Different steps of sexual development are differentially regulated by the Sec8p and Exo70p exocyst subunits

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Abstract

In this paper we show that in Schizosaccharomyces pombe, mating-specific cell adhesion is dependent on the exocyst subunit Sec8p, but independent of the exocyst subunit Exo70p. In the absence of Exo70p, the forespore membrane does not develop properly and the leading edge protein Meu14p is abnormally distributed. Additionally, the spindle pole body is aberrant in a significant number of exo70Δ ascis. In both the sec8Δ and the exo70Δ mutants, the development of the spore cell wall is impaired. These results show that different steps of sexual development are differentially regulated by the exocyst and suggest the existence of exocyst subcomplexes with distinct roles in mating.

Introduction

Schizosaccharomyces pombe cells belong to either of two mating types: h+ or h−. Homothallic h90 strains are self-fertile because a mating-type switching allows them to form colonies containing h+ and h− cells. When nitrogen is scarce, the Ste11p transcription factor induces the expression of genes essential for sexual development, including those coding for pheromones (see Yamamoto et al., 1997; Nielsen, 2004; Shimoda & Nakamura, 2004; Yamamoto, 2004). The binding of these pheromones to receptors in cells of the opposite mating type initiates a signaling pathway that requires a mitogen-activated protein (MAP) kinase cascade consisting of Byr2p, Byr1p, and Spk1p. As a result, cells differentiate into shmoos with a polarized growth pattern (Nielsen, 2004). Then the Mam3p and Map4p agglutinins (Yamamoto et al., 1997; Mata & Bhahler, 2006; Sharifmoghadam et al., 2006) facilitate and strengthen the union of the shmoos, producing prezygotes (Calleja & Johnson, 1971). Later on, the cell walls between the two mating partners degrade, allowing fusion of the membranes, diffusion of the cytoplasmic material, and karyogamy producing a diploid zygote (Calleja et al., 1977; Nielsen, 2004; Yamamoto, 2004).

The diploid nucleus immediately undergoes meiosis and gives rise to four haploid nuclei (Shimoda & Nakamura, 2004; Yamamoto, 2004). The leading edge protein (LEP) Meu14p accumulates besides the spindle pole body (SPB), which acts as a center for the organization of the forespore membrane (FSM), and forms ring-shaped structures that promote the development of the membrane around the nuclei (Ikemoto et al., 2000; Okuzaki et al., 2003; Shimoda, 2004; Shimoda & Nakamura, 2004; Nakamura et al., 2008; Nakase et al., 2008). FSM development requires the secretory pathway and vesicle docking (Shimoda, 2004; Shimoda & Nakamura, 2004; Nakamura et al., 2008). Spores maturate with the building of a specialized cell wall, which involves the synthesis of α- and β-glucan and chitin (Arellano et al., 2000; Liu et al., 2000; Martín et al., 2000; Matsuo et al., 2005; García et al., 2006; de Medina-Redondo et al., 2008).

The exocyst is a protein complex involved in the tethering and spatial targeting of post-Golgi vesicles to the plasma
membrane before vesicle fusion (TerBush et al., 1996; Guo et al., 1999; Mehta et al., 2005). In S. pombe, this complex participates in cell separation because it is required to target hydrolytic enzymes to the septum (Wang et al., 2002; Martin-Cuadrado et al., 2005). The only viable exocyst mutants in this organism are sec8-1 and exo70A (Wang et al., 2002, 2003). The term exomer refers to a Saccharomyces cerevisiae coat complex required for the transport of certain membrane proteins from the trans-Golgi network to the plasma membrane (Wang et al., 2006; Barfield et al., 2009). The exomer subunit Chs5p is required for chitin synthesis and mating (Santos et al., 1997). In S. pombe, the Chs5p-homologue Crf1p is required for cell wall digestion during mating (Cartagena-Lirola et al., 2006).

