap1 C278 and C501 that causes Pap1 to disassociate from the Crm1-Hba1 nuclear export machinery (Castillo *et al.* 2002, 2003; Vivancos *et al.* 2004, 2005; Bozonet *et al.* 2005). The oxidation of Txl1 by Tpx1 also prevents Txl1 from reducing Pap1, hence retaining oxidized Pap1 in the nucleus (Vivancos *et al.*

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2004; Day *et al.* 2012; Brown *et al.* 2013). Nuclear Pap1 then activates ~40–80 antioxidant defense and multidrug-resistance genes in response to H_2O_2 (Chen *et al.* 2008; Calvo *et al.* 2012). However, when H_2O_2 concentration exceeds 1 mM, Tpx1 becomes inactivated by hyperoxidation and fails to oxidize Txl1. This permits Txl1-mediated reduction of Pap1 to become a substrate for Crm1-mediated export. During this transition, the Sty1-Atf1 pathway (an MAPK pathway) becomes activated (Vivancos *et al.* 2005; Calvo *et al.* 2012). The effect of an activated Sty1-Atf1 pathway leads Srx1 to reduce hyperoxidized Tpx1 and other antioxidant enzymes to scavenge excess H_2O_2. Subsequently, as the H_2O_2 concentration drops, Tpx1 oxidation of Pap1 reinitiates the Pap1 activation pathway under low H_2O_2 stress (Biteau *et al.* 2003; Vivancos *et al.* 2004, 2005).

Compared with the well-described H_2O_2 stress response, much less is known of the molecular responses to other oxidative stressors. Diamide is a thiol-reactive electrophile that reacts directly and rapidly with glutathione or protein thiols, leading to the formation of disulfides or *S*-thiolated proteins (Hochgräfe *et al.* 2007). We recently described a Pap1-Oxs1 pathway specific for the diamide-induced disulfide stress response in *S. pombe* (He *et al.* 2017). Oxs1 is a 207 aa high-mobility-group protein required for tolerance to diamide or cadmium. Overproduction of Oxs1 enhances tolerance to these chemicals, but only in the presence of Pap1. Oxs1 and Pap1 physically interact to coregulate the transcription of at least nine diamide-responsive genes. With *hsp90*$^+$, *ssa2*$^+$, *wis2*$^+$, or *SPBC36.02c*, Oxs1 or Pap1 can each upregulate transcription, and the presence of both exerts an additional positive effect. With *sro1*$^+$, *SPBC1347.14c*, or *SPAC23D3.12*, Oxs1 or Pap1 alone suffices to repress transcription, and derepression requires loss of both proteins. With *gal10*$^+$ or *ght5*$^+$, Pap1 can repress transcription but the presence of Oxs1 prevents Pap1 repression. Pap1 and Oxs1 bind these nine gene promoters in a diamide- or Cd-dependent manner, and promoter occupancy by Oxs1 in these nine genes is enhanced by but does not require Pap1, whereas occupancy by Pap1, except for *gal10*$^+$ or *ght5*$^+$ promoters, depends on Oxs1.

Like Pap1, Oxs1 is a conserved nucleocytoplasmic shuttling protein that enters the nucleus during stress, but is exported by exportin Crm1 in the absence of stress. Also like Pap1, Oxs1 appears to be part of an evolutionarily conserved stress-response pathway, as functional conservation has been implicated through the ability of heterologous Oxs1 homologs to enhance diamide stress tolerance in *S. pombe*, as well as the ability of *S. pombe*, human, and *Arabidopsis* homologs to interchangeably bind Pap1 or Pap1 homologs human cJun and *Arabidopsis* bZIP10 (He *et al.* 2017).

In this study, we report that overproduction of a peptide conjugate, the Oxs1 NES (^{107}IDDALDLLSL^{116}, henceforth referred to as o1NES) coupled to green fluorescent protein (GFP), is sufficient to enhance tolerance to diamide in either the wild-type (WT) or *oxs1*Δ background. As with Oxs1, the elevated tolerance to diamide by overproduction of the o1NES conjugate also depends on Pap1. The o1NES conjugate retains Pap1 in the nucleus prior to stress, presumably by competing for Crm1-mediated nuclear export (Figure 1B). Nuclear Pap1 then activates at least three drug-resistance genes, *SPCC663.08c*, *caf5*$^+$, and *obr1*$^+$, priming the cells to tolerate a higher level of disulfide stress. Interestingly, overproduction of Oxs1 primes diamide tolerance through direct binding to the drug-resistance gene promoters for Pap1-mediated activation, rather than serving as a competitive substrate for nuclear export. It is tempting to speculate that overproduction of NES-peptide conjugates may find possible uses in gene therapy, much like chemicals that inhibit the nuclear export of proteins and RNAs involved in human diseases (Cullen 2003; Turner and Sullivan 2008; Mutka *et al.* 2009).

Materials and Methods

Yeast strains and plasmids

S. pombe strains derived from JS23 are listed in Supplemental Material, Table S1, and strains not previously described were created by homologous recombination as shown in Figure S1. Plasmids derived from *S. pombe* expression vectors pART1 or pSLF173 are listed in Table S2. *S. pombe* strains were grown under standard conditions described in the Fission Yeast Handbook (Nurse Lab) (http://www.dieter-wolf-lab.org/protocols/).

Sensitivity assay

For survival on solid plates, *S. pombe* strains were grown, diluted, and spotted onto EMM media agar plates containing indicated concentration of diamide, H_2O_2, $CdCl_2$, NaCl, KCl, or sorbitol. Plates were incubated at 30° for 2–3 days.

In vitro GST pull-down assay

In vitro GST pull-down assays were performed as in He *et al.* (2017). His-Pap1 was incubated with GST beads bound to GST or GST fused to GFP as control, GST fused to Oxs1, GST fused to Oxs1 variants with mutations in the NES, or to GST fused with an Oxs1-truncated peptide. Proteins bound to the beads were analyzed by SDS-PAGE and subjected to Western blotting with α-His antibodies and Coomassie Brilliant Blue staining. Input represents 2% of the total amount of His-Pap1 used in each binding reaction.

Fluorescence microscopy

Single-colony inoculated cultures were diluted to OD_{600}~0.1 and grown overnight to OD_{600}~0.5–1.0. Cells collected by centrifugation from 1 ml cultures were washed at least three times with 1× PBS (137 mM sodium chloride, 2.7 mM potassium chloride, and 11.9 mM phosphate buffer; pH 7.4). After suspending in 30~50 μl 1× PBS, 5 μl of the cell solution was loaded on a slide for fluorescence microscopy (Zeiss Axiophot fluorescence microscope equipped with an AxioCam MRc camera using AxioVision Rel. 4.8 software) or confocal microscopy (Zeiss LSM 800 confocal microscope with Airyscan using Zen imaging software). When used, DAPI

(1 μg/ml; Sigma, St. Louis, MO) was added just prior to fluorescence microscopy to minimize exposure as it can cause a higher percentage of cells with nuclear localization of Pap1-mCherry, presumably due to stress caused by this chemical. For fluorescence microscopy, signals were detected using filters for GFP (excitation 470/40 nm; emission 525/50 nm), mCherry (excitation 546/12 nm; emission 575–640 nm), and DAPI (excitation 365 nm; emission 445/50 nm). For confocal microscopy, GFP or mCherry were excited with 488 or 561 nm lasers; fluorescence emission was collected at 495–555 nm to detect the presence of GFP and at 596–650 nm to detect mCherry.

