Sro7 and Sro77, the yeast homologues of the *Drosophila* lethal giant larvae (Lgl), regulate cell proliferation via the Rho1–Tor1 pathway

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Saccharomyces cerevisiae Sro7 and Sro77 are homologues of the *Drosophila* tumour suppressor lethal giant larvae (Lgl), which regulates cell polarity in *Drosophila* epithelial cells. Here, we showed that double mutation of *SRO7/SRO77* was defective in colony growth. The colony of the *sro7Δ/sro77Δ* double deletion was much smaller than the WT and appeared to be round with a smooth surface, compared with the WT. Analysis using transmission electron microscopy revealed multiple defects of the colony cells, including multiple budding, multiple nuclei, cell lysis and dead cells, suggesting that the double deletion caused defects in cell polarity and cell wall integrity (CWI). Overexpression of *RHO1*, one of the central regulators of cell polarity and CWI, fully recovered the *sro7Δ/sro77Δ* phenotype. We further demonstrated that *sro7Δ/sro77Δ* caused a decrease of the GTP-bound, active Rho1, which in turn caused an upregulation of *TOR1*. Deletion of *TOR1* in *sro7Δ/sro77Δ* (*sro7Δ/sro77Δ/tor1Δ*) recovered the cell growth and colony morphology, similar to WT. Our results suggested that the tumour suppressor homologue *SRO7/SRO77* regulates cell proliferation and yeast colony development via the Rho1–Tor1 pathway.

INTRODUCTION

*Saccharomyces cerevisiae* Sro7 and Sro77 are functional homologues, and are involved in docking and fusion of post-Golgi vesicles with plasma membrane. Double deletion of *SRO7/SRO77* induces a severe defect in exocytosis as evidenced by the accumulation of post-Golgi vesicles (Lehman et al., 1999). Sro7 and Sro77 are homologues of the *Drosophila* tumour suppressor lethal giant larvae (Lgl), which regulates the cell polarity in epithelial cells (Bilder, 2004; Gibson & Perrimon, 2003; Harihar & Bilder, 2006). Loss of Lgl results in defects of cell polarization and carcinoma (Agrawal et al., 1995).

In *S. cerevisiae*, polarized cell growth is regulated primarily by Rho-like GTPases, including Cdc42 and the Rho proteins (Rho1–Rho5) (Madden & Snyder, 1998; Bi & Park, 2012). Defects of cell polarization can be categorized into four classes: secretory block, secretory mistargeting, endocytic block and cell wall synthesis block (Pruyne & Bretscher, 2000). Cdc42 functions in the organization of actin filaments and septin. It also helps vesicle delivery to the site of bud growth and helps bud site selection (Adamo et al., 2001; Pruyne & Bretscher, 2000). Cdc42 mutation results in budding polarization loss and isotropic growth at multiple random sites on the cell membrane. Rho1, an essential gene for polarized growth (Levin, 2011), plays an important role in the reorganization of the actin cytoskeleton and cell wall synthesis during cytokinesis. Rho1 controls the localization of the exocyst and actin filament assembly towards the budding sites with Cdc42.

The fact that Sro7/Sro77 are homologues of the *Drosophila* Lgl suggests that Sro7/Sro77 might also be involved in regulation of cell polarity in yeast. Transmission electron microscopy (TEM) was used to further examine *sro7Δ/sro77Δ* colony cells, which have a more profound phenotype than those of liquid culture. We showed that in a 3 day colony, *sro7Δ/sro77Δ* cells displayed multiple defects, including cell lysis, multiple budding, multiple nuclei and cell death. Moreover, we identified that Sro7/Sro77 are upstream regulators of the small GTPase Rho1, which in turn regulates the *TOR1* complex that controls asymmetrical cell growth. Our study sheds light on a possible role of Sro7/Sro77 as a cancer repressor and its mechanism of repressing cell growth.

