Molecular Microbiology (2018) 110(5), 831–846

doi:10.1111/mmi.14128
First published online 28 October 2018

A role for *Saccharomyces cerevisiae* Centrin (Cdc31) in mitochondrial function and biogenesis

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Summary

Centrins belong to a family of proteins containing calcium-binding EF-hand motifs that perform well-established roles in centrosome and spindle pole body (SPB) duplication. Yeast encodes a single Centrin protein (Cdc31) that binds components in the SPB. However, further studies revealed a role for Centrins in mRNA export, and interactions with contractile filaments and photoreceptors. In addition, human Centrin-2 can bind the DNA-lesion recognition factor XPC, and improve the efficiency of nucleotide excision repair. Similarly, we reported that yeast Cdc31 binds Rad4, a functional counterpart of the XPC DNA repair protein. We also found that Cdc31 is involved in the ubiquitin/proteasome system, and mutations interfere with intracellular protein turnover. In this report, we describe new findings that indicate a role for Cdc31 in the energy metabolism pathway. Cdc31 and cdc31 mutant proteins showed distinct interactions with proteins in energy metabolism, and mutants showed sensitivity to oxidative stress and poor growth on non-fermentable carbon. Significant alteration in mitochondrial morphology was also detected. Although it is unclear how Cdc31 contributes to so many unrelated mechanisms, we propose that by controlling SPB duplication Centrin proteins might link the cellular responses to DNA damage, oxidative load and proteotoxic stresses to growth control.

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Introduction

The single Centrin gene (*CDC31*) in the yeast *Saccharomyces cerevisiae* encodes a calcium-binding protein with strong similarity to calmodulin (Baum *et al.*, 1986). Cdc31 bears strong similarity to human Centrins (Sullivan *et al.*, 1998). Four Centrin genes have been described in human (Errabolu *et al.*, 1994; Friedberg, 2006; Hsu *et al.*, 2016), of which three (CETN1, CETN2 and CETN3) have been characterized. CETN1 and CETN2 are nearly identical, and may have arisen through gene duplication (Hart *et al.*, 1999). CETN3 is more divergent and shares stronger sequence similarity with yeast Cdc31 (Middendorp *et al.*, 1997). Centrin proteins show unique tissue-specific expression in human and mouse (Hart *et al.*, 1999).

Centrins and Calmodulin contain two pairs of EF-hand motifs that can bind calcium and magnesium (Baum *et al.*, 1986; Wiech *et al.*, 1996; Veeraraghavan *et al.*, 2002; Hu *et al.*, 2004). The pairs of EF-hands are separated by an alpha-helical linker whose sequence is not conserved. The linker keeps the two pairs of EF-hands separated, and permits mobility of the N- and C-terminal domains (Veeraraghavan *et al.*, 2002). Unlike Calmodulin, only one pair of the EF-hands in Centrin shows significant binding to calcium (Friedberg, 2006; Gifford *et al.*, 2007), suggesting that the other pair of EF-hands may perform a regulatory role (Matei *et al.*, 2003; Hu *et al.*, 2004; Sheehan *et al.*, 2006; Yang *et al.*, 2006; Miron *et al.*, 2011).

Centrins interact with the microtubule organizing center/MTOC (such as centrosomes and basal bodies) (Salisbury *et al.*, 1986; Salisbury *et al.*, 1988; Sanders and Salisbury, 1989; Salisbury *et al.*, 2002; Sun *et al.*, 2002; Martinez-Sanz *et al.*, 2006). Similarly, in yeast Cdc31 is present in the SPB, whose duplication is required for entry into the cell cycle (Baum *et al.*, 1986; Biggins and Rose, 1994; Vallen *et al.*, 1994; Sullivan *et al.*, 1998; Khalfan *et al.*, 2000; Ivanovska and Rose, 2001). Cdc31 interactions with Kar1 (Biggins and Rose, 1994), Sfi1 (Kilmartin, 2003) and Mps3 (Jaspersen *et al.*, 2002) are important for SPB duplication. Centrin is also detected in photoreceptors (Wolfrum, 1995), and can regulate cytoskeletal dynamics by binding contractile fibers (Salisbury *et al.*, 1984; Salisbury *et al.*, 1986).

Yeast Sfi1 functions as a scaffold within the half-bridge structure of the SPB to promote assembly of other components, including Centrin (Kilmartin, 2003). The Mps3 protein is also present in the half-bridge and appears to couple this structure to other components in the core SPB that is anchored to the nuclear envelope. These interactions are not altered by Ca^{2+}, although it is possible that other functions of Cdc31 may be regulated by calcium (Cox *et al.*, 2005). Intriguingly, Cdc31 binds many additional proteins that do not contain the repeated motif that is present in Sfi1, raising important questions about the mechanism of its interaction with structurally dissimilar proteins. Calmodulin also binds many structurally unrelated proteins (Tidow and Nissen, 2013).

The single Centrin-encoding gene in yeast (*CDC31*) is essential for viability, and many temperature-sensitive (*ts*) mutants fail to initiate entry into the mitotic cycle (Ivanovska and Rose, 2001). However, the biochemical defect in SPB duplication is not well understood. Centrin is also detected in photoreceptors (Wolfrum, 1995), and can regulate cytoskeletal dynamics by binding contractile fibers (Salisbury *et al.*, 1984; Salisbury *et al.*, 1986). Cdc31 is also involved in regulating DNA repair (Araki *et al.*, 2001; Popescu *et al.*, 2003; Nishi *et al.*, 2005; Krasikova *et al.*, 2012), as it can improve DNA excision in a reconstituted system (Nishi *et al.*, 2005). We described an interaction with the proteasome, which predicts a previously unknown role for Cdc31 in protein degradation (Chen and Madura, 2008). Centrin mutants showed defects in protein turnover and caused accumulation of polyubiquitylated substrates (Chen and Madura, 2008). Cdc31 also functions in mRNA and protein export (Fischer *et al.*, 2004; Resendes *et al.*, 2008). Certain nuclear proteins are degraded only after they are exported from the nucleus (Chen and Madura, 2014), and it is intriguing that Cdc31 interacts with proteasomes in the nuclear pore (Rout *et al.*, 2000; Fischer *et al.*, 2004; Niepel *et al.*, 2013). These findings raise the possibility that *Cdc31* might contribute directly to the transport and degradation of nuclear substrates. Remarkably, catalytically active proteasomes were also co-localized with purified human centrosomes (Wigley *et al.*, 1999; Fabunmi *et al.*, 2000), predicting a proteolytic function in the MTOC. A requirement for Centrin in photoreceptors (Wolfrum, 1995), and in regulating cytoskeletal dynamics has been known for a long time (Salisbury *et al.*, 1984; Salisbury *et al.*, 1986). A major fraction of Centrin is not present in the SPB/MTOC, consistent with its involvement in diverse mechanisms (Spang *et al.*, 1993; Biggins and Rose, 1994).

We investigated Cdc31 interaction with cellular proteins by mass spectrometry. In agreement with previous finding, we detected Cdc31 interaction with Rad4, as well as other DNA repair proteins. Cdc31 also co-purified numerous factors involved in energy metabolism. To validate these results, we tested the interactions biochemically and also examined *cdc31* mutants (Table 1) harboring defects in SPB duplication and cell morphology (Ivanovska and Rose, 2001). Specific *cdc31* mutant proteins (cdc31-1; cdc31-2) showed distinct interactions with proteins involved in energy metabolism. *cdc31* mutants also showed unique sensitivities to oxidative stress, and growth in the medium containing non-fermentable carbon. Allele-specific effects of *cdc31* mutants were also described previously (Ivanovska and Rose, 2001).

The stability of a number of regulatory proteins that promote mitochondrial fusion and fission is controlled by the proteasome (Fritz *et al.*, 2003; Escobar-Henriques *et al.*, 2006; Karbowski *et al.*, 2007; Cohen *et al.*, 2008; Deng *et al.*, 2008). These morphological transitions are affected by stress, which could promote the elimination of damaged mitochondria. Proteasome and *cdc31* mutants share common defects in mitochondrial morphology, which could reflect their interaction. Growth arrest can occur during stress conditions, and Cdc31's role in SPB duplication could define its requirement in DNA damage, oxidation stress, carbon deprivation and proteotoxic stress.