The analysis of how cell wall-modifying enzymes required for sexual development reach the cell surface is not only interesting for the characterization of the mating process in yeast but also represents a model system to study intracellular trafficking during a developmental process. The initial goal of this work was to study the regulation of cell adhesion by genes that have already been implicated in the mating and/or the cell wall remodeling processes. To do so, we analyzed agglutination in several mutants; the mutants selected were spk1Δ (defective in the mating signal transduction pathway; Nielsen, 2004), spm1Δ (deleted for a MAP kinase that regulates morphogenesis, cell integrity, and mating; Zaitsevskaya-Carter & Cooper, 1997), dni1Δ (deleted for a claudin-like tetraspan protein required for cell wall reorganization and membrane fusion during mating; Clemente-Ramos et al., 2009), cfr1Δ (deleted for an exomer component; Cartagena-Lirola et al., 2006), sec8-1 (bearing a point mutation in sec8+; Wang et al., 2002), and exo70Δ (deleted for exo70+; Wang et al., 2003). Surprisingly, our results showed that agglutination is dependent on Sec8p, but independent of Exo70p. This result prompted us to analyze in detail the role of these exocyst subunits in mating. Our results suggest that Sec8p and Exo70p participate in different subcomplexes that are differentially required during sexual development.

Materials and methods

Strains and growth conditions

All techniques for S. pombe growth and manipulation have been described elsewhere (http://www.biotwiki.org/bin/view/Pombe/NurseLabManual). The relevant genotype of the strains used is listed in Supporting Information, Table S1.

Genetic manipulations

All the tagged proteins were integrated into the chromosome under the control of their own promoters. The combination of mutated alleles with green fluorescent protein (GFP)-tagged proteins was performed either by plasmid transformation or by ‘random spore’ selection from genetic crosses. Exo70p was tagged at its chromosomal locus as described before (Bahler et al., 1998) using the oligonucleotides eexo70up (5’-tataca aatttacaagctgatttagatttttttataagcgcttgctctccccctccagatgcc cgggtttaa3’) and eexo70do (5’-caatatagttgctagttacgtctc aacgagaaagcagggtaaacaagctcattaaaaaggggaaaggaattctac gtttaaa3’) and a plasmid bearing the red fluorescent protein (RFP; a generous gift from P. Perez).

Agglutination and sporulation analyses

Agglutination, mating, and sporulation were analyzed using h300 strains. Agglutination was performed in liquid minimal medium without nitrogen and mating efficiency was calculated from cultures that had been induced to mate on sporulation agar (SPA) plates (1% glucose, 0.1% KH2PO4, 3% agar, and vitamins as in minimal medium) for 15 h, as described before (Arellano et al., 2000; Sharifmoghadam et al., 2006; Sharifmoghadam & Valdivieso, 2008). Because the efficiency of sexual adhesion and sporulation is reduced at temperatures above 28 °C (Clemente-Ramos et al., 2009 and our unpublished data), the experiments were performed at 32 °C, a temperature at which the sec8-1 mutant grows in a rich medium exhibiting its characteristic multiseptation phenotype. The agglutination index (AI) was calculated as the 1/OD600 nm of the culture supernatant (Sharifmoghadam & Valdivieso, 2008).

Microscopy

Hoechst 33258 was used for nuclear staining. Images were captured using a Leica DM RXA microscope equipped with a Photometrics Sensys CCD camera, using the QFISH 2.3 program.

Western blotting

Western blotting was performed as described (Sharifmoghadam & Valdivieso, 2008). Briefly, cells from 50-mL cultures (about 109 cells) were collected by centrifugation after 5 h of incubation in minimal medium without nitrogen with gentle shaking in 500-mL flasks. Culture media were concentrated to a volume of 200 μL using Amicon Ultra-15 (ultracel 10 K, Millipore); 200 μL of 2× Laemmli sample buffer was added, and the samples were boiled for 5 min. Cells were washed with Buffer B (50 mM Tris–HCl, pH 7.5; 50 mM EDTA; 150 mM NaCl) supplemented with protease inhibitors (1 mM PMSF; 1 μg/mL Aprotinin, Leupeptin, and Pepstatin) and broken in 100 μL of the same buffer in a FastPrep (Savant). Total protein was estimated using the Biorad protein assay kit (Bradford method) and cell extracts were adjusted to the same protein concentration in a final volume of 200 μL. Cell extracts were centrifuged for 1 min at 16 200 g in a cold centrifuge to pellet cell walls. Supernatants