METHODS

Yeast strains and culture conditions. Yeast cells were grown on YPD plates (1% yeast extract, 2% peptone, 2% glucose and 1.7%...
ag) at 30 °C or as otherwise noted in the text. The WT S. cerevisiae strain BY4742 (Mata his3Δ leu2Δ met15A ura3A) was purchased from Thermo Scientific. All other strains were derived from BY4742. Deletion of SRO7 was introduced by the standard PCR-mediated gene replacement method (Wach et al., 1994). For SRO7/SRO7 double deletion, SRO7 was deleted from the sro77A single-deleted strain using URA3 as a selection marker. The triple deletion of sro77A/sro77A/tor1Δ was constructed using the sro77A/sro77A double mutant. RHO1 and CDC42 overexpression plasmids were purchased from Thermo Scientific. The genes were cloned into vector p5472 carrying a selectable marker and a yeast centromere (Ho et al., 2009). The expression of each gene was driven by its endogenous promoter and its normal 3′ untranslated region. The C-terminal Rho1-haemagglutinin (HA) tag was generated as previously described (Janke et al., 2004).

TEM. Cells were cultured at 30 °C on YPD plates. Colonies were covered with a thin layer of 1 % low-melting-point agarose at 37 °C. The colony was then cut from the plate into small cubes, and fixed with 2 % glutaraldehyde and 2.5 % paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature overnight. The cubes were washed in the following order: 0.1 M cacodylate buffer (five times; 10 min each), deionized water (three times; 15 min each), 2 % sodium metaperiodate (one time; 50 min) and deionized water (two times; 15 min each). The cubes were then fixed in freshly prepared 2 % potassium permanganate overnight at room temperature. Fixed colonies were stained with 2 % uranyl acetate overnight at room temperature and dehydrated with serial ethanol [30, 50, 75, 85, 95 and 100 % (three times), each for 30–40 min]. Cells were transitioned with propylene oxide, infiltrated in EMBed 812 resin (Electron Microscopy Sciences). Ultrathin sections (50 nm) were cut and stained with lead citrate. Sections were examined using a Hitachi H-7000 electron microscope, equipped with a high-resolution (4K x 4K) digital camera (Gatan). The sections were imaged at x1000 and the images were stitched together manually with Adobe Photoshop software.

Fluorescence microscopy. Cells were fixed with 4 % paraformaldehyde for 20 min. For visualization of chitin, cells were stained with 25 μM fluorescent brightener 28 (Sigma-Aldrich) in PBS (pH 7.4) for 30 min in the dark. For visualization of nuclei, cells were stained with DAPI (0.5 mg ml⁻¹). All images were taken using a Zeiss 710 laser scanning confocal microscope.

Semiquantitative reverse transcription (RT)-PCR. Total RNA from yeast cells was extracted using the RNeasy Protect Mini kit (Qiagen). The RT-PCR and the amplification procedures were performed as described previously (Gao et al., 2009). The yeast actin gene (ACT1) was used as a control, and the primers were 5′-GGATTGCCG-3′ and 5′-GGATTCGACGTAATTCGGG-3′. Primers used for RHO1 amplification were 5′-GCAAAAGAATAATGGATCG-3′ and 5′-ACGGGTTGTTGACCTTCTTG-3′. TOR1 primers were 5′-GCGAGCGCTTACACTCGGTA-3′ and 5′-ATATCCC-CACTCCTCAACCC-3′. TOR2 primers were 5′-GCAAAAGAATTCT- GCCGTGCTA-3′ and 5′-GCATAAACACTGGAGGGGA-3′.

Western blot analysis. Cells were cultured at 30 °C in YPD to early exponential phase. Cell lysate preparation was according to Ren et al. (2012). Equivalent amounts of protein were separated by 10 % SDS-PAGE and transferred to PVDF membranes. The membranes were blocked in 5 % BSA for non-specific binding. The membranes were then incubated with rat anti-HA (1:1000 dilution; Roche Diagnostics) or rat anti-tubulin (1:2000 dilution; Abcam) as a loading control. The membrane was washed with blocking buffer, followed by goat anti-rat IgG conjugated with horseradish peroxidase (Thermo Scientific). Immunoreactive bands were visualized using a ChemiDoc system (Bio-Rad).