Table 1. Yeast strains harboring mutations in Centrin and proteasome subunits

Strains	Genotype	Reference	Figures
LCY1050	*MAT***a** *ura3 his3 leu2 trp1 ade2*	Lab stock	4C
LCY1015	*MATα cdc31-1 ura3, his3, leu2 trp1*	Ivanovska and Rose (2001)	4ABCD
LCY1016	*MATα cdc31-2 ura3 leu2 trp1 ade2 cyh2-r*	Ivanovska and Rose (2001)	4ABCD
LCY1262	*MAT***a** *cdc31-5 ura3, ade2 met2*	Ivanovska and Rose (2001)	4BCD
KMY576	*MAT***a** *ura3 his3 trp1 leu2 lys2*	Lab stock	1AB, 2B, 3ABCDEFG, 4ABC, 6A
KMY851	*MATα pre1-1 pre2-2, ura3 his3 leu2*	Lab stock	6ABC
KMY977	*MAT***a** *ura3 leu2 his3 lys2 trp1 ade2*	Lab stock	6BC
KMY978	*MAT***a** *cim5-1 ura3 leu2 his3 ade2*	Lab stock	6BC
KMY1171	*MAT***a** *cim3-1 ura3 leu2 his3 ade2*	Lab stock	6BC
LCY1266	*MAT***a** rho-*ura3 his3 trp1 leu2 lys2*	Lab stock	4B

Results

Cdc31 binds proteins involved in energy metabolism

Wild-type Cdc31, and two mutant proteins (cdc31-1; cdc31-2), were fused to an epitope tag (FLAG) and expressed in wild-type yeast cells (Table 2). Protein extracts were prepared from exponential phase cells that were either untreated (-), or exposed to ATP-depleting (+) conditions (Fig. 1A). Protein extracts were incubated with FLAG-agarose and the bound proteins were separated by SDS/PAGE and detected by staining with Coomassie blue. Protein bands were excised and identified by mass spectrometry. In agreement with previous studies (Araki *et al*., 2001), we confirmed Cdc31 interaction with DNA repair protein Rad4, and also detected binding to repair factors Rad50 and Xrs2. Unexpectedly, we also detected an interaction with a significant number of proteins in energy metabolism. We identified both cytosolic (pyruvate kinase/Cdc19 and S-adenosylmethionine synthetase/Sam1), and mitochondrial factors (ferrochelatase/Hem15, prohibitin/Phb1 and ubiquinol-cytochrome c1 reductase catalytic subunit/Cyt1, cytochrome c oxidase subunits/Cox1, Cox2 and Cox3) in association with Cdc31. Additional mitochondrial factors included Fmp26, Faa1, Tdh3, Pet127, Mdm35 and OM45 (Table 2).

The C-terminal domain of yeast and human Centrins has been previously shown to confer target specificity (Geier *et al*., 1996; Hu and Chazin, 2003; Matei *et al*., 2003). In agreement, we reported that the C-terminal domain in Cdc31 binds Rad4, polyubiquitin and proteasomes (Chen and Madura, 2008). We therefore examined the interactions between the amino- and carboxy-domains of Cdc31 and factors functioning in energy metabolism. Only the carboxy-terminal domain (Fig. 1B; lane 4) was required for these interactions. The amino-terminal domain showed no binding (lane 5), similar to the control (Mock, lane 2). cdc31-2 protein showed weaker binding

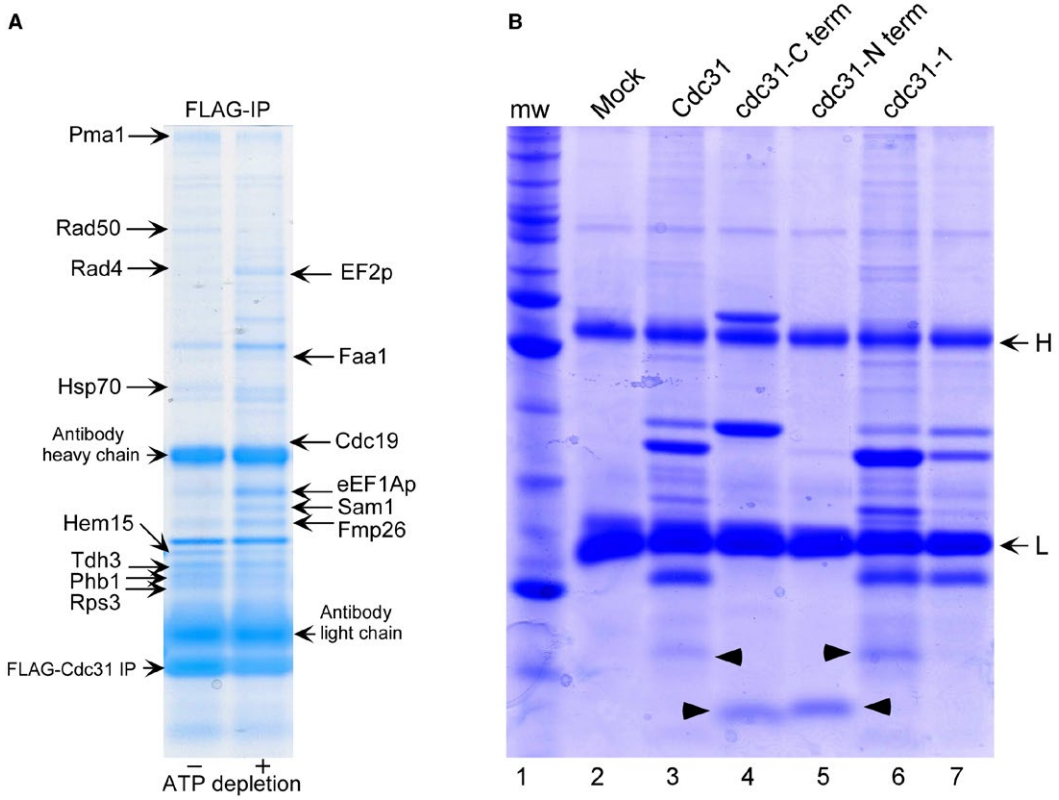

Fig. 1. Yeast Centrin binds proteins involved in energy metabolism.
A. FLAG-Cdc31 was isolated from untreated yeast cells (–), and from cultures treated with 2-deoxyglucose + 2-4, dinitrophenol to deplete ATP levels (+). FLAG-Cdc31 was purified and the bound proteins examined by SDS-PAGE. Co-purified proteins (Coomassie staining) were excised and identified by mass spectrometry. The identities of a subset of co-purifying proteins are labeled on the sides of the panel.
B. To determine if the amino- or carboxy-terminal EF-hands contributed to protein::protein interactions we expressed the N-terminal or the C-terminal domains in wild-type yeast. Arrowheads show the positions of the relevant FLAG-tagged Cdc31 and mutant proteins in the Coomassie stained gel. cdc31-1 mutant (lane 6) showed a similar binding pattern as Cdc31 (lane 3), whereas cdc31-2 (land 7) showed weaker binding. The C-terminal domain showed strong binding to several proteins (lane 4). In agreement with previous studies, the N-terminal domain failed to bind any protein (lane 5), and was similar to the mock control (lane 2). The positions of heavy (H) and light (L) antibody chains are indicated in the right margin. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2. DNA plasmids expressing Cdc31 derivatives, Cyt1, and mito-GFP

Plasmid log #	Description	Reference
TEP36	P_{CUP1}-FLAG-Cdc31::LEU2::2u	Chen and Madura (2008)
TEP37	P_{CUP1}-FLAG-Cdc31::TRP1::2u	Chen and Madura (2008)
LEP291	P_{CUP1}-FLAG-cdc31-1::TRP1::2u	Chen and Madura (2008)
LEP292	P_{CUP1}-FLAG-cdc31-2::TRP1::2u	Chen and Madura (2008)
LEP293	P_{CUP1}-FLAG-cdc31-1::LEU2::2u	Chen and Madura (2008)
LEP294	P_{CUP1}-FLAG-cdc31-2::LEU2::2u	Chen and Madura (2008)
LEP336	P_{CUP1}-FLAG-cdc31-5::TRP1::2u	This study
LEP438	P_{CUP1}-Cyt1-HAHA::LEU2::2u	This study
LEP355	P_{CUP1}-FLAG-cdc31-C term::LEU2::2u	This study
LEP356	P_{CUP1}-FLAG-cdc31-N term::LEU2::2u	This study
LEP444	P_{CUP1}-FLAG-cdc31$^{P\text{-}T}$::TRP1::2u	This study
LEP528	pYX232-Mt-GFP::TRP1::2u	B. Westermann