RNA analysis

Cells grown to OD_{600}~0.3 were treated with 1.5 mM diamide, collected at indicated time points by centrifugation of 50 ml cultures, and washed at least two times with 1× PBS prior to preparation of total RNA (RNeasy Mini Kit 74104; Qiagen, Valencia, CA). Reverse transcription was conducted using PrimeScript RT Reagent Kit with gDNA Eraser (RR047A; TaKaRa); quantitative RT-PCR was performed with GoTaq qPCR Master Mix (A6002; Promega, Madison, WI) on LightCycler480 II (Roche). The messenger RNA (mRNA) level of each gene was normalized to the level of *act1*$^+$ (*SPBC32H8.12c*) of the control strain with the plasmid empty vector (EV) or a plasmid overproducing GFP. Primers for quantitative RT-PCR are listed in Table S3.

Chromatin immunoprecipitation

A total of 100 ml of cells grown to OD_{600}~1.0 were cross-linked by adding fresh 30% paraformaldehyde to a final concentration of 1% and incubated at 30° for 20 min. Cross-linking was stopped with 125 mM glycine for 10 min. Cell pellets were washed three times with 1× PBS, resuspended in 0.5 ml of nuclear lysis buffer [50 mM HEPES-potassium hydroxide (pH 7.5), 500 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% NaDoc, 1% SDS, 1 mM PMSF, 1× protein inhibitor cocktail] and transferred to 2 ml capped tubes containing 0.5 ml glass beads. During lysis intervals [FastPrep-24 (MP): 6.5 M/sec, 40 sec/pulse, 5 min interval, 6~8 pulses], tubes were kept cold in ice/water. Additional pulses were performed until microscopic examination of cell aliquots showed >95% cell breakage (lysed cells appear dark under phase/contrast). Cell lysates were separated from glass beads and debris by passing through a 25 gauge syringe needle. Chromatin from cell lysates was released and sheared using M220 sonicator (Covaris). Anti-HA (H3663; Sigma) immunoprecipitation and DNA recovery procedures were as described (Sansó *et al.* 2011). Recovered DNA fragments were amplified by quantitative RT-PCR using GoTaq qPCR Master Mix (A6002; Promega) on LightCycler480 II (Roche). Specific primers amplifying the promoter region of *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$ and control primers amplifying the coding region of *gpd3*$^+$ (*SPBC354.12.1*) or the intergenic region (*S. pombe* chromosome I: position 465226–465326) are listed in Table S3.

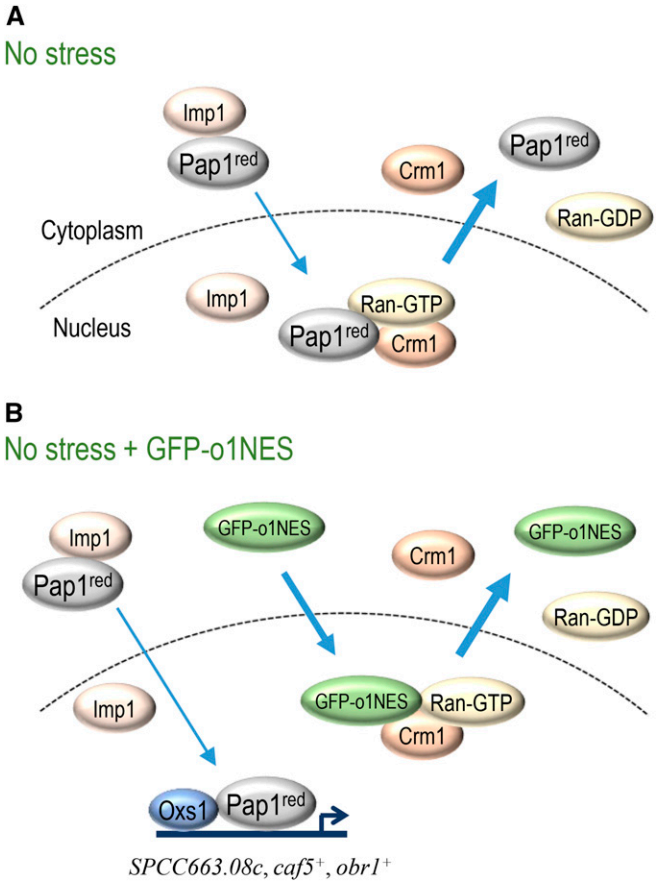

Figure 1 Pap1 nucleocytoplasmic shuttling altered by Crm1 competitive substrate. (A) In the absence of stress, Crm1-mediated Pap1^{red} (reduced form of Pap1) export predominates over Pap1^{red} entry into nucleus. (B) Overproduction of GFP-o1NES competes for Crm1-mediated export, leading to Pap1^{red} accumulation in the nucleus and activation of drug-resistance genes in which Oxs1 can be but not required as a coregulator.

Data availability

All data supporting the conclusions of this article are included in this published article and its supplemental files, and materials reported in this study are freely available upon request. Supplemental material available at Figshare: https://doi.org/10.6084/m9.figshare.7043366.

Results

o1NES is necessary for diamide tolerance

To test what sequences within Oxs1 are critical for conferring stress tolerance, random mutations within the *oxs1*$^+$ coding region were generated by error-prone PCR and inserted into the *S. pombe* vector pART1 by *in vivo* gap repair (Figure S2). After DNA transformation, 1545 *S. pombe* clones were obtained and screened for diamide sensitivity. From this collection, 410 clones showed lower tolerance to diamide than the WT *oxs1*$^+$ complementary DNA. Mutagenized *oxs1*$^+$ coding region fragments were amplified by PCR from these 410 diamide-sensitive clones, but after sequence analysis,

only 56 were found to harbor Oxs1 amino acid substitutions. As for the other 354 clones without amino acid changes, we did not investigate whether mutations in other parts of the plasmid might have affected gene expression or copy number. However, it is also likely that many of those were false negatives, as after subsequent rounds of reintroduction into WT cells, only 20 of the 56 clones with amino acid substitutions could reproducibly confer lower tolerance to diamide. Distribution of the mutations show that 16 of the 20 diamide-sensitive clones harbor changes in 107, 110, 111, 113, 114, 115 and/or 116 aa, all within 7 of the 10 NES amino acid sequence (o1NES) defined by the loose consensus sequence of ϕ-$X_{(2-3)}$-ϕ-$X_{(2-3)}$-ϕ-X-ϕ, where ϕ is a hydrophobic residue (L, V, I, F, or M) critical for NES function and X is any amino acid (Figure 2A) (Dong *et al.* 2009). This suggests that o1NES is important for ameliorating diamide stress.

Since each of the Oxs1 mutants generated by error-prone PCR contains more than a single amino acid change within the coding region, we decided to test single amino acid substitutions within the NES. A codon change was introduced into each of the same seven amino acids uncovered by the mutant screen to generate the Oxs1 variants: $Oxs1^{I107S}$, $Oxs1^{A110V}$, $Oxs1^{L111W}$, $Oxs1^{L113F}$, $Oxs1^{L114F}$, $Oxs1^{S115R}$, $Oxs1^{S115G}$, and $Oxs1^{L116F}$. As shown in Figure 2B, each of these seven single amino-acid-substitution mutants of Oxs1 failed to confer diamide tolerance to the level seen with WT Oxs1, and the loss of function was more severe with the four hydrophobic residues 107, 111, 114, and 116 aa. This shows that the NES is necessary for Oxs1-mediated diamide tolerance.

o1NES conjugate is sufficient for diamide tolerance

To ask whether peptides containing the NES would enhance diamide tolerance, we compared truncated Oxs1 derivatives with or without the NES. Whereas the Oxs1 1–131 aa or Oxs1 100–207 aa fragment containing the NES was capable of enhancing diamide tolerance, the Oxs1 1–106 aa or Oxs1 117–207 aa fragment lacking the NES lost this ability (Figure 3A). Furthermore, the native Oxs1 protein is not required for this o1NES-mediated stress tolerance, as Oxs1 1–131 aa and Oxs1 100–207 aa fragments were effective in the *oxs1*Δ background (Figure 3A), even outperforming the overexpression of $oxs1^+$.