Rho1 pull-down assay. The active Rho1 (Rho1-GTP) was measured by using the Rho1-GTP pull-down kit (Cytoskeleton). Proteins were prepared in the same way as for Western blot analysis and normalized with the Bradford assay (Bradford, 1976). Normalized proteins were incubated with GTP-γ-S for 15 min as a positive control. Equal amounts of proteins were incubated with Rhotekin-RBD beads (Cytoskeleton) for 1 h at 4 °C. Pelleted beads were washed with washing buffer and the Rho1-GTP bound to beads was eluted with 2× Laemmli buffer and boiled for 2 min. The eluted Rho1-GTP protein was analysed by Western blotting using anti-HA antibody.

RESULTS

Double deletion of SRO7 and SRO77 (sro7Δ/sro77Δ) causes defects of colony development

It has been reported that the double deletion of SRO7 and SRO77 (sro7Δ/sro77Δ) is cold sensitive and has a defect in exocytosis (Lehman et al., 1999). The sro7Δ/sro77Δ double deletion appeared to grow normally at 30 or 37 °C, but grew poorly at 24 °C. Overexpression of SRO7 (sro7Δ/sro77Δ + SRO7) or SRO77 (sro7Δ/sro77Δ + SRO77) recovered the growth defect of the sro7Δ/sro77Δ double deletion (Fig. 1a). sro7Δ/sro77Δ also affected colony growth, possibly due to its defect in exocytosis. As shown in Fig. 1b, even at 30 °C, the sro7Δ/sro77Δ colony was much smaller than the WT. The colony appeared to be round with a smooth surface, compared with the WT, suggesting that the double deletion was defective in colony differentiation. To better understand its phenotype, we examined the internal structure of sro7Δ/sro77Δ colony cells using TEM. When grown on YPD for 3 days, the WT showed a normal cellular structure (Fig. 2a, b). In contrast, many dead cells were observed inside the sro7Δ/sro77Δ colony, especially near the middle part of the colony (Fig. 2c–g). One possibility was that the dead cells were older cells and they died to release nutrients to support the growth of the other cells (Váchaová & Palková, 2005).

RHO1 overexpression recovers the sro7Δ/sro77Δ phenotype

TEM analysis revealed that some colony cells underwent cell lysis (Fig. 2f), suggesting a defect in the cell wall integrity (CWI) in the sro7Δ/sro77Δ mutant (Levin & Bartlett-Heubusch, 1992). Multiple nuclei and multiple buds were also observed in sro7Δ/sro77Δ colony cells (Fig. 2g, h), suggesting that the double deletion was defective in polarized cell growth. Staining with fluorescent brightener 28 showed that chitin was concentrated mainly on the bud scars in WT, but in sro7Δ/sro77Δ the chitin was spread across the cell wall (Fig. 3), further suggesting its defect in CWI. All these phenomena pointed to possible misregulation of small GTPases, RHO1 and/or CDC42, the
centrally regulated cell polarity and CWI. To test this possibility, we overexpressed CDC42 or RHO1 in sro7Δ/sro77Δ (sro7Δ/sro77Δ + CDC42 or sro7Δ/sro77Δ + RHO1). Overexpression of CDC42 showed no apparent effect on colony growth. Overexpression of RHO1, however, fully recovered the sro7Δ/sro77Δ phenotype (Fig. 4). The colony of sro7Δ/sro77Δ + RHO1 was similar to WT and no multiple nuclei or multiple budding was observed. Overexpression of RHO1 also recovered the chitin localization in sro7Δ/sro77Δ, whilst no apparent difference was observed when CDC42 was overexpressed (Fig. 3).