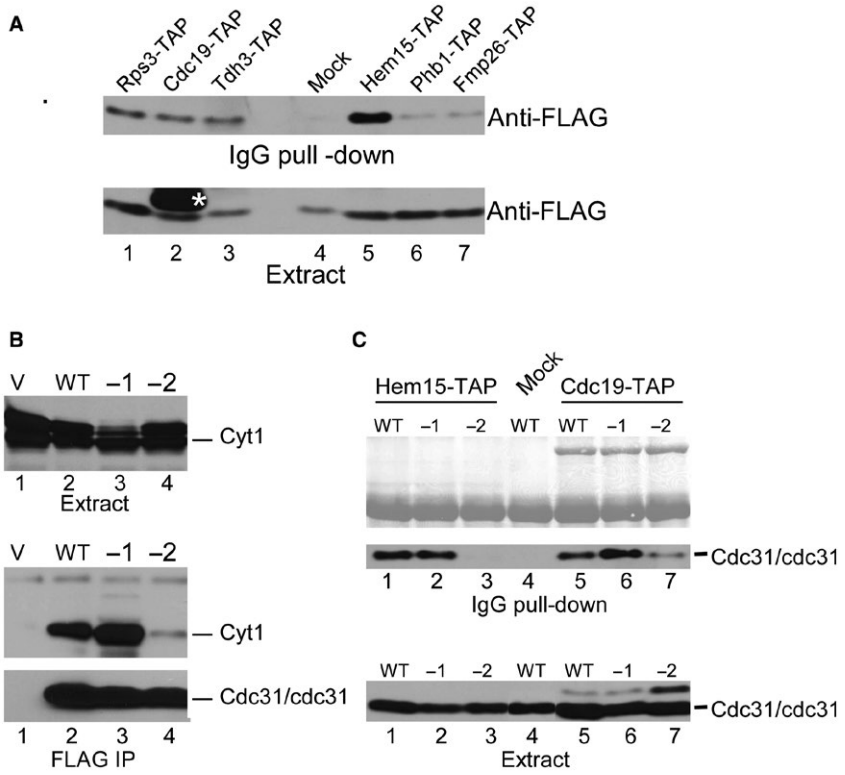

Fig. 2. Specific proteins involved in energy metabolism can be isolated in complex with Cdc31. Based on the mass spectrometry results, we investigated the interaction between epitope-tagged proteins and FLAG-Cdc31.
A. Rps3, Cdc19, Tdh3, Hem15, Phb1 and Fmp26, were among a large number of proteins that could be co-purified with Cdc31. FLAG-Cdc31 was expressed in individual strains expressing TAP-tagged derivatives of these proteins at physiological levels. Protein lysates were incubated with IgG agarose and the bound proteins analyzed by SDS-PAGE and immunoblotting. All the TAP-tagged proteins were detected in complex with FLAG-Cdc31, although their binding efficiency varied. FLAG-Cdc31 was expressed well in all the strains. A strong cross-reacting band in lane 2 (*) is a nonspecific reaction in this particular strain, and is not related to Cdc19.
B. Mass spectrometry also revealed an interaction between the cytochrome oxidase subunit Cyt1 and Cdc31. We reexamined this binding to Cdc31, and compared it with mutant forms of Centrin. cdc31-1 formed a stronger interaction with Cyt1-HA compared to wild-type Cdc31; however cdc31-2 bound very weakly (lane 4).
C. Based on the allele-specific binding proteins described in panel B, we investigated Cdc31 interaction with Hem15 and Cdc19 (see panel A). Protein lysates were incubated with IgG-agarose to purify Hem15-TAP and Cdc19-TAP. Their interaction with Cdc31 (WT), cdc31-1 (-1) and cdc31-2 (-2) was investigated. In agreement with the unique allele-specific effects, Hem15 formed a very weak interaction with cdc31-2.

compared to cdc31-1 (compare lanes 6 and 7), consistent with the presence of this mutation in the carboxy-terminal domain (Ivanovska and Rose, 2001).

Interaction between Cdc31 and mitochondrial factors

The proteins that were co-purified with Cdc31 are abundant cellular factors raising concern that the interactions might be nonspecific. To validate these interactions, we transformed FLAG-Cdc31 into yeast cells that expressed TAP-tagged proteins at physiological levels (Fig. 2). FLAG-Cdc31 interaction with Rps3-TAP (ribosomal subunit), Cdc19-TAP (pyruvate kinase), Tdh3-TAP (glyceraldehyde-3-phosphade dehydrogenase), Hem15-TAP (ferrochelatase), Phb1-TAP (prohibitin subunit) and Fmp26-TAP (mitochondrial structural component) was tested (Table 2). Protein extracts were incubated with IgG-Sepharose and the bound proteins were isolated, resolved by SDS/PAGE and transferred to nitrocellulose. Following treatment of the filter with anti-FLAG antibodies, we confirmed that FLAG-Cdc31 was co-purified with the TAP-tagged candidates (Fig. 2A), in agreement with the mass spectrometry results. Similar levels of FLAG-Cdc31 were expressed in all strains (Extract).

Mass spectrometry revealed an interaction between Cdc31 and Cyt1 (cytochrome-c1). To test allele-specific effects, we expressed Cyt1-HA in a wild-type strain containing FLAG-Cdc31, FLAG-cdc31-1 or FLAG-cdc31-2 (Fig. 2B). The mutation in *cdc31-1* (A48->T) lies in the amino terminal domain between EF1 and EF2, whereas the mutation in *cdc31-2* (E133->K) lies in the carboxy-terminal domain between EF3 and EF4. Because both mutants are temperature-sensitive for growth at 37°C, we characterized them at a semi-permissive temperature (30°C). Protein lysates were prepared and binding between Cyt1-HA and Cdc31/cdc31 proteins was tested. Equal amount of extract was incubated with anti-FLAG agarose and immunoblotting showed that the Cdc31/cdc31 proteins displayed unique binding properties to Cyt1-HA. Higher levels of Cyt1-HA were isolated with FLAG-cdc31-1 (lane 3), compared to FLAG-Cdc31 (lane 2). In contrast, much lower levels of Cyt1-HA were bound to cdc31-2 (lane 4), although Cdc31 and mutants proteins were expressed at similar levels. This finding is consistent with the findings described in Fig. 1B, where cdc31-2 showed weaker interactions with many cellular proteins.

To confirm these allele-specific effects we examined Cdc31/cdc31 interactions with Hem15 and Cdc19 (Fig. 2C). Hem15-TAP and Cdc19-TAP were efficiently co-purified with both FLAG-Cdc31 and FLAG-cdc31-1 (Fig. 2C, upper panel; lanes 1, 2 and 5, 6), consistent with their strong binding to Cyt1 (Fig. 2B). Compared to the wild-type Cdc31 protein, FLAG-cdc31-1 formed a stronger interaction with Cdc19-TAP (lane 6), similar to its binding to Cyt1-HA (panel B, lane 3). Although Cdc31, cdc31-1 and cdc31-2 were expressed at similar levels much lower levels of FLAG-cdc31-2 were co-purified with either Hem15 (lane 3) or Cdc19 (lane 7). Since the cdc31 mutant proteins differ from Cdc31 by only a single amino acid substitution we think the binding defect of cdc31-2 is authentic. Moreover, the robust expression of the cdc31 mutant proteins (lower panel: Extract) indicates that protein misfolding, or instability is not likely to cause the weak interactions shown by cdc31-2. Taken together, these studies confirmed the mass spectrometry results, and revealed distinct binding properties by cdc31 mutant proteins. These results are consistent with the allele-specific effects described previously (Ivanovska and Rose, 2001).

A Cdc31-interacting protein (Cyt1) is conjugated to ubiquitin

Cdc31 can bind proteins in diverse pathways suggesting that these targets may share a common protein fold, or modification. Similarly, Calmodulin can bind many proteins that lack sequence similarity (Tidow and Nissen, 2013). We previously showed that Cdc31 forms a weak interaction with polyUb proteins (Chen and Madura, 2008), a unique modification that could provide a way to bind unrelated cellular proteins. For instance, both Rad4 and XPC can bind Cdc31, and are known to be conjugated to ubiquitin. Similarly, many regulators of mitochondrial biogenesis, including dynamin-related protein Dnm1, and the mitofusin Fzo1, are conjugated to ubiquitin and degraded by the proteasome (Karbowski *et al.*, 2007; Cohen *et al.*, 2008). These observations led us to determine if the Cdc31-interacting proteins (Fig. 1) were conjugated to ubiquitin (Ub).