To further delineate the extent of NES-containing DNA needed to enhance diamide tolerance, we examined WT or *oxs1*Δ cells overexpressing the small NES-containing peptide Oxs1 100–131 aa. However, Oxs1 100–131 aa was only slightly effective for enhancing tolerance to diamide (Figure 3B). Assuming that this could be due to the instability of such a small peptide, we fused Oxs1 100–131 aa to the C or N terminus of GFP, yielding GFP-Oxs1 100–131 aa and Oxs1 100–131 aa-GFP, respectively. Both GFP fusions displayed a strong ability to enhance diamide tolerance. When we truncated this peptide further to only the NES motif comprising of 107–116 aa, the NES conjugate GFP-o1NES or o1NES-GFP was still effective (Figure 3B).

To ask whether this o1NES conjugate-mediated stress tolerance is specific for disulfide stress, we examined the tolerance of WT or *oxs1*Δ cells overexpressing GFP-o1NES to other cytotoxic compounds. However, overproduction of GFP-o1NES failed to yield cells resistant to stresses elicited by H_2O_2, $CdCl_2$, NaCl, KCl, or sorbitol (Figure S3B).

Nuclear exclusion capability associated with diamide tolerance

To test whether any of these seven amino acid substitutions introduced into the full-length Oxs1 protein would affect its subcellular localization, each variant was fused to the C terminus of GFP. As described previously (He *et al.* 2017), GFP-Oxs1 fluorescence can be observed in the nucleus upon treatment with diamide but not in the absence of stress (Figure 4A). For the substitution mutants in the absence of diamide, only GFP-$Oxs1^{A110V}$, GFP-$Oxs1^{L113F}$, or GFP-$Oxs1^{S115G}$, like the GFP-Oxs1 control, retained the ability to be excluded from the nucleus (Figure 4Ba–c). For GFP-$Oxs1^{I107S}$, GFP-$Oxs1^{L111W}$, GFP-$Oxs1^{L114F}$, or GFP-$Oxs1^{L116F}$, nuclear export of these Oxs1 variants was abolished (Figure 4Bd–g).

Given that amino acid substitutions in these same four hydrophobic residues showed a more severe impairment of the ability for diamide tolerance (Figure 2B), we chose to confirm this finding through a second set of amino acid substitutions. Rather than using the original amino acid substitutions that may have resulted in gaining a harmful amino acid residue, we chose instead to replace each residue with alanine that has a smaller and chemically inactive side chain. In this manner, a loss of function may more likely be due to loss of a critical amino acid. Hence, we generated GFP-$o1NES^{I107A}$, GFP-$o1NES^{L111A}$, GFP-$o1NES^{L114A}$, and GFP-$o1NES^{L116A}$. As shown in Figure 4C, none of them were able to enhance diamide tolerance, and this most likely is not due to harmful effect by alanine because the alanine substitution in GFP-$o1NES^{L113A}$ was effective for diamide tolerance in both the WT and *oxs1*Δ background. Loss of diamide tolerance was also seen with GFP-$o1NES^{S115G}$ but not with GFP-$o1NES^{S115R}$. With GFP-$o1NES^{A110V}$, diamide tolerance was found in the WT background, but not in the *oxs1*Δ background (Figure 4C), suggesting that its stress tolerance effect may be due to interaction with the native Oxs1.

o1NES conjugate-mediated diamide tolerance is dependent on Pap1

Previously, we reported the Oxs1 overproduction that confers enhanced tolerance to diamide and Cd depends on Pap1. Pap1 and Oxs1 form a complex *in vivo* upon diamide or Cd treatment, which then regulates certain diamide-response genes. This regulation includes coactivation of some genes, corepression of other genes, and in a third class of genes, Oxs1 keeps Pap1 from repressing transcription (He *et al.* 2017). To ask whether the NES peptide functions similarly to that of the full-length Oxs1, we tested the effect of $pap1^+$ on o1NES conjugate-enhanced diamide tolerance. As with Oxs1, enhanced tolerance to diamide was found when GFP-o1NES

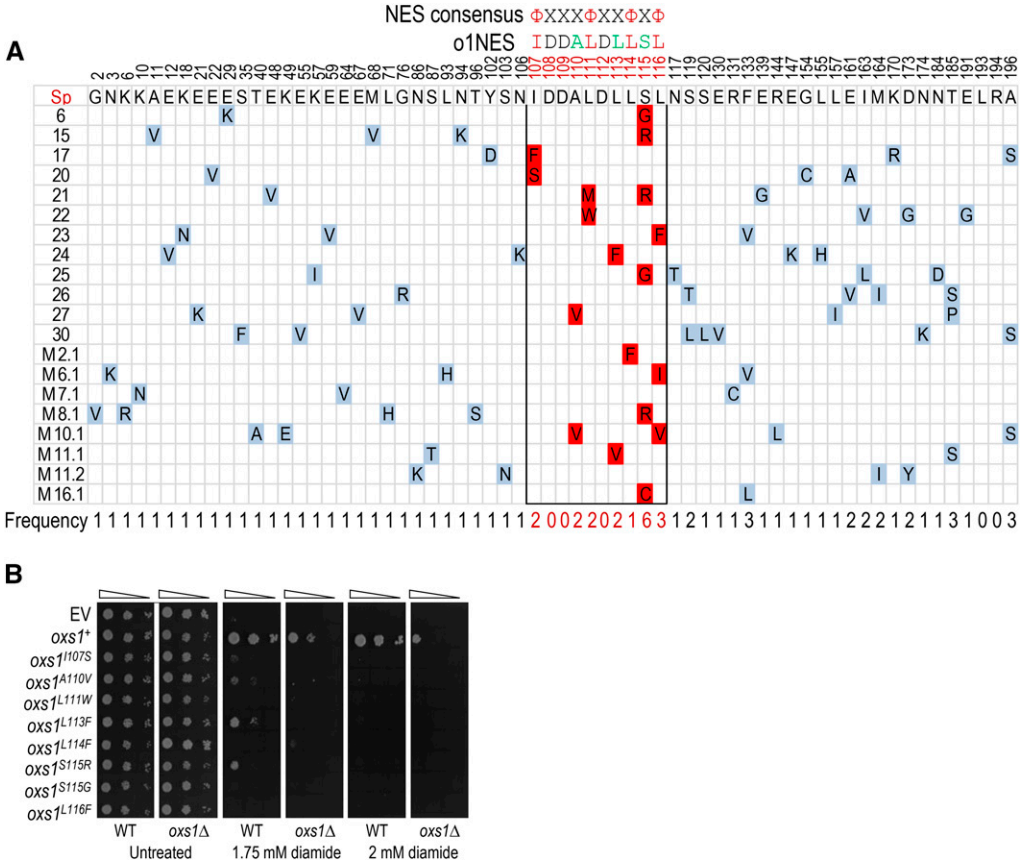

Figure 2 Mutation screen indicates o1NES in diamide tolerance. (A) Distribution of aa substitutions in 20 diamide-sensitive clones (only residues with changes shown in protein sequence). Number of clones containing amino acid substitution(s) at each residue indicated below table. (B) WT or *oxs1*Δ strains overproducing Oxs1 with single amino acid substitutions in the NES. Empty vector (EV) and *oxs1*$^{+}$ serve as negative and positive controls, respectively. Serial dilutions of WT or *oxs1*Δ cells overexpressing indicated clones spotted onto EMM + Ura plates with or without diamide (*oxs1*Δ cells are in a *HA-pap1*$^{+}$ background, but are phenotypically identical to those in a *pap1*$^{+}$ background; see Figure S3A).

was overexpressed in a *pap1*$^{+}$ genotype but not in a *pap1*Δ mutant (Figure 4D).