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(cytosols) were transferred to clean tubes and boiled in a final volume of 400 μL in the presence of Laemmli sample buffer (50 mM Tris-HCl, pH 6.8; 1% SDS; 143 mM β-mercaptoethanol; 10% glycerol). Pellets (cell walls) were washed twice with 1 mL of 2 M NaCl and three times with 1 mL of Buffer B and boiled in a final volume of 400 μL in the presence of Laemmli sample buffer. The volume corresponding to 200-μg protein from the cytosols was loaded from the culture media, cytosols, and cell walls in 6.5% polyacrylamide gels to perform the electrophoresis. The α-HA and α-Cdc2 antibodies were used at a 1 : 5000 dilution.

**Results**

**Agglutination depends totally on Spk1p and Sec8p and partially on Smp1p and Cfr1p**

In order to gain information about the requirements for agglutination, the AI of spk1Δ, smp1Δ, dni1Δ, cfr1Δ, sec8-1, and exo70Δ mutants induced to mate in liquid medium was estimated. Wild-type (WT) and map4Δ strains were used as controls. As shown in Fig. 1a, in the cultures from the dni1Δ and exo70Δ mutants, agglutination took place as efficiently as in the WT. In the case of the cfr1Δ and smp1Δ mutants, the AI was lower than that in the WT; however, mating aggregates were observed in these cultures, indicating that agglutination had taken place. The AI for the spk1Δ and the sec8-1 mutants was similar to that of the negative control map4Δ (Fig. 1a). We then determined whether the agglutination efficiency was correlated with the level of Map4p by observing under the fluorescence microscope cells from the mutants and the WT strain that had been induced to mate in liquid medium. Map4p localizes at the tip of the shmoos and at the mating bridge of the zygotes in the WT strain (Sharifmoghadam et al., 2006; Sharifmoghadam & Valdivieso, 2008). As shown in Fig. 1b, Map4p was readily observed in the cfr1Δ, exo70Δ, smp1Δ, and dni1Δ mutants; agglutinin exhibited a weak fluorescent signal in the sec8-1 cells, and it

![Fig. 1](https://academic.oup.com/femsle/article-abstract/305/1/71/442037)
could not be observed in the \( spk1 \Delta \) cells. Western blot analyses were performed to determine the level of Map4p in the culture media, the cytosols, and cell walls of the WT, \( exo70 \Delta, sec8 \Delta -1 \), and \( spk1 \Delta \) cells more precisely. Map4p was not detected in the culture media from any of the strains (Sharifmoghadam & Valdivieso, 2008 and results not shown). As shown in Fig. 1c, the level of agglutinin in the cytosol from the WT and \( sec8 \Delta -1 \) strains was low; it was undetectable in the \( spk1 \Delta \) strain, and was high in the \( exo70 \Delta \) strain. In the cell walls of the WT and \( exo70 \Delta \) strains, the level of Map4p was similar, while this level was lower in the \( sec8 \Delta -1 \) mutant and was undetectable in the \( spk1 \Delta \) mutant. These results suggest that in the WT strain, Map4p is incorporated rapidly into the cell wall, where the protein accumulates; thus, most of the protein is detected in the cell walls. In the \( exo70 \Delta \) mutant, Map4p incorporates into the cell wall less efficiently than in the WT strain, and so it accumulates in the cytosol, although the amount of agglutinin that accumulates in the cell wall is similar in both strains. In the \( sec8 \Delta -1 \) mutant, a low amount of Map4p incorporates into the cell wall.