We co-expressed Cyt1-HA with FLAG-Cdc31 and FLAG-cdc31 mutants (Ivanovska and Rose, 2001) in a wild-type strain (Fig. 3A). The *cdc31-5* mutant contains a single amino acid substitution (P94->S) in the linker that separates the two pairs of EF-hand motifs. We independently generated an analogous mutation at the same position (cdc31$^{P94->T}$) in an attempt to structurally alter the linker. The expression of FLAG-Cdc31, FLAG-cdc31-5 and FLAG-cdc31$^{P94->T}$ was similar in cells expressing Cyt1-HA (Fig. 3A; Extract). In contrast, FLAG-cdc31-1 levels were moderately lower, and FLAG-cdc31-2 levels were significantly reduced (lanes 3, 4). Equal amount of protein lysates was incubated with HA antibodies to purify Cyt1-HA (Fig. 3B). Higher molecular weight forms of Cyt1, resembling polyubiquitylation, were detected in cells expressing Cdc31, and cdc31-1 (lanes 2 and 3). In contrast, lower levels of these high molecular weight species were observed in cells expressing FLAG-cdc31-2,

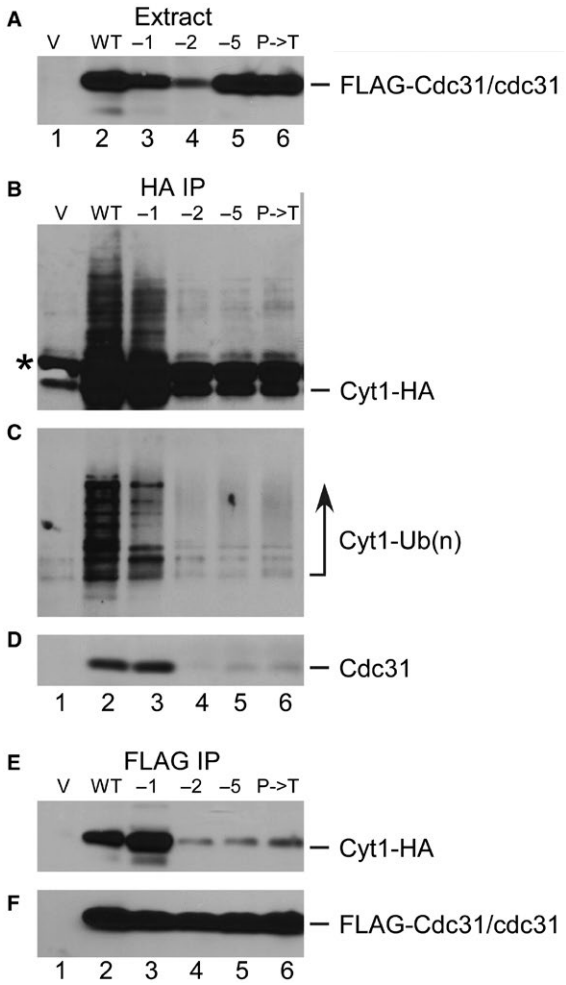

Fig. 3. A target of Cdc31 is conjugated to ubiquitin. We investigated if polyubiquitin chains allowed Cdc31 to bind unrelated proteins.
A. Cyt1-HA was isolated from cells expressing wild-type and Centrin mutants. Protein extracts were incubated with antibody against HA and the purified proteins separated by SDS/PAGE.
B. Immunoblotting showed high molecular weight forms of Cyt1-HA in cells expressing either FLAG-Cdc31 or FLAG-cdc31-1 (lanes 2, 3). High molecular weight forms of Cyt1-HA were weakly detected in cells expressing cdc31-2, cdc31-5 and cdc31$^{P->T}$ mutants (lanes 4–6).
C. the same filter was treated with antibodies against ubiquitin, and high molecular weight species were detected only in lanes 2 (FLAG-Cdc31) and 3 (FLAG-cdc31-1).
D. the filter was incubated with antibody against the FLAG epitope, and co-purification of FLAG-Cdc31 (lane 2) and FLAG-cdc31-1 (lane 3) with Cyt1-HA was confirmed.
E. in a reciprocal study the co-purification of Cyt1-HA with FLAG-tagged Cdc31/cdc31 proteins was investigated. Whereas, both FLAG-Cdc31 and FLAG-cdc31-1 formed a strong interaction with Cyt1-HA, much weaker binding was observed with cdc31-2, cdc31-5 and cdc31$^{P->T}$ mutants (lanes 4-6).
F. The level of FLAG-Cdc31 and FLAG-cdc31 mutant proteins in the immunoprecipitates was determined.

FLAG-cdc31-5 or FLAG-cdc31$^{P94->T}$ (lanes 4–6). Protein extract prepared from a strain expressing only vector did not react to anti-HA antibodies (lane 1), although some spill-over from the adjacent lane is seen (*). To characterize the high molecular weight species we incubated the immunoblot with anti-ubiquitin antibody (Fig. 3C), and a strong reaction was detected in cells expressing FLAG-Cdc31 and FLAG-cdc31-1 (lanes 2 and 3), consistent with polyubiquitylation. Because lower levels of Cyt1-HA were detected in cells expressing FLAG-cdc31-2, FLAG-cdc31-5 and FLAG-cdc31$^{P94->T}$ (Fig. 3B, lanes 4–6), polyubiquitylation was correspondingly reduced (Fig. 3C, lanes 4–6). The immunoblot was reacted with anti-FLAG antibodies and FLAG-Cdc31 and FLAG-cdc31-1 were both co-purified with Cyt1-HA (Fig. 3D; lanes 2, 3). However, much lower levels of FLAG-cdc31-2, FLAG-cdc31-5 and FLAG-cdc31$^{P94->T}$ were detected (lanes 4–6).

In a reciprocal study lysates were incubated with anti-FLAG antibodies, and Cyt1-HA was detected with FLAG-Cdc31 and FLAG-cdc31-1 (Fig. 3E; lanes 2 and 3). Consistent with earlier results (Figs 2B and 3D), Cyt1-HA formed a strong interaction with FLAG-Cdc31 and FLAG-cdc31-1, but weaker binding to cdc31-2, cdc31-5 and cdc31$^{P->T}$ (Fig. 3E; lanes 4-6).

A role for Centrin in mitochondrial biogenesis and function

The interactions between Cdc31 and multiple factors involved in energy metabolism (Fig. 1) led us to determine if *cdc31* mutants harbored defects in mitochondrial function. Mitochondria undergo extensive reorganization; oscillating between a fusion stage representing large, interconnected mitochondrial networks and fission, where fragmentation is thought to facilitate removal of defective mitochondrial structures. The coordination of these transitions involves degradation of regulatory factors by the proteasome. Fzo1, Fis1 and Dnm1, control these transitions between fusion and fission, and counterparts of these proteins are evolutionarily conserved. Mito-GFP is a well-studied reporter construct containing a mitochondrial targeting signal fused to GFP (Westermann and Neupert, 2000). We expressed Mito-GFP in wild-type (*CDC31*) cells and as expected detected a tubular network (Fig. 4A). In contrast, large aggregates, consistent with excessive fusion, were seen in *cdc31-1*, and extensive fragmentation was detected in *cdc31-2*.

We also examined the ability of cdc31 mutants to utilize non-fermentable carbon (Fig. 4B), and their sensitivity to oxidative stress (Fig. 4C), since these are affected by mitochondrial activity. *cdc31-2* and *cdc31-5* showed moderately weaker growth on acetate medium (a poor carbon source), whereas *cdc31-1* grew as well as the wild-type strain. As expected, a respiration deficient (rho-) mutant failed to grow on medium containing acetate. *cdc31-1* also showed strong sensitivity to H_2O_2, as revealed by a