Next, we tested whether the o1NES is essential for the interaction with Pap1. Using fusions to GST, pull-down assays found that like WT Oxs1 (He *et al.* 2017), each of the Oxs1 variants with a single amino acid substitution in the NES motif (Oxs1^{I107S}, Oxs1^{A110V}, Oxs1^{L111W}, Oxs1^{L113F}, Oxs1^{L114F}, Oxs1^{S115G}, or Oxs1^{L116F}) was still able to interact with Pap1 *in vitro* (Figure S4A). Furthermore, whereas the NES itself failed to pull down Pap1, the Oxs1 amino terminus (Oxs1 1–106 aa) or carboxyl terminus (Oxs1 117–207 aa) that lack the NES still interacted with Pap1 *in vitro* (Figure S4B). These data show that the NES is not the Oxs1 segment responsible for Pap1 interaction.

Because physical interaction between the o1NES and Pap1 does not correlate with diamide tolerance, this leaves the possibility that o1NES has a different mode of function than full-length Oxs1. We tested the possibility that overexpression of the o1NES conjugate might increase expression of *pap1*$^{+}$, but the data showed otherwise; overproduction of the o1NES conjugate could not increase steady-state *pap1*$^{+}$ mRNA (Figure S5).

Overproduction of o1NES conjugate renders nuclear retention of Pap1

Given that both Pap1 and GFP-o1NES are NES-bearing proteins, we hypothesized that overproduction of the o1NES conjugate might compete with Pap1 for nuclear export. If that were the case, then this might trigger nuclear retention of Pap1 and the consequent up-regulation of the Pap1-dependent genes. To test this possibility, we checked the subcellular localization of Pap1-mCherry in WT cells coproducing the GFP control or GFP-o1NES. As shown in the confocal images of Figure 5A, when cells were grown under nonstressed conditions, Pap1-mCherry was excluded from nuclei when co-overproduced with GFP. With GFP-o1NES, however, Pap1-mCherry can be seen in the nucleus.

For a more quantitative analysis, we counted the number of cells under fluorescence microscopy that showed both GFP and mCherry signals, scoring how many showed mCherry nuclear fluorescence (nucleus and cytoplasm, N+C) *vs.* those lacking nuclear fluorescence (cytoplasm only, C). Consistent with previous observations (He *et al.* 2017), in the absence of stress Pap1-mCherry also displayed cytoplasmic-only localization in strains co-overproducing GFP-Oxs1 (Figure 5B and Table S4). So too was cytoplasmic-only localization found in most of the cells co-overproducing GFP-o1NES^{I107A}, GFP-o1NES^{L111A}, GFP-o1NES^{L114A}, or GFP-o1NES^{L116A} (Figure 5B and Table S4)—the same clones that could not enhance diamide tolerance in WT or *oxs1*Δ strains (Figure 4C). In contrast, nearly all of the cells co-overproducing GFP-o1NES scored positive for nuclear (as well as cytoplasmic) fluorescence.

This observation that GFP-o1NES could cause Pap1-mCherry to be retained in the nucleus is consistent with the interpretation that it could be a competitive substrate of Pap1

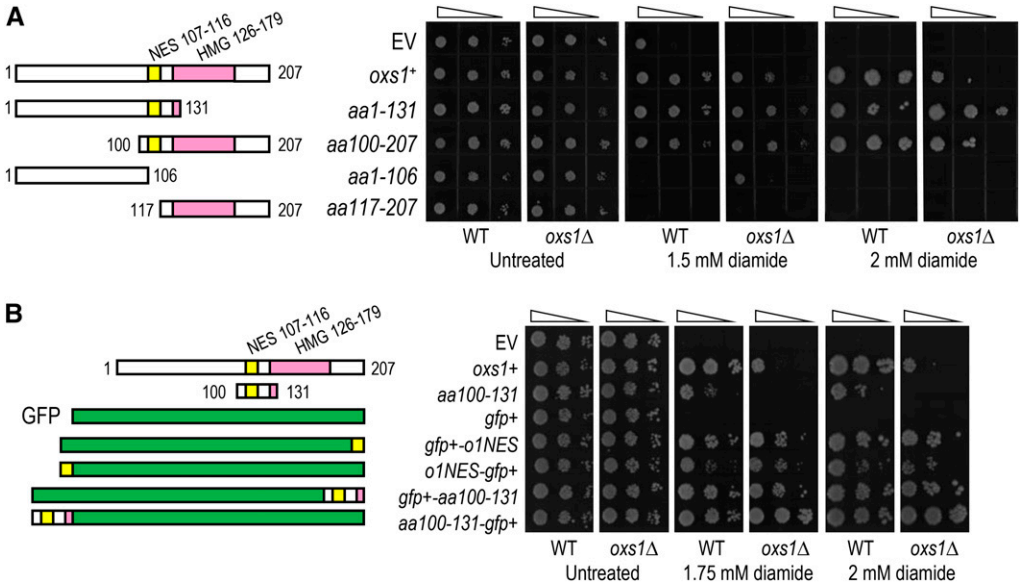

Figure 3 o1NES conjugate is sufficient for diamide tolerance. (A) Diamide tolerance of strains overproducing Oxs1 derivatives with or without the NES, or (B) overproducing GFP or GFP fused to o1NES or o1NES-containing peptide. Plating conditions as in Figure 2B (*oxs1*Δ cells in *HA-pap1*$^+$ background).

for Crm1-mediated export. If that were the case, its overproduction might also affect other nucleocytoplasmic proteins. Hence, we tested another Crm1 export substrate, Wis1, which is an NES-containing MAPKK in the Sty1-Atf1 pathway. Like Pap1, Wis1 is located in the cytoplasm prior to stress, but enters the nucleus in response to stress (Nguyen *et al.* 2002). As shown in Figure 5C, in the absence of stress the Wis1-mCherry signal was cytoplasmic-only in ~83% of the cells that overproduced GFP, but were found in the nucleus as well as the cytoplasm in ~87% of the cells overproducing GFP-o1NES (Table S4). This shows that the nuclear retention induced by overproduced GFP-o1NES is not restricted to Pap1, and may affect the nuclear export of other similarly regulated proteins.