All the above results showed that the MAP kinase Spk1p and the exocyst subunit Sec8p were required for proper Map4p synthesis and delivery to the cell wall, while the exocyst subunit Exo70p was not.

**The \( sec8 \Delta -1 \) and the \( exo70 \Delta \) mutations produce different mating defects**

It has been described that Map4p is required for agglutination in liquid medium and for mating on solid medium (Mata & Bahler, 2006; Sharifmoghadam et al., 2006; Sharifmoghadam & Valdivieso, 2008). In order to determine whether Sec8p and the Exo70p might play some role in the mating process on solid medium, \( b^{90} \ sec8 \Delta -1 \) and \( b^{90} \ exo70 \Delta \) cells were induced to mate on SPA plates at 32°C for 15 h. Under these conditions, it was found that the mating efficiency (the number of zygotes plus asci with respect to the number of zygotes, asci, and cells) was 45% for the WT strain, while this value was 6% for the \( map4 \Delta \) mutant. As described previously (Mata & Bahler, 2006; Sharifmoghadam et al., 2006), a significant number of shmoos were detected in the \( map4 \Delta \) mating mixtures (Fig. 1d) and the asci produced by the \( map4 \Delta \) mutant had a WT appearance (not shown; Sharifmoghadam et al., 2006). In the \( sec8 \Delta -1 \) mutant, mating efficiency was 10%; in the mating mixtures from this mutant, a significant number of enlarged shmoos were observed, and about half of the asci contained non-refractile spores with a heterogeneous size (Fig. 1d). In the \( exo70 \Delta \) mating mixtures, mating efficiency was 42% and mature asci were scarce (< 10% of the asci contained four refringent spores; Fig. 1d). This phenotype was even more drastic when the cells were induced to mate on solid minimal medium with supplements (under these conditions, no spores could be detected in the \( exo70 \Delta \) asci; not shown).

**Exo70p is essential for FSM development, but Sec8p is not**

We wished to determine the step in sporulation at which the exocyst was required. Initially, Hoechst staining was performed to determine whether meiosis took place in the \( sec8 \Delta -1 \) and \( exo70 \Delta \) mutant strains. As shown in Figs 2 and 3, four nuclei were observed in the asci from both mutants, showing that nuclear division was not defective in the absence of either Sec8p or Exo70p.

Then, we analyzed the development of the FSM in the WT, \( sec8 \Delta -1 \), and \( exo70 \Delta \) strains. To do so, the localization of the syntaxin-like Psy1p was analyzed in the WT strain and in the mutants. As described previously (Nakamura et al., 2008), in the WT control, GFP-Psy1p was observed as cup-shaped structures [Fig. 2a(i)] that developed to form sacs around the nucleus [Fig. 2a(ii)]. In the \( sec8 \Delta -1 \) mutant, the behavior of Psy1p was similar to that observed in the WT strain (Fig. 2b), showing that Sec8p is not required for the development of the FSM. In the \( exo70 \Delta \) asci, Psy1p was detected as amorphous membranous structures in the cytoplasm of binucleated or tetranucleated asci [Fig. 2c(i) and (ii)] or as vesicle-like or even tubular structures that failed to engulf the nuclei [Fig. 2c(iii) and (iv)]. This result showed that Exo70p is essential for the FSM development.

**Distribution of the LEP Meu14p and the SPB protein Sad1p is abnormal in the absence of Exo70p**

Next, we wished to study in more detail the defect in FSM development exhibited by the \( exo70 \Delta \) mutant. To do so, we analyzed the behavior of the LEP Meu14p in the WT and the \( exo70 \Delta \) strains. Cells bearing Meu14p fused to the GFP were induced to mate and were observed under the fluorescence microscope. As reported previously (Okuzaki et al., 2003) and shown in Fig. 3a, in the WT strain Meu14p was observed as four rings of different diameters that were situated in the vicinity of the nuclei, but apart from them. In the \( exo70 \Delta \) asci, the four Meu14p rings seemed to be attached to the nuclei [Fig. 3b(i) and (ii)] or as vesicle-like or even tubular structures that failed to engulf the nuclei [Fig. 3b(iii) and (iv)]. This result showed that Exo70p is essential for the FSM development.