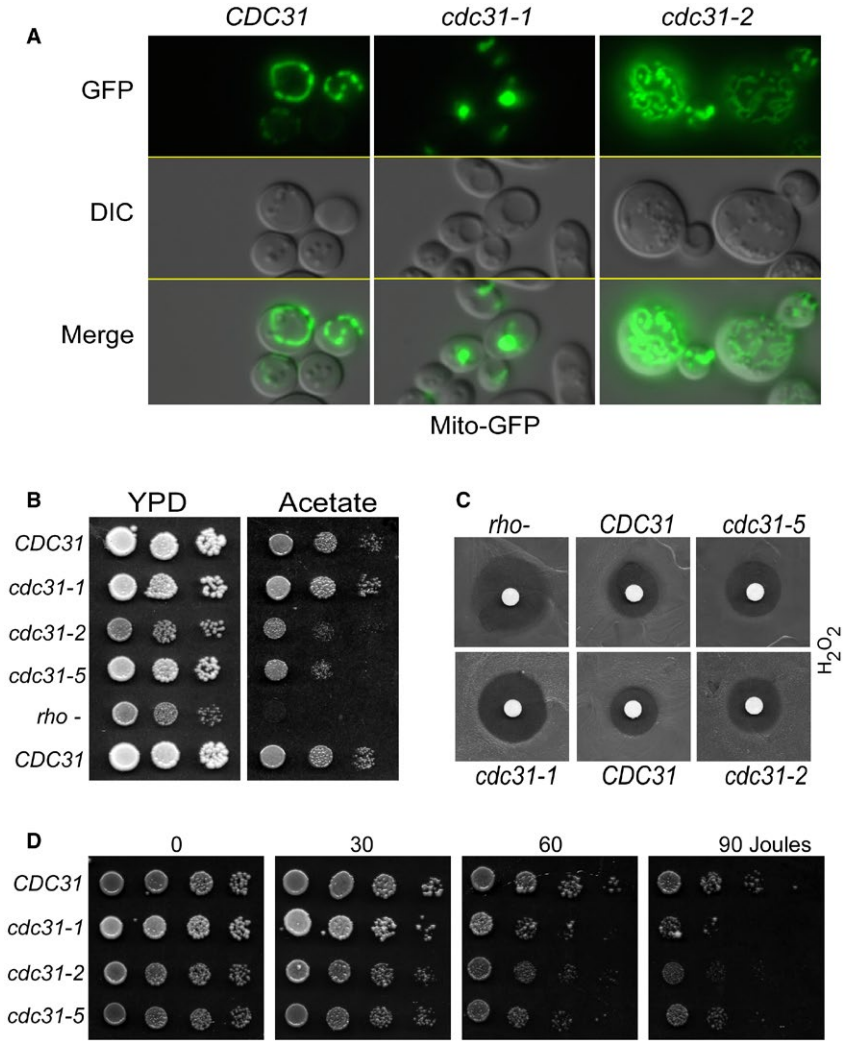

Fig. 4. Centrin mutants display specific defects in mitochondrial morphology and function.
A. we expressed a reporter (Mito-GFP) in wild-type and Centrin mutants. Fluorescence microscopy revealed extensive tubular networks in the wild-type strain, as expected. However, large aggregates were detected in *cdc31-1*, and extensive fragmentation and dispersed localization of mitochondria were observed in *cdc31-2*, suggesting that yeast Cdc31 affect mitochondrial fission/fusion dynamics.
B. to determine if the altered morphology affected mitochondrial function we examined yeast growth on a non-fermentable carbon. Both cdc31-2 and cdc31-5 showed weaker growth on acetate medium. In contrast, the growth of *cdc31-1* was not affected. As expected, a rho- (respiration deficient) yeast strain failed to grow on this medium. The allele-specific growth defect is consistent with the distinct morphological deficiencies in the Centrin mutants (panel **A**).
C. we examined the sensitivity of Centrin mutants to oxidative damage. A uniform density of yeast cultures was spread on agar plates, and 3µlof H_2O_2 (0.03%) was deposited on a filter in the middle of the plate. The zone of growth inhibition is a sensitive measurement of the relative growth inhibition. In contrast to two different wild-type strains (*CDC31*), both *cdc31-1* and rho- showed strong growth inhibition.
D. we previously reported that *cdc31-1* mutant harbored moderate sensitivity to 254 nm UV light. To determine if UV sensitivity was allele-specific we compared *cdc31* mutants. We determined that both *cdc31-1* and *cdc31-2* showed UV sensitivity at high dose of UV (90 Joules). In contrast, *cdc31-5* showed similar survival to the wild-type (*CDC31*) strain. [Colour figure can be viewed at wileyonlinelibrary.com]

large zone of growth inhibition (Fig. 4C), comparable to rho-. In contrast, *cdc31-2* and *cdc31-5* were weakly sensitive. It is presently not known if the altered mitochondrial morphology seen in *cdc31* mutants (Fig. 4A) is related to their different responses to oxidative stress, and growth in acetate medium.

Cdc31 can bind the essential nucleotide excision repair factor Rad4. Whereas, mutations in *RAD4* cause extreme sensitivity to UV light, *cdc31-1* is only weakly sensitive (Chen and Madura, 2008), suggesting that it performs an ancillary role. In agreement with this view Nishi *et al*, reported that Centrin improved XPC-dependent DNA excision, although NER occurred efficiently in its absence. We exposed *CDC31*, *cdc31-1*, *cdc31-2* and *cdc31-5* to 254 nm UV light, and detected slightly reduced survival after exposure to UV treatment (Fig. 4D). Collectively, these findings (panels A–D) may represent the minimal defect because these mutants were grown at a permissive

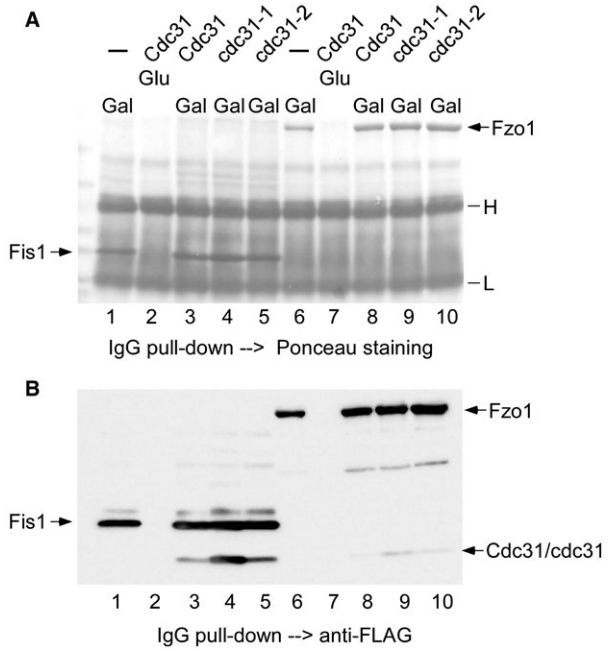

Fig. 5. Cdc31 can bind proteins that regulate mitochondrial fission and fusion. Specific regulatory proteins that control the cycling between fusion and fission are degraded by the proteasome. Because Cdc31 interacts with both mitochondrial factors and the UPS, we investigated its interaction with key promoters of fission and fusion.
A. FLAG-Cdc31 was expressed in yeast strains that expressed either TAP-Fis1 or TAP-Fzo1. Protein lysates were incubated with IgG-agarose and the immobilized proteins analyzed by SDS/PAGE. Both Fis1 (18 kDa) and Fzo1 (98 kDa) were expressed from the galactose-inducible P_{GAL1} promoter. Consequently, no expression was detected by Ponceau S staining in glucose medium (lanes 2 and 7). The positions of antibody heavy (H: 52 kDa) and light (L: 20 kDa) chains are indicated.
B. immunoblotting confirmed the galactose-specific expression of both TAP-Fis1 and TAP-Fzo1. The filter was also incubated with anti-FLAG antibody. An interaction between Fiz1 and Cdc31 was detected (lane 3). Interestingly, this interaction was noticeably stronger with cdc31-1 (lane 4), consistent with the stronger binding between this Centrin mutant and many other proteins. Higher molecular weight forms of TAP-Fis1 were detected in cells expressing Cdc31/cdc31 (lane 3–5), which could represent post-translational conjugation to ubiquitin. Confirmation has not been possible due to the weak signal. Low levels of Cdc31/cdc31 proteins were also detected in association with Fzo1 (lanes 8–10), although the binding was much weaker than with Fis1.

growth temperature (30°C). Stronger defects may occur at the non-permissive temperature; however, growth arrest at 37°C precludes this analysis. Similarly, we previously described the partial stabilization of proteasome substrates in *cdc31* mutants (Chen and Madura, 2008). The variable phenotypes arising from mutations in *cdc31* are fully consistent with the previously described allele-specific effects (Ivanovska and Rose, 2001). However, it is unclear how the biochemical differences of cdc31 mutant proteins contribute to their variable responses to DNA damage, oxidation and carbon depletion.

Regulators of mitochondrial fusion and fission can bind Centrin

The yeast mitofusin, encoded by *FZO1*, promotes mitochondrial fusion and is degraded by the proteasome (Fritz *et al.*, 2003; Karbowski *et al.*, 2007; Cohen *et al.*, 2008). Given the involvement of Centrin in both ubiquitin/proteasome system (UPS) (Chen and Madura, 2008) and mitochondrial function (this report), we investigated if Cdc31 interacted with Fzo1. We also examined Cdc31 interaction with another regulatory factor, Fis1, which promotes mitochondrial fission (Chan, 2006a; 2006b). FLAG-Cdc31 was co-expressed in yeast cells in which Fzo1-TAP or Fis1-TAP was expressed from the chromosome using a regulated P_{GAL1} promoter. The cells were grown in either glucose or galactose medium, and protein extracts were incubated with IgG-agarose (Fig. 5A). As expected both Fzo1-TAP and Fis1-TAP were detected in galactose, but not glucose medium (lanes 2 and 7). The immunoblot was incubated with anti-FLAG antibodies and FLAG-Cdc31, FLAG-cdc31-1 and FLAG-cdc31-2 were co-purified with Fis1 (Fig. 5B; lanes 3–5). FLAG-cdc31-1 formed a stronger interaction with Fis1, consistent with its enhanced binding to other proteins described in Figs 2 and 3. It is presently not known if this stronger binding is related to the formation of large aggregates in *cdc31-1* mutant (Fig. 4A). The interaction between Cdc31 and Fzo1-TAP was much weaker (lanes 8-10).