It is worth noting that unlike GFP-Oxs1 in the absence of stress, GFP-o1NES is not exclusively cytoplasmic, as is also the case with GFP (Figure 5, A and B). Previous studies indicate that proteins with a molecular weight below 60 kDa are able to cross the nuclear pore complex by passive diffusion (Nachury and Weis 1999). GFP with a molecular mass of 27 kDa can diffuse freely between the nucleus and the cytoplasm and is commonly used as a benchmark for passive diffusion (Wei *et al.* 2003; Cardarelli *et al.* 2007) because it does not interfere with biological processes or contain a nuclear localization signal or NES capable of triggering active nuclear transport (Ormö *et al.* 1996). Likewise, GFP-o1NES being 28 kDa raises the possibility that diffusion of GFP-o1NES prevails over its NES-mediated nuclear export. To test this possibility, a GST tag was added to the N terminus of GFP-o1NES, as described for the study of Pap1 NES (Kudo *et al.* 1999), yielding a 55 kDa GST-GFP-o1NES. Whereas GST-GFP without an NES exhibited pancellular localization (Figure 5B), the GST-GFP-o1NES displayed cytoplasmic-only localization (Figure 5B), which indicates that NES-mediated export prevailed over its diffusion into the nucleus.

Overproduction of this 55 kDa GST-GFP-o1NES also failed to keep Pap1-mCherry in the nucleus or to confer enhanced tolerance to diamide (Figure 5, B and D). To rule out that the larger GST-GFP-o1NES was not produced at a comparable level, we examined the protein levels of GFP, GFP-o1NES, o1NES-GFP, GST-GFP and GST-GFP-o1NES by western blotting. As shown in Figure 5E, a significant difference in the steady state level among these proteins was not found. These data indicate that a functional o1NES conjugate capable of diffusing into the nucleus is essential for the nuclear retention of Pap1 in the absence of stress as well as for enhanced diamide tolerance.

Nuclear Pap1 caused by overproduced o1NES conjugate directly activates SPCC663.08c, caf5$^+$*, and obr1*$^+$ *prior to stress*

We next asked whether nuclear localized Pap1 is able to turn on its target genes in the absence of stress. To eliminate the effect of native Oxs1, we conducted the following experiments in an *oxs1*Δ strain. Quantitative RT-PCR was performed prior to or after 1 hr of diamide treatment on 15 genes previously reported as Pap1 targets, among which three are involved in drug resistance (*SPCC663.08c*, *caf5*$^+$, *obr1*$^+$) (Calvo *et al.* 2012), three are upregulated by H_2O_2 (*srx1*$^+$, *trr1*$^+$, *ctt1*$^+$) (Calvo *et al.* 2012), and nine upregulated by diamide (*hsp90*$^+$, *ssa2*$^+$, *wis2*$^+$, *SPBC36.02c*, *sro1*$^+$, *SPBC1347.14c*, *SPAC23D3.12*, *gal10*$^+$, *ght5*$^+$) (He *et al.* 2017). The results showed that the mRNA levels of the drug-resistance genes (*SPCC663.08c*, *caf5*$^+$, and *obr1*$^+$) increased in cells overproducing Oxs1 or GFP-o1NES prior to diamide treatment, but not in cells with the plasmid EV, overproducing GFP, any of the four GFP-o1NES mutants (Figure 6A), or GST-GFP-o1NES that fails to retain Pap1 in the nucleus (Figure S6). With the H_2O_2- or diamide-responsive genes, a change of the mRNA level was not detected among the different strains regardless of diamide treatment (Figure 6, B and C).

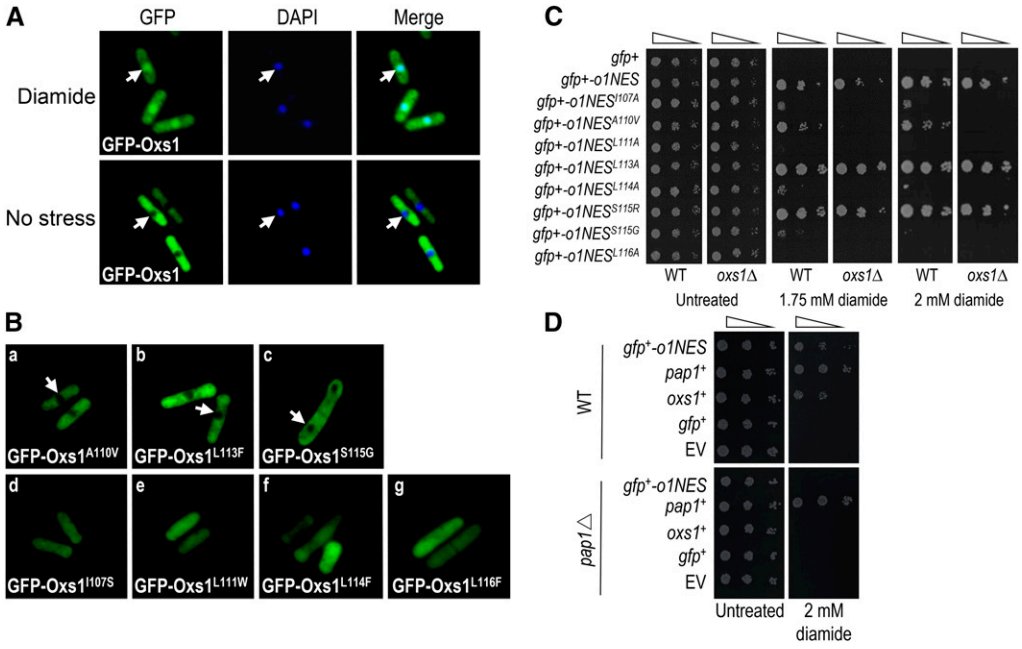

Figure 4 Nuclear exclusion associated with diamide tolerance. (A and B) Fluorescence microscopy of WT cells show subcellular localization of GFP fused to Oxs1 or to Oxs1 variant with mutation within NES. (A) Cells overproducing GFP-Oxs1 with or without diamide treatment. White arrows point to nuclei indicated by DAPI staining (1 μg/ml added just before fluorescence microscopy). (B) Cells without diamide treatment overproducing GFP-Oxs1 NES mutants as indicated. Because DAPI causes a higher percentage of cells with GFP-Oxs1 nuclear localization, presumably due to stress caused by this chemical, it was not included in the experiment shown in B; white arrows point to nuclei. (C) WT or *oxs1*Δ strains overproducing GFP fused to o1NES mutants; *gfp⁺-o1NES* gene product structure shown in Figure 3B. (D) WT or *pap1*Δ strains overexpressing indicated genes show that o1NES conjugate-mediated diamide tolerance requires Pap1. In C and D, plating conditions are as in Figure 2B (*oxs1*Δ cells in a *HA-pap1⁺* background).

Previous research has shown that nuclear Pap1, irrespective of its redox state, binds and activates the drug-resistance genes *SPCC663.08c*, *caf5*$^+$, and *obr1*$^+$ (Calvo *et al.* 2012). To test whether nuclear Pap1 directly activates these genes in the o1NES conjugate-overproducing cells, we performed a chromatin immunoprecipitation (ChIP) assay. Indeed, Pap1 was bound to all three promoters prior to stress in the strains overproducing GFP-o1NES (Figure 6D), but not in the strains overproducing Oxs1. Therefore, we conclude that even if high abundance of Oxs1 could compete for nuclear export of Pap1, it most likely could not be effective enough to score positive in this ChIP assay.

To further investigate whether upregulation of *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$ is involved in diamide tolerance, we overexpressed each complementary DNA from a multicopy plasmid. As shown in Figure 6E, overexpression of each gene was able to enhance tolerance to diamide in the WT or *oxs1*Δ background. When tested in a *pap1*Δ background, however, only *caf5*$^+$ conferred diamide resistance comparable to the overexpression of *pap1*$^+$ itself (Figure 6F). This suggests that Pap1 was still needed when overexpressing *SPCC663.08c* or *obr1*$^+$.