**Rho1-GTP level is lower in the sro7Δ/sro77Δ deletion**

The fact that RHO1 overexpression recovered the sro7Δ/sro77Δ phenotype suggested that the RHO1 level may be lower in the sro7Δ/sro77Δ strain. RT-PCR was used to compare the RHO1 levels between WT and sro7Δ/sro77Δ. As shown in Fig. 5(a), the RHO1 mRNA levels of WT and sro7Δ/sro77Δ were similar. Western blot showed that the Rho1 proteins of WT and sro7Δ/sro77Δ were also similar (Fig. 5b). Rho1 is a small GTPase, which switches from its GTP-bound, active form to a GDP-bound, inactive form. To examine if sro7Δ/sro77Δ was involved in the activation of RHO1, a pull-down assay was performed and the results showed that GTP-bound Rho1 (Rho1-GTP) was much lower in the sro7Δ/sro77Δ deletion than in the WT (Fig. 5c, top panel). To further confirm this, we overexpressed ROM2 and TUS1, the guanine nucleotide-exchange factors of Rho1 that activate Rho1 by catalysing the dissociation of GDP, allowing GTP to bind to Rho1. The sro7Δ/sro77Δ grew slightly better when TUS1 was overexpressed, while ROM2 significantly recovered the sro7Δ/sro77Δ growth defect (Fig. 5d). Overexpression of ROM2 also partially recovered the Rho1-GTP level of sro7Δ/sro77Δ (Fig. 5c, middle panel), further suggesting that the lack of active Rho1, rather than the total amount of Rho1, was responsible for the sro7Δ/sro77Δ phenotype.

TOR (target of rapamycin) proteins are protein kinases that play a central role in cell metabolism and polarized growth. Recent studies have shown that TOR is a direct target of the small GTPase Rho1 (Yan et al., 2012). We speculated that SRO7/SRO77 might regulate cell polarity via the Rho1→TOR pathway. To test this possibility, we first measured the mRNA level of TOR1 and TOR2 in the sro7Δ/sro77Δ double deletion. Whilst no obvious change was observed for TOR2 mRNA levels, the TOR1 mRNA level was much higher in sro7Δ/sro77Δ than in WT (Fig. 6a). Overexpression of RHO1 reduced the TOR1 level of sro7Δ/sro77Δ to the WT level, indicating that the TOR1 mRNA increase in sro7Δ/sro77Δ was a consequence of a decreased level of active Rho1 in this strain (Fig. 6b). The sro7Δ/sro77Δ double deletion was also more sensitive to rapamycin, further suggesting its higher TOR1 level, and overexpression of RHO1 in sro7Δ/sro77Δ partially recovered its sensitivity to rapamycin (Fig. 6c). Deletion of TOR1 in the sro7Δ/sro77Δ background (sro7Δ/sro77Δ/tor1Δ) recovered the cell growth and colony morphology, similar to WT (Fig. 6d).

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**Fig. 1.** Double deletion of SRO7 and SRO77 caused a defect of colony growth. (a) Spot assay showing that sro7Δ/sro77Δ cells are cold sensitive. Aliquots of cells were spotted on YPD plates using 4 µl serial 1:5 dilutions. Cells were cultured for 2 days at 24, 30 or 37 °C. (b) Single-colony morphology of sro7Δ/sro77Δ, which was much smaller and round with a smooth surface, compared with WT. The colony was grown on YPD plates at 30 °C for 3 days. Bar, 1 mm.
DISCUSSION

Sro7 and Sro77 were identified originally as high-copy suppressors of Rho3 mutants. They play important roles in the late stages of exocytosis (Lehman et al., 1999). Consistent with previous studies (Lehman et al., 1999; Zhang et al., 2005), we observed that sro7Δ/sro77Δ displayed a multiple budding and multiple nuclei phenotype, which is more profound when grown on solid media than in liquid media. Cell lysis, which had been studied traditionally by examining the release of cellular material into the medium (Levin & Bartlett-Heubusch, 1992), was also observed in the sro7Δ/sro77Δ colony. Traditionally, when TEM is used to examine yeast cells, cells are fixed and processed in liquid, and therefore it is difficult to ‘capture’ the lysis process. The ‘in situ’ fixation and process for TEM preparation enabled us to observe the cell lysis process directly. The phenomena of cell lysis (Fig. 2f) and the thickened, irregular cell wall between mother–daughter cells (Fig. 2g) strongly suggest that sro7Δ/sro77Δ is defective in the CWI pathway, which is regulated by the small GTPase Rho1 (Levin, 2005).