Proteasome mutants display unique defects in mitochondrial biogenesis and function

Cdc31 can bind the proteasome and polyubiquitylated proteins (Chen and Madura, 2008). In addition, the degradation of a proteasome substrate was inhibited in *cdc31-1*, but not *cdc31-2*; consistent with allele-specific functional differences. Previous studies also showed that an *rpn11* proteasome mutant displayed a defect in mitochondrial morphology (Rinaldi *et al.*, 1998; Joshi *et al.*, 2011), although the significance of this effect was not determined. We expressed Mito-GFP in proteasome mutants *cim5-1* and *cim3-1*, which encode defective AAA-type ATPases in the 19*S* regulatory particle. We also examined *pre1-1 pre2-2*, in which peptidase activity of the 20*S* catalytic particle is significantly reduced. Large aggregates detected in *pre1-1 pre2-2* (Fig. 6A) could be caused by a failure to degrade Fzo1 (Fritz *et al.*, 2003; Escobar-Henriques *et al.*, 2006; Cohen *et al.*, 2008; Deng *et al.*, 2008). In contrast, both *cim5-1* and *cim3-1* showed excessive fission, which can be explained by a failure to degrade Dnm1, which promotes fragmentation. All three mutants were sensitive to H_2O_2 (Fig. 6B). Intriguingly, only *cim3-1* showed a severe growth defect in non-fermentable carbon media (Fig. 6C). Because

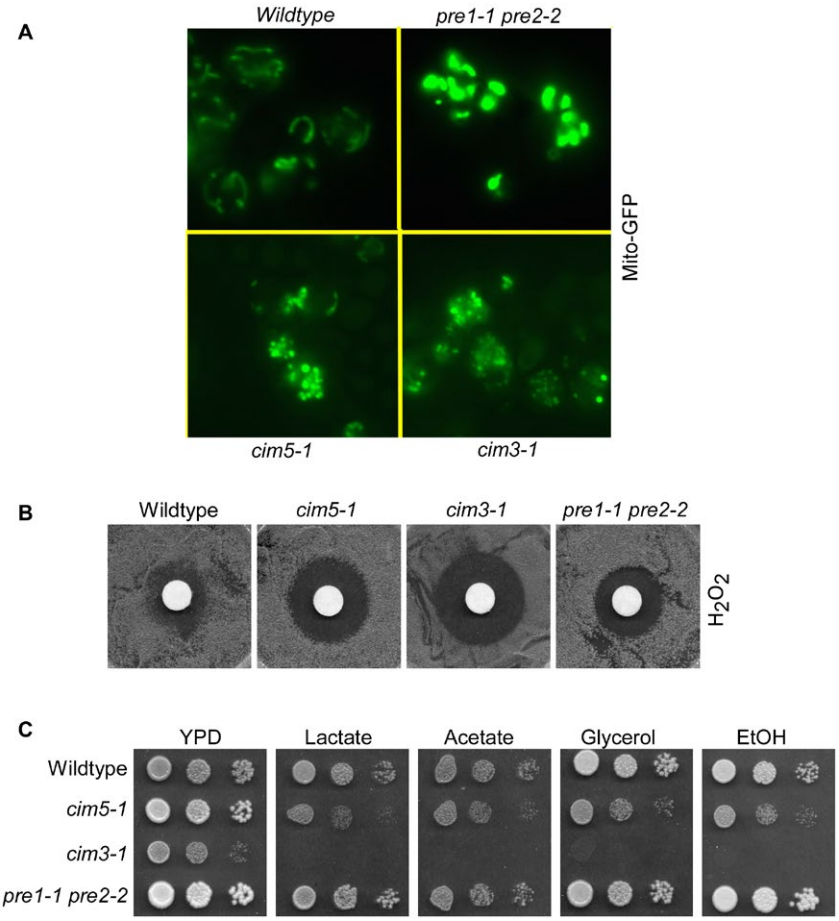

Fig. 6. Proteasome mutants show variable deficiencies in mitochondrial morphology and function. The diverse phenotypic effects displayed by Centrin mutants led us to question if proteasome mutants would also harbor complex defects related to mitochondrial functions.
A. we expressed Mito-GFP in *pre1-1 pre2-2* (mutant in the catalytic 20S core particle), as well as in *cim5-1* and *cim3-1* (mutants in the 19S regulatory particle). Whereas, normal tubular distribution was observed in the wild-type strain, significant large aggregates were detected in *pre1-1 pre2-2*. Mito-GFP was detected in numerous, dispersed aggregates in *cim5-1*, and appeared to reveal extensive fragmentation in *cim3-1*.
B. we examined the sensitivity of proteasome mutants to oxidative damage, as described in Fig. 4B. In contrast to the wild-type strain, all three proteasome mutants were sensitive to H_2O_2, particularly *cim3-1*.
C. Growth on medium containing non-fermentable carbon revealed that *cim3-1* was respiratory deficient, whereas *cim5-1* and *pre1-1 pre2-2* showed efficient growth. Collectively, these studies showed that both proteasome and Centrin mutants show variable phenotypic differences.
[Colour figure can be viewed at wileyonlinelibrary.com]

the proteasome is required for the turnover of regulators in competing morphological pathways (fusion; fission), it is not unexpected that these mutants also display allele-specific effects.

Human Centin 3 (CETN3) interacts with proteins involved in DNA repair, nuclear export and energy metabolism

In an effort to understand the role of human Centrin-3, we examined its interactions in primary breast cancer specimens. CETN3 was expressed in *Escherichia coli* as a fusion to glutathione S-transferase, and purified. Protein extracts prepared from primary tissue, as well as unaffected neighboring tissue, were incubated with immobilized GST-CETN3. The bound proteins were separated by SDS/PAGE and identified by mass spectrometry. We determined that CETN3 formed reproducible binding to cellular proteins in breast cancer specimens (Fig. 7, lanes 4, 6 and 8), prepared from three subjects. Distinct interactions were observed in non-affected neighboring tissue (lanes 3, 5 and 7). In agreement with previous studies we detected human Rad23 (hHR23B), which was detected in a DNA repair complex containing Centrin (Araki *et al.*, 2001). GST-CETN3 also co-purified Ddi1, a protein that is structurally and functionally similar to hHR23B. Yeast and human Centrins are required for nuclear export of mRNA and protein (Fischer *et al.*, 2004; Resendes *et al.*, 2008). Significantly, nuclear export factors (NXF5 and Mdn1) that contribute to mRNA and

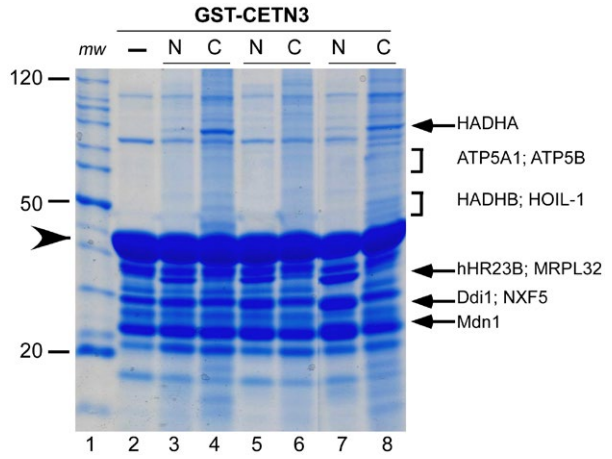

Fig. 7. Human Centrin (CETN3) binds proteins involved in DNA repair, nuclear export and energy metabolism. GST-CETN3 was expressed in *E. coli* and purified on glutathione Sepharose. Protein extracts were prepared from primary tissues derived from breast cancer patients and incubated with the GST-CETN3 beads (labeled 'C'). Protein extracts were also prepared from neighboring unaffected tissues (labeled 'N'). Untreated beads examined in lane 2 shows the position of GST-CETN3 (large arrowhead on left), and numerous degradation products. Samples from three patients were examined in lanes 3–8. Bands and regions of interest were excised and characterized by mass spectrometry. Among the proteins we identified were factors involved in DNA repair (hHR23B), and protein degradation (hHR23B; Ddi1), nuclear mRNA export (NXF5) and nuclear protein export (Mdn1) and mitochondrial energy metabolism (HADHA/B; ATP5A/B). Similarly, yeast Cdc31 binds proteins involved in DNA repair, nuclear export and energy metabolism, and is required for nuclear export. [Colour figure can be viewed at wileyonlinelibrary.com]

protein transport were isolated with GST-CETN3. GST-CETN3 also purified mitochondrial factors, including HADHA, HADHB, MRPL32, ATP5A1 and ATP5B. These interactions by human CETN3 are fully consistent with our characterization of yeast Cdc31, and its previously reported role in nuclear export, DNA repair, and protein turnover. GST-CETN3 formed similar interactions with cellular proteins in all three cancer, and unaffected tissue specimens. A control lane containing only GST-CETN3 (but no extract) shows GST-CETN3 and degradation fragments (which are also detected in lanes 3-8).