As for whether downregulation of *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$ would affect diamide tolerance, we created deletion alleles in each of these genes (Figure S1). Whereas GFP-o1NES–mediated tolerance was unaffected in an *obr1*Δ or a *SPCC663.08c*Δ mutant, it failed to do so in *caf5*Δ background (Figure 6G). This effect was also observed in the double mutant *caf5*Δ *obr1*Δ and in the triple mutant *caf5*Δ *obr1*Δ *SPCC663.08c*Δ, showing that loss of *caf5*$^+$ alone can impair GFP-o1NES–mediated diamide tolerance.

All of the above point to a model whereby diffusion into the nucleus by the o1NES conjugate retains Pap1 in the nucleus, presumably by competing for Crm1-mediated export (Figure 1B). Pap1 retention then activates several key genes to prime the cells for a subsequent challenge by diamide stress.

Oxs1 can activate SPCC663.08c, caf5⁺, and obr1⁺ by binding to their promoters

However, there were some conflicting data. As shown in Figure 6A, expression of *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$ was upregulated in cells overproducing Oxs1 in the absence of stress. Yet our Figure 5B data show that Pap1 is cytosolic in strains overproducing GFP-Oxs1 prior to stress (He *et al.* 2017). Moreover, in contrast to Oxs1 or GFP-o1NES, GFP-Oxs1 was not able to enhance diamide tolerance (Figure S7A) or activate the drug-resistant genes (*SPCC663.08c*, *caf5*$^+$, and *obr1*$^+$) (Figure S7B). We first considered the possibility that this may be due to the size difference between the 51 kDa GFP-Oxs1 and the 24 kDa Oxs1, with the smaller Oxs1 more freely able to enter the nucleus to cause the same effect as the 28 kDa GFP-o1NES conjugate. Because we were not able to visualize the localization of Oxs1 without the GFP tag, we could only follow Pap1-mCherry in a strain that also overproduces Oxs1. However, we failed to observe significant mCherry fluorescence in the nucleus, indicating that Oxs1 was not effective in retaining Pap1-mCherry in the nucleus (Figure S8 and Table S4), which is also consistent with our ChIP data that overexpression of untagged Oxs1 failed to induce Pap1 association with the *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$promoter.

To reconcile these seemingly contradictory data, we considered a second possibility that Oxs1, like Pap1, may also be able to activate *SPCC663.08c*, *caf5*$^+$, and *obr1*$^+$ directly. In a ChIP experiment similar to that conducted with Pap1, in

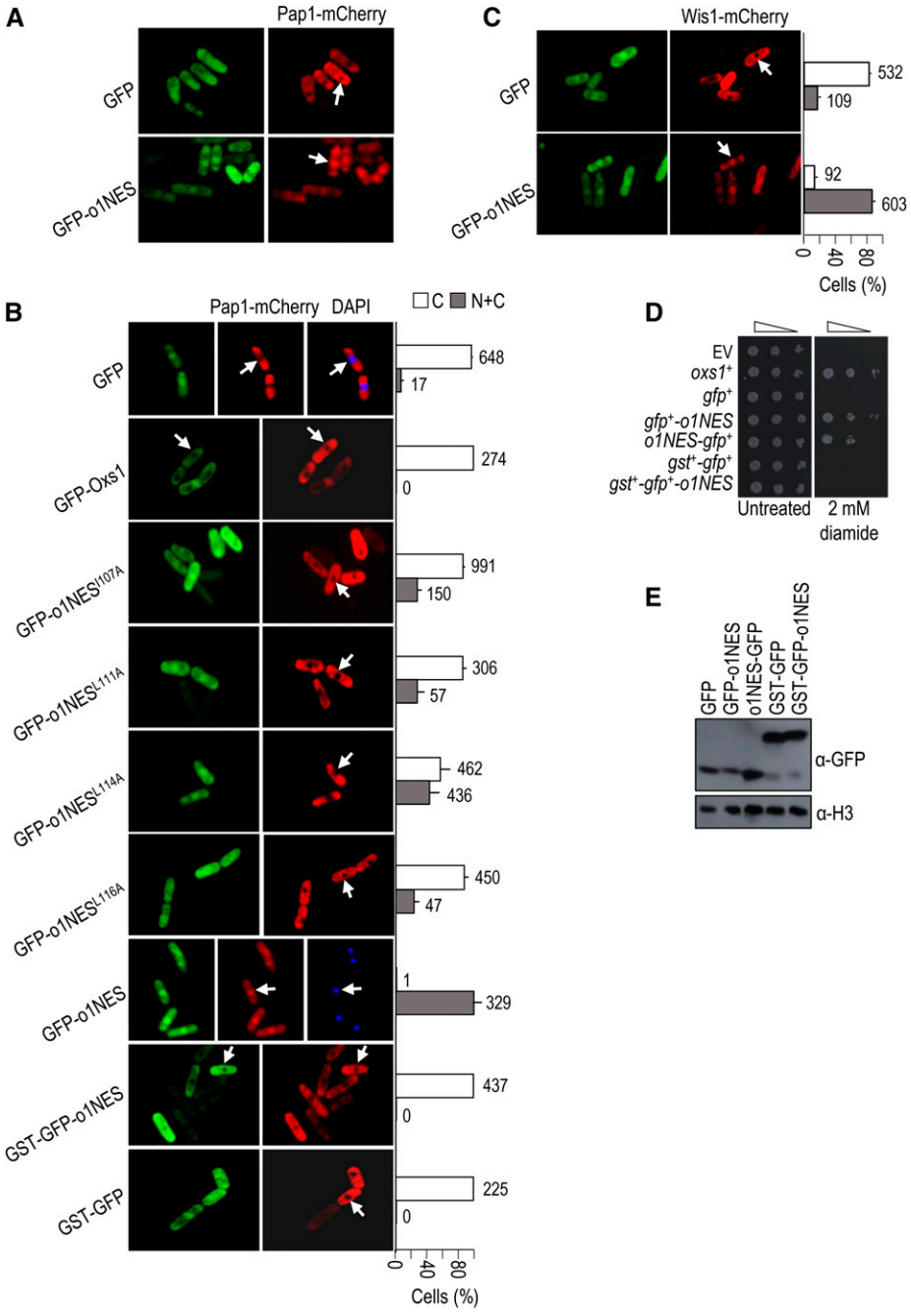

Figure 5 o1NES-conjugate retains Pap1 in the nucleus prior to stress. (A–C) Subcellular localization of Pap1-mCherry or Wis1-mCherry from (A) confocal microscopy or (B and C) fluorescence microscopy on WT cells co-overproducing indicated clones in the absence of stress; white arrows point to nuclei. In B, images from GFP or GFP-o1NES were produced after adding DAPI to 1 μg/ml just before fluorescence microscopy to confirm locations of nuclei. Because DAPI causes a higher percentage of cells with Pap1-mCherry nuclear localization, presumably due to stress caused by this chemical, it was not used in cell count experiments shown by the bar graphs. Cells that showed GFP signals were scored for Pap1-mCherry (or Wis1-mCherry) signals in C (cytoplasmic-only) or in N+C (observable nuclear and cytoplasmic). Data displayed in the bar graph represent mean + SEM from three independent experiments with total number of cells scored shown (from Table S4). (D) GST-GFP fused to o1NES fails to enhance diamide tolerance in WT cells. Plating conditions as in Figure 2B. (E) Western blot shows similar levels of GFP and GFP-derivatives. Total protein extracted from cells detected by α-GFP or α-H3 antibodies used as loading control.