RHO1 is an essential gene and its main function is to regulate polarized growth and the organization of the actin cytoskeleton (Madden & Snyder, 1998). We demonstrated that overexpression of RHO1 recovered the sro7Δ/sro77Δ phenotype, which is different from a previous report which showed that a high dose of CDC42 or RHO1 could not rescue the sro7Δ/sro77Δ phenotype (Zhang et al., 2005). This difference is likely caused by different strain backgrounds. Overexpression of ROM2, a guanine nucleotide-exchange factor of Rho1, also recovered the sro7Δ/sro77Δ phenotype. In addition, the Rho1p-GTP level was

Fig. 2. Microscopy analysis of WT and sro7Δ/sro77Δ cells. (a, b). TEM cross-section of a WT, 3 day colony, showing normal structure of yeast cells in the colony. (a) Outline of a colony (half) grown on a YPD plate at 30 °C for 3 days. (b) TEM image of inset in (a). (c). TEM cross-section of a sro7Δ/sro77Δ colony on a YPD plate at 30 °C for 3 days. (d). TEM image of the boxed area in (c). (e–g). Cells at high magnification from (d), showing a dead cell (e), cell lysis (f), and multiple buds and multiple nuclei (g). Arrow in (f) indicates lysis of the cell wall. N, nucleus. (h) Confocal microscopy images showing multiple buds and multiple nuclei of sro7Δ/sro77Δ cells. DIC, differential interference contrast.
much lower in the sro7Δ/sro77Δ strain and was restored partially when ROM2 was overexpressed (Fig. 5c). These results indicate that SRO7/SRO77 are upstream regulators for Rho1 activation. This notion is further supported by
the fact that neither RHO1 mRNA nor the Rho1 protein levels were decreased in sro7Δ/sro77Δ (Fig. 5a, b).

It has been reported recently (Yan et al., 2012) that the Rho1 GTPase regulates directly the TOR complex 1 (TORC1) under stress conditions. Stresses, such as rapamycin or caffeine, induce the activation of Rho1, which in turn binds directly to and downregulates TORC1 activity. Consistent with these findings, we demonstrated that the lower level of the GTP-bound, active Rho1 in sro7Δ/sro77Δ caused an increased level of TOR1, which stimulates cell growth. Our results suggest strongly that Sro7/Sro77 may repress cell growth by regulating the TOR1 pathway via the GTPase Rho1. The results also suggest that Sro7/Sro77 are upstream of the Rho1-regulated polarized cell growth (Fig. 7). These findings may shed light on the molecular mechanism of cell growth regulated by cancer repressors.

Fig. 6. TOR1 mRNA was increased in sro7Δ/sro77Δ. (a) RT-PCR analysis showing TOR1 mRNA was increased, whilst TOR2 mRNA was unchanged in sro7Δ/sro77Δ. ACT1 was used as a loading control. (b) RT-PCR analysis showing overexpression of RHO1 reduced TOR1 mRNA to the WT level. (c) Spot assay showing sro7Δ/sro77Δ was more sensitive to rapamycin compared with WT. RHO1 overexpression partially recovered its sensitivity to rapamycin. Cells were cultured on YPD plates at 30 °C for 2 days. (d) Spot assay showing that the triple deletion sro7Δ/sro77Δ/tor1Δ recovered the growth defect of sro7Δ/sro77Δ at 24 and 30 °C on YPD for 2 days.
Fig. 7. Diagram of the possible pathway by which Sro7/Sro77 regulates cell polarity through Rho1 and Tor1. References: 1, Yan et al. (2012); 2, Levin (2005).

ACKNOWLEDGEMENTS

This study was supported in part by an Institutional Development Award from the National Institute of General Medical Sciences of the National Institutes of Health (grant P30 GM103398).

REFERENCES


Edited by: H. Sychrova