Discussion

The yeast *S. cerevisiae* encodes a single Centrin protein (Cdc31) that is required for SPB-duplication (Baum *et al.*, 1986). Cdc31 binds Sfi1, which contains ~ 17 binding sites for Cdc31 (Kilmartin, 2003). It is likely that the level of Cdc31 bound to Sfi1 in the SPB is a determinant factor in triggering entry into the cell cycle. Many *cdc31* mutants show temperature sensitive growth arrest; often the result of a failure to duplicate the SPB (Ivanovska and Rose, 2001). The instability of cdc31-1 and cdc31-2 mutant proteins could partly explain their temperature-sensitive growth defects (Chen and Madura, 2008).

In addition to their requirement in SPB/centrosome duplication, Centrin proteins also function in DNA repair (Araki *et al.*, 2001; Nishi *et al.*, 2005), protein degradation (Chen and Madura, 2008) and nuclear export (Fischer *et al.*, 2004; Resendes *et al.*, 2008). Initial evidence predicting multiple roles for Cdc31 emerged from protein-binding studies (Table 3), which showed that it could bind and stimulate Kic1 kinase activity. Kic1 and Cdc31 promote cell integrity, a role that is independent of its function in SPB duplication (Sullivan *et al.*, 1998). In agreement with its diverse roles a significant amount of Cdc31 is not present in the SPB (Spang *et al.*, 1993; Biggins and Rose, 1994).

A role for Centrin in nuclear export is supported by our finding that human CETN3 can bind components of the export system (NXF5; Mdn1). This finding is significant because Centrin is bound to proteasomes in the nuclear pore complex (NPC) (Jani *et al.*, 2009; Niepel *et al.*, 2013). Since many nuclear substrates are degraded after export from the nucleus (Chen and Madura, 2014), Cdc31 might contribute to their export and degradation within the NPC (Chen and Madura, 2008).

Rad23 is a specific type of *shuttle factor* that can transport polyubiquitylated substrates to the proteasome (Chen and Madura, 2002). Human Rad23 (hHR23) is structurally and functionally related to yeast Rad23, and can be co-purified with human Centrin (Fig. 7, and (Araki *et al.*, 2001)). Well-studied targets of Rad23 and hHR23 include the DNA repair factors Rad4 and XPC, respectively, which are polyubiquitylated and degraded by the proteasome (Ng *et al.*, 2003; Ortolan *et al.*, 2004). We determined that the degradation of Rad4 required export from the nucleus (Chen and Madura, 2014). Because Rad4 is stabilized in *rad23*Δ mutant we believe Rad23 translocates Rad4 to proteasomes in the NPC. Shuttle factors are also detected in the SPB (Biggins *et al.*, 1996), predicting a role for proteolysis in SPB function.

A role for Centrin in nucleotide excision repair was anticipated by its interaction with the DNA repair factor XPC (Araki *et al.*, 2001), and confirmed by its stimulation of DNA incision in a reconstituted system (Nishi *et al.*, 2005; Krasikova *et al.*, 2012). Similarly, we detected Cdc31 in a complex with Rad4 (Chen and Madura, 2008), a functional counterpart of XPC (Guzder *et al.*, 1999), and showed that *cdc31* mutants are moderately sensitivity to UV light (Chen and Madura, 2008). Both XPC and Rad4 are degraded by the proteasome (Ng *et al.*, 2003; Ortolan *et al.*, 2004). Although the significance of Rad4/XPC degradation is unknown, we determined that Rad4 destabilization caused UV sensitivity (Ortolan *et al.*, 2004).

We describe here a new role for Cdc31 in mitochondrial function and morphology. These and other findings

Table 3. Verification of Cdc31 mass spectrometry results

Mass Spec	Function	Biochemical verification	Reference
Yeast			
Rad4	Nucleotide excision repair	+	Araki *et al.* (2001) Chen and Madura (2008)
Rad23	Nucleotide excision repair Protein degradation	+	Araki, *et al.* (2001) Chen and Madura (2008)
Hem15	Ferrochelatase		Kilmartin (2003) This study
Cyt1	Cytochrome c1	+	This study
Phb1	Prohibitin	+	This study
Fmp26	Mito inner membrane	+	This study
Cdc19	Pyruvate kinase	+	This study
Tdh3	Glyceraldehyde-3-phosphate dehydrogenase	+	This study
Cox1	*Cytochrome oxidase (subunit I)*		
Cox2	*Cytochrome oxidase (subunit II)*		
Pma2	*Mito ATPase*		
Mdm35	*Mito inner membrane*		
Pet9	*Mito ADP/ATP carrier*		
Faa1	*Mito fatty acyl-coA synthetase*		
Human			
XPC	Nucleotide excision repair	+	Araki, *et al.* (2001) Chen and Madura, unpublished data
HR23B	*Nucleotide excision repair*	+	
Ddi1	*Protein degradation*		
ATP5A, B	*ATP synthase F1 subunits*		
HADHA, B	*Mito trifunctional protein*		
NXF5	*Nuclear export factor*		
Mdn1	*Nuclear export factor*		

Summary of mass spectrometry results: Yeast proteins identified in association with Cdc31 are indicated, and interactions that were confirmed biochemically are shown (+). Cdc31 co-purification with Rad4 and Rad23 were previously described. Interacting proteins shown in italic have not been confirmed biochemically. Similar mass spectrometry analyses of human proteins that bind CETN3 are shown. Centrin binding to XPC and HR23B were described previously. We detected HR23B and a related protein Ddi1 in association with CETN3. In addition the mitochondrial factors ATP5A/B and HADHA/B, as well as nuclear export factors MXF5 and MDN1 were detected. Similarly, Cdc31 functions in DNA repair, nuclear export and mitochondrial function.

(Rinaldi *et al.*, 1998; Joshi *et al.*, 2011), suggest that both Cdc31 and the proteasome are required for the cellular response to oxidative stress, and mitochondrial function. It is noteworthy that the stability of key regulators of mitochondrial fission and fusion (Fzo1; Dnm1) is controlled by the proteasome (Karbowski *et al.*, 2007; Cohen *et al.*, 2008). These proteins promote opposing mechanisms and therefore it is not surprising that *cdc31* mutants have distinct allele-specific effects (Ivanovska and Rose, 2001).

Calmodulin shares strong structural similarity with Centrin, and also binds many cellular proteins that lack sequence similarity (Tidow and Nissen, 2013). The primary evolutionary divergence among Centrin proteins is present at the amino terminus. However, Rose and co-workers reported that the carboxy terminus binds Kic1 kinase (Sullivan *et al.*, 1998). Remarkably, other interactions also involve the carboxy-terminal domain in Cdc31. We showed that this domain interacted with polyubiquitylated proteins, proteasomes and Rad4 (Chen and Madura, 2008). In agreement, only the carboxy domain binds mitochondrial factors. Similarly, X-ray studies showed that the carboxy-terminus of human CETN2 interacted with XPC (Thompson *et al.*, 2006), and to small peptides representative of various target proteins (Hu and Chazin, 2003; Hu *et al.*, 2004).

A mutation in the carboxy terminus of cdc31-2 ($133^{E\rightarrow K}$) severely reduced binding to various factors (Fig. 2). In contrast, a mutation in the amino terminal domain in cdc31-1 ($52^{A\rightarrow T}$) did not reduce Cdc31 interactions with other proteins. These findings are consistent with the importance of the carboxy-terminal domain for protein::protein interactions. The allele-specific effects of *cdc31* mutants is also noted by mutations in the amino terminal half that impaired cell integrity, but did not affect SPB duplication (Ivanovska and Rose, 2001).

Centrins may form a closed structure that shields the amino-terminal region. However, even when separated the amino-terminal domain (FLAG-cdc31-$^{N\text{-terminus}}$) did not bind target proteins (Fig. 1B). Binding to Ca^{2+} was proposed to

enable the EF2-hand motifs to operate as sensors (Cox *et al*., 2005), and provide a mechanism for substrate targeting and selectivity (Matei *et al*., 2003). Because the release of calcium from mitochondria is closely linked to the stress-induced escape of cytochrome c, it will be important to determine if Cdc31/Cyt1 (cytochrome c1) interaction is induced by stress and affects SPB duplication.