Oxs1-overproducing strains grown in the absence of stress, we found that Oxs1 binds all three promoters (*SPCC663.08c*, *obr1*$^+$, and *caf5*$^+$) in both the WT and the *oxs1*Δ mutant background, but not in the *pap1*Δ mutant (Figure 7A). This requirement for Pap1 is also reflected in the Oxs1 or o1NES conjugate-mediated transcription activation of *SPCC663.08c*, *obr1*$^+$, and *caf5*$^+$ in the *oxs1*Δ, but not in the *pap1*Δ background (Figure 7B). The fact that Oxs1 can bind to target gene promoters when it is in the nucleus even in the absence of stress may also explain why it may not be effective in retaining Pap1 in the nucleus. Promoter occupancy would reduce the number of nonbound proteins to serve as a Crm1 export substrate.

Discussion

Oxs1 and o1NES conjugate activate the drug-resistance genes by different mechanisms

During the course of trying to find how Oxs1 imparts enhanced tolerance, we unexpectedly found that overproduction of just the o1NES conjugate is sufficient to produce the enhanced tolerance effect, including its requirement for Pap1. This led to the finding that o1NES conjugate can retain Pap1 in the nucleus, leading to the Pap1-directed activation of three genes (*SPCC663.08c*, *obr1*$^+$, and *caf5*$^+$) described for drug resistance. Although overexpression of each of these genes alone enhances diamide tolerance, only the loss of function of *caf5*$^+$

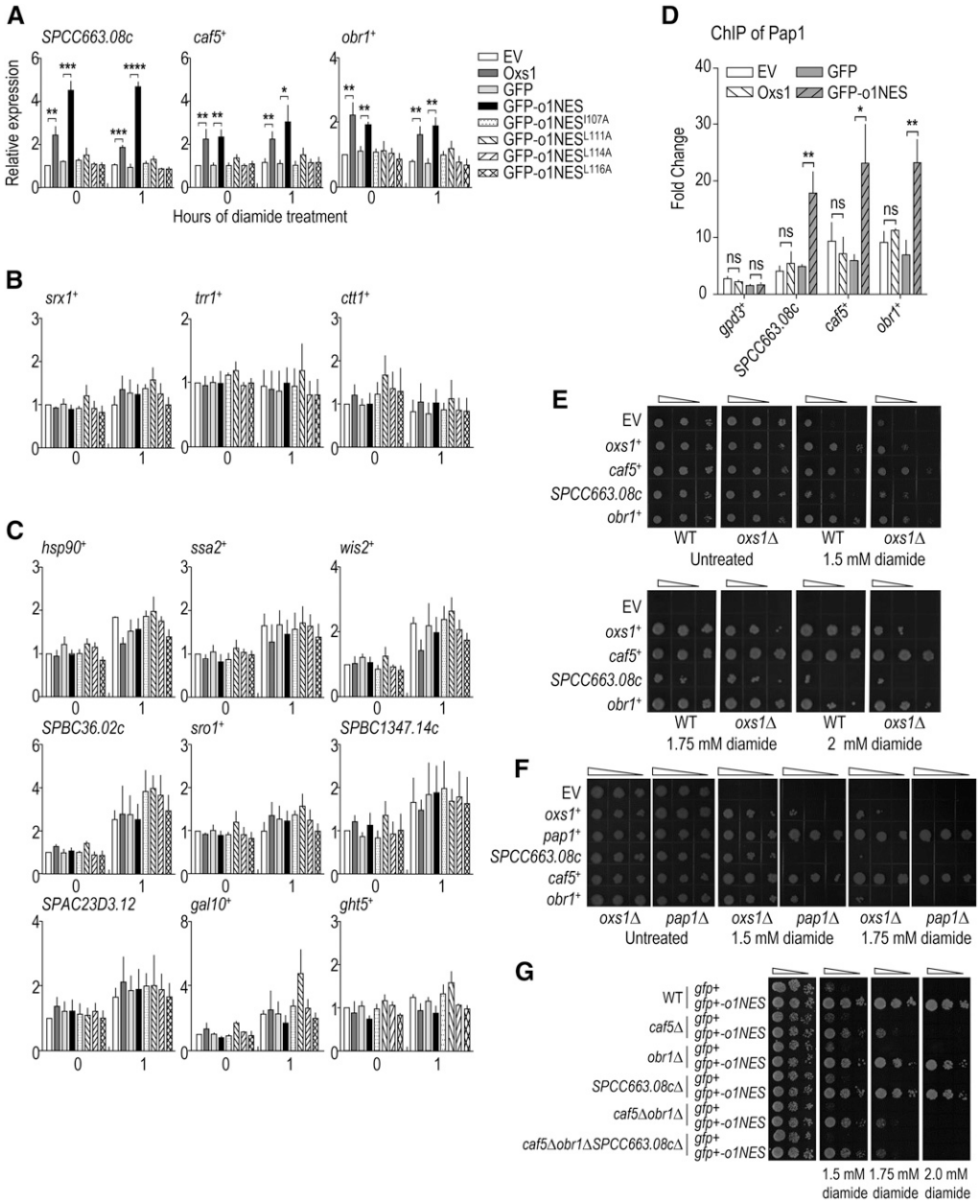

Figure 6 Nuclear accumulation of Pap1 caused by overproduced o1NES conjugate binds and activates *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$ in the absence of stress. (A–C) Relative expression examined by quantitative RT-PCR on Pap1-dependent genes in *HA-pap1*$^+$ *oxs1*Δ cells overproducing Oxs1, GFP, GFP-o1NES, or indicated GFP-o1NES mutant before or after 1 hr of diamide treatment. Transcript level of each gene normalized to *act1*$^+$ (*SPBC32H8.12c*) from strain with the empty vector (EV) control. (D) ChIP shows Pap1 associated with *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$ promoter prior to stress in *HA-pap1*$^+$ *oxs1*Δ cells overproducing GFP-o1NES. Results expressed as fold enrichment above background (enrichment relative to no-antibody control). (E) Overexpression of *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$ enhances diamide tolerance. (F) Diamide tolerance of *oxs1*Δ or *pap1*Δ cells overexpressing *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$. (G) Diamide tolerance of *caf5*Δ, *obr1*Δ, *SPCC663.08c*Δ, *caf5*Δ *obr1*Δ, or *caf5*Δ *obr1*Δ *SPCC663.08c*Δ strains overproducing GFP or GFP-o1NES. In A–D, data represent mean + SEM from three or more independent experiments. Significant differences from unpaired Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns, not significant). In E–G, plating conditions are as in Figure 2B (*oxs1*Δ cells in *HA-pap1*$^+$ background).

could impair the o1NES effect. This highlights the essential role of *caf5*$^+$, however, it does not necessarily diminish the roles of *SPCC663.08c* and *obr1*$^+$ because there may be other stress pathways compensating for their loss of function. At least one other Crm1 export substrate we tested, the Wis1 MAPKK, was found to exhibit the same nuclear retention effect when GFP-o1NES conjugate was overproduced, which suggests that perhaps other Crm1 export substrates could have been affected as well.

Interestingly, we were unable to find direct evidence for Pap1 nuclear retention when the full-length Oxs1 was overproduced. Although this does not rule out that high abundance of Oxs1 had some effect in retaining Pap1, it nonetheless forced us to consider alternative explanations. Given that Oxs1 can activate expression of the same three drug-resistance genes, we hypothesized that Oxs1 might activate these genes directly; indeed, we found that Oxs1 can bind these promoters except when in a *pap1*Δ background. This suggests Oxs1 can act as a positive regulator of these three genes, although as in the case of o1NES conjugate, activation of these genes requires Pap1. Prior production of stress proteins that prime a cell for a higher level of tolerance is an established doctrine, and we surmise that this also applies in our case. Furthermore, this could also explain why overproduction of Oxs1 does not produce the Pap1 nuclear retention effect because proteins bound to target gene promoters would be less available as a competitive substrate for export.

To conclude, we can now explain the molecular basis of the various observations. First, overproduction of o1NES

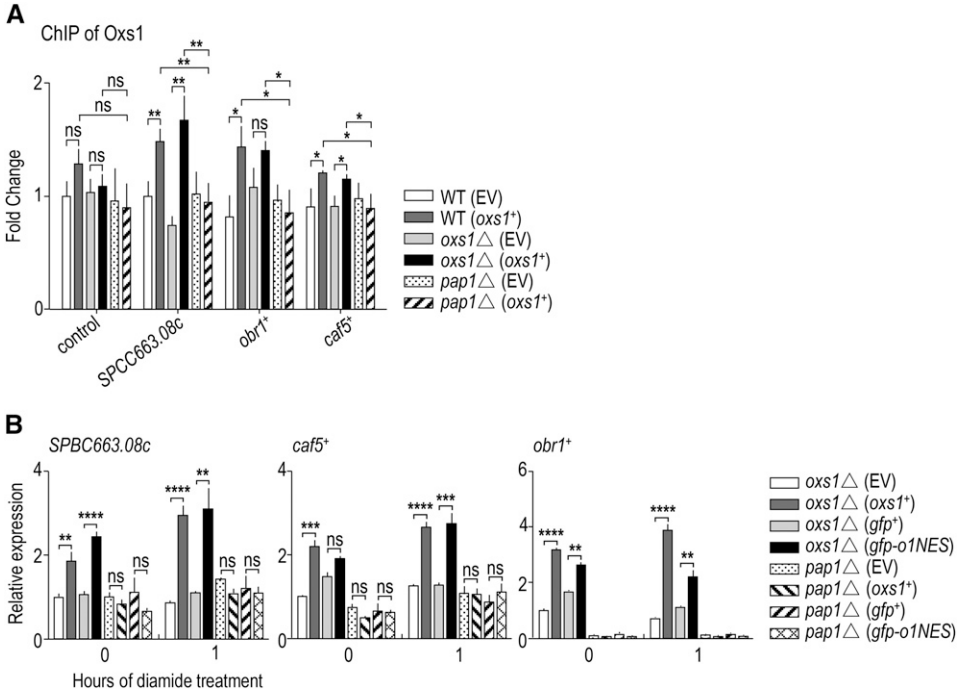

Figure 7 Oxs1 is a coactivator of *SPCC663.08c*, *caf5*$^+$, and *obr1*$^+$ but requires Pap1. (A) Oxs1 physically associates with *SPCC663.08c*, *caf5*$^+$, or *obr1*$^+$ promoter in the absence of stress in WT or *oxs1*Δ strains that overexpress *oxs1*$^+$. Anti-Oxs1 polyclonal antiserum used to immunoprecipitate Oxs1. Fold enrichment normalized against negative control intergenic region of WT cells harboring empty vector (EV) set as 1. (B) mRNA analysis of *SPCC663.08c*, *caf5*$^+$, and *obr1*$^+$ in *oxs1*Δ or *pap1*Δ cells overexpressing indicated genes before or after 1 hr of diamide treatment. mRNA level of each gene normalized to *act1*$^+$ mRNA of the *oxs1*Δ strain with EV. Mean + SEM from three or more independent experiments. Significant differences from unpaired Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns, not significant).

conjugate competes as a Crm1 substrate to retain Pap1 in the nucleus, and nuclear Pap1 activates at least three drug-resistance genes to enhance diamide tolerance even in the absence of Oxs1. However, despite previous reports of enhanced Cd tolerance by a Pap1-producing plasmid, Cd tolerance is unaffected by the overproduction of an o1NES conjugate. This difference may be due to a Pap1 dosage effect, as Pap1 produced by the single genome copy cannot be comparable to the production from a multicopy plasmid. Second, overproduction of Oxs1 primes higher diamide tolerance through activation of at least the same three drug-resistance genes by direct binding to their promoters, although that binding requires Pap1. Whether Oxs1 plays a minor role in retaining Pap1 in the nucleus remains a possibility. Third, Oxs1 cannot simply be a nuclear protein to activate target genes, or a mere NES carrier with potential nuclear retention of Pap1. Shuttling out of the nucleus appears to be critical for function, as without its NES, it is unable to enhance diamide tolerance. In contrast to Pap1, where nuclear export is also essential for Pap1 oxidation by H_2O_2 to a more active form (Calvo *et al.* 2012), the underlying reason why Oxs1 needs to shuttle out of the nucleus remains to be resolved.

Potential application of the NES peptide in disease therapy

Dysregulation of nucleocytoplasmic shuttling of disease-related regulators has been shown in many types of cancer (Fabbro 2003; Kau *et al.* 2004). Hyperactivity of Crm1, the conserved exportin for NES-bearing proteins and RNAs from the nucleus, is a prognostic indicator of different malignancies such as breast cancer (Yue *et al.* 2018), ovarian cancer (Noske *et al.* 2008), osteosarcoma (Yao *et al.* 2009), pancreatic cancer (Huang *et al.* 2009), and cervical cancer (van der Watt *et al.* 2009). For example, overproduction of Crm1 in cervical cancer cells induces excessive nuclear export of p53 (van der Watt *et al.* 2009), a tumor suppressor that functions in the nucleus to inhibit growth of abnormal or stressed cells (Sionov and Haupt 1999). Treatment with drugs that inhibits Crm1 function enhances p53 nuclear accumulation and reduces cancer cell proliferation (Hietanen *et al.* 2000). Crm1-mediated nuclear export is also utilized by various viruses (*e.g.*, HIV-1 and influenza) to export their newly synthesized products (Nagai-Fukataki *et al.* 2011; Pickens and Tripp 2018). This means inhibiting Crm1-mediated nuclear export holds promise for antiviral therapy as well.

Leptomycin B (LMB) is a prototypical inhibitor of Crm1 (Mutka *et al.* 2009), but with high cytotoxicity and limited efficacy (Newlands *et al.* 1996). The failure of phase 1 clinical trial of LMB led to the development of natural and synthetic LMB alternatives. In recent years, a new class of Crm1-selective inhibitors of nuclear export (SINEs) with similar mechanism of action to LMB has been developed. SINEs include KPT-185, KPT-249, KPT-251, KPT-276, KPT-330, and KPT-335 (Parikh *et al.* 2014), among which KPT-330 (selinexor) is a first-in-class oral SINE (Gerecitano 2014; Abdul Razak *et al.* 2016) undergoing phase 1 and phase 2 clinical trials in many human malignancies (Clinicaltrials.gov identifier: NCT03095612, NCT01986348, NCT02227251).

This study suggests an additional possibility to suppress Crm1-mediated export of nuclear molecules. Viral vectors delivered to target cells might be able to overexpress an NES conjugate to serve as a competitive Crm1 substrate. The use of natural peptides as anticancer or antiviral agents may have fewer side effects and better cell penetration, overcoming limitations such as low oral bioavailability, easy

degradation, and off-target effects (Vlieghe *et al.* 2010). Whether this approach will find its way to medical applications remains to be tested.

Acknowledgments

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