Centrin-binding to Kar1 (Hu *et al*., 2004) predicted a one-site binding model with K_d values of ~ 5×10^{-7} M. Similarly, studies by Craescu and co-workers found the interaction between human Centrin-3 and a Kar1 peptide had a K_d value of ~260 nM (Cox *et al*., 2005). Calcium-dependent interactions between Cdc31 and peptides derived from other proteins including XPC (K_d of ~ 4.5 nM), Sac3 and Sfi1 have been reported (Miron *et al*., 2011), although detailed binding studies with intact physiological targets have not been described.

Human Centrin proteins display unique tissue-specific distribution (Hart *et al*., 1999), and the expression of multiple Centrin isoforms suggests that they perform non-redundant roles. Centrin proteins allow contractile fibers to respond to calcium levels (Salisbury *et al*., 1984; Salisbury *et al*., 1986; Sanders and Salisbury, 1989; Sanders and Salisbury, 1994), and are implicated in cell signaling. We compared the interaction between human CETN3 and proteins in primary human breast cancer specimens to neighboring unaffected tissue derived from three subjects (Figure 7). We detected unique binding properties in cancer tissues, and in unaffected neighboring tissues. Strikingly, mass spectrometry revealed CETN3 interaction with factors involved in DNA repair, nuclear export, protein turnover and mitochondrial function, consistent with our characterization of yeast Cdc31.

Specific *cdc31* mutants show a defect in protein turnover, and display increased sensitivity to drugs that cause protein misfolding (Chen and Madura, 2008). We showed previously that incompletely synthesized polypeptides, generated by drugs or ATP depletion, were rapidly degraded by the proteasome. This mechanism requires the translation elongation factor eEF1A (Chuang *et al*., 2005). To investigate a role for Cdc31 during stress we examined its binding properties in metabolically inhibited yeast cells. ATP levels were depleted by adding 2-deoxyglucose and 2,4-dinitrophenol to the growth medium, and Cdc31 interactions with various cellular proteins was altered. Protein synthesis is acutely sensitive to mitochondrial function, since ATP depletion disrupts translation elongation. Strikingly, ATP depletion revealed an interaction between Cdc31 and eEF1A (Fig. 1A). Cdc31 interactions with other factors, including DNA repair proteins, were also altered following ATP depletion. Collectively, our characterization of primary breast cancer tissues, and ATP-depleted yeast extracts, showed that stress conditions can alter Centrin interactions with its cellular partners.

The co-localization of Cdc31 with proteasomes in the nuclear pore has been described (Rout *et al*., 2000; Fischer *et al*., 2004; Resendes *et al*., 2008; Niepel *et al*., 2013). In addition, centrosomes purified from Hela cells contained catalytically active proteasomes that can degrade polyubiquitylated substrates (Fabunmi *et al*., 2000). Remarkably, substrates accumulated in the centrosome when proteasome function was inhibited. Proteasome inhibition also caused higher levels of proteasomes to co-localize with the centromere, predicting an important role for proteolysis in the MTOC. Taken together these studies suggest that Centrins may integrate the cellular response to diverse stresses, including DNA damage, mitochondrial dysfunction and proteotoxicity. Further study will be required to determine if Centrins regulate SPB duplication following stress.

Experimental procedures

Yeast strains

Yeast Centrin mutants (*cdc31-1, cdc31-2, cdc31-5*) were kindly provided by Dr. M. Rose (Princeton Univ.). A rho- respiratory mutant was generated by treating wild-type strains with ethidium bromide from 30 min. Mutant colonies were identified by their inability to grow on non-fermentable carbon medium (including 2% glycerol; 2% lactate; and 2% ethanol).

Plasmids

Plasmids encoding FLAG-Cdc31, FLAG-cdc31-1, FLAG-cdc31-2 and FLAG-cdc31-5 were expressed from a 2 micron plasmid that contained the copper-inducible *CUP1* promoter. The gene encoding Cyt1 was amplified by polymerase chain reaction, cleaved with *Eco*R1 and *Bgl*II DNA restriction enzymes and ligated into Yeplac181. The detection of Cyt1 was achieved by fusing its carboxy-terminus to two hemagglutinin (HA) epitopes. All constructs were authenticated by DNA sequencing. A plasmid encoding Mito-GFP was provided by Dr. B. Westermann (Univ. Bayreuth).

Preparation of protein extracts and immunological methods

Plasmid-containing yeast strains were grown to late logarithmic stage (A_{600} ~ 2) in synthetic medium (SM) supplemented with 100 μM $CuSO_4$. Cell pellets were suspended in 0.5 ml of lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and a cocktail of protease inhibitors (Roche). Cells were lysed by glass bead disruption using Fastprep. Protein extracts were centrifuged at 12,000 × *g* at 4°C and the concentration

determined using the Bradford reagent. For immunoprecipitation, equal amounts of protein were incubated with affinity beads, including anti-FLAG-M2 Agarose (Sigma), IgG Sepharose 6 Fast Flow (GE Healthcare) and anti-HA agarose (Sigma), and incubated at 4°C for 4 h. The matrices were washed extensively and then boiled in SDS-containing electrophoresis buffer.

Gel electrophoresis and immunoblotting

Total extracts, and proteins released from affinity matrices were separated in SDS-Tris/Tricine polyacrylamide gels, and transferred to nitrocellulose (Hoefer semi-dry apparatus). Efficiency of transfer was confirmed by staining the filter with GelCode (Pierce), and subsequently reacted with specific antibodies. Fluorescence signal was captured using enhanced chemiluminescence using a Kodak imager.

Mass spectrometry and protein identification

TAP-tagged proteins were similarly resolved, and co-purified protein bands were identified by staining (Coomassie blue) and excised. The gel bands were diced into 1 mm^3 pieces, washed with 30% acetonitrile (ACN) in 50 mM ammonium bicarbonate, reduced with DTT and then subject to iodoacetamide alkylation. Trypsin was used for digestion at 37°C overnight. The peptides were extracted in 30 µl 1% trifluoroacetic acid (TFA) and desalted using C_{18}-Ziptip. For MS analysis the peptides were mixed with 7 mg ml^{-1} c-cyano-4-hydroxy-cinnamic acid matrix (in 60% ACN) at a 1:1 ratio, and then spotted onto a matrix-assisted laser desorption/ionization (MALDI) plate. The peptides were analyzed on a 4700 MALDI TOF/TOF analyzer (Applied Biosystem, Framingham, MA). Mass spectra (m/z 880-3,200) were acquired in positive ion reflector mode. Fifteen of the most intense ions were sequenced (MS/MS) in 1 kV mode. Protein identification was performed by searching the combined MS and MS/MS spectra against the yeast NCBI database using a local MASCOT search engine (V. 1.9) on a GPS (V. 3.5, ABI) server. The following search parameters were used: mass accuracy, 50 ppm in MS and 0.3 Da in MS/MS. Iodoacetamide alkylation of cysteine and oxidation of methionine were set as variable modifications. Proteins containing at least two peptides with Confidence Interval (C.I.) values no less than 95% were considered.

ATP depletion

Yeast cells expressing FLAG-Cdc31 was grown to late exponential phase and split into two flasks. We added 0.2 mM 2,4-dinitrophenol (2DNP) and 20 mM 2-deoxyglucose (2DG) to one flask, and incubated for 30 min to deplete intracellular ATP.

H_2O_2 sensitivity

We prepared YP-dextrose (YPD) plates by pipetting an equal volume of 1X YPD + 1.5% agar to ensure that each plate was of equal thickness. An equal number of cells were plated. Sterile 0.5 cm diameter Whatman filters were placed in the middle of the plate, and 3 µl of 0.03% H_2O_2 was spotted. The plates were incubated at 30°C and photographed after 24 h.

Microscopy

A plasmid encoding Mito-GFP was transformed into *CDC31*, *cdc31-1* and *cdc31-2* strains. The cells were examined using a Zeiss AxioImager microscope (filter set #38). The exposure settings for wild-type and mutants strains were identical (with exposure time limited to 75 ms).

Cancer specimens

Patient-matched breast cancer and neighboring unaffected tissue specimens were purchased from the Cancer Institute of New Jersey Tissue Repository.

Acknowledgements

These studies were supported by grants awarded to KM from the National Institutes of Health (GM104968) and National Cancer Institute (CA083875). We thank Benedikt Westermann, Andrew Vershon, Mark Rose and John Kilmartin for generously providing yeast strains and plasmids.

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