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mutant strain, 34% of the asci exhibited multiple Sad1-GFP fluorescent dots (Fig. 3c), while this value was 11% for the WT strain. This result suggested that the SPBs are unstable in the \textit{exo70} \textit{D} mutant.

**Proper localization of the \(\alpha\)-glucan synthase Mok12p at the spore surface requires both Sec8p and Exo70p**

Finally, we analyzed the distribution of the \(\alpha\)-glucan synthase homologues Mok12p and Mok13p, which are required for the synthesis of the spore cell wall. Mok13p is expressed earlier than Mok12p (Garcia \textit{et al.}, 2006). As reported previously, in the WT strain, Mok13p localized to the FSM, forming cup-shaped structures and sacs around the nuclei (Garcia \textit{et al.}, 2006). The same result was obtained for the \textit{sec8-1} mutant (not shown). In the \textit{exo70A} mutant, Mok13p formed amorphous structures or small sacs, like those formed by Psy1p, which did not surround the nuclei (not shown). This result was in agreement with an inability of the \textit{exo70A} mutant to develop the FSM properly.

The \(\alpha\)-glucan synthase Mok12p localizes at the surface of the developing spores (Garcia \textit{et al.}, 2006). Because the spore cell wall is not permeable to Hoechst, we analyzed the localization of the Mok12-GFP protein with respect to the spore surface photographed under a phase-contrast microscope. In the control strain, Mok12p was observed at the spore periphery (Fig. 4; WT). In the \textit{sec8-1} mutant, the distribution of this protein was heterogeneous; in those asci that had refringent spores, Mok12p localized at the spore surface (Fig. 4; \textit{sec8-1}), while in those asci that exhibited immature spores, Mok12p could not be observed. In the \textit{exo70A} mutant, the signal corresponding to Mok12p was hardly observed in the asci interior (Fig. 4; \textit{exo70A}). These results suggest that both exocyst subunits participate in the maturation of the spore cell wall.

### Sec8p and Exo70p colocalize partially at the shmoo tip and totally at the spore surface

All the results described above confirmed that the exocyst was required for mating in \textit{S. pombe} and that different steps of this process are differentially regulated by these exocyst subunits. In order to know whether the different requirements of Sec8p and Exo70p for agglutination and sporulation were a consequence of a different distribution of these proteins, cells carrying a GFP-tagged Sec8p and an RFP-tagged Exo70p were induced to mate in liquid medium and were observed under the microscope. As shown in Fig. 5, both proteins were observed at the cell–cell contact area of the zygotes. However, the localization of both proteins was not the same and their fluorescent signals only overlapped partially in some zygotes [Fig. 5a(ii)]. During sporulation, Sec8-GFP and Exo70-RFP were observed at the surface of the spores. At this localization, the signal from both proteins was mostly overlapping.
Discussion

The initial goal of this work was to study the regulation of sexual agglutination by certain genes that have been implicated in mating and/or cell wall remodeling. As expected, we found that the MAP kinase Spk1p, which is necessary for the mating signal transduction pathway (Nielsen, 2004), was required for agglutination. It has been shown that sporulation is retarded in an \textit{spm1}\textit{D} mutant, and it has been suggested that this delay would probably be due to a defect.

![Fig. 3. Meu14p and SPBs are abnormal in the exo70\textit{D} mutant. Nuclear (Hoechst) and LEP complex (Meu14-GFP) morphology in WT (a) and exo70\textit{D} (b) asci. (c) Nuclear (Hoechst) and SPB (Sad1-GFP) morphology in an exo70\textit{D} ascus.](https://academic.oup.com/femsle/article-abstract/305/1/71/442037)
in some event before cytoplasmic mixing (Zaitsevskaya-Carter & Cooper, 1997). We have confirmed that in this mutant, agglutination indeed proceeds more slowly than in the WT control. A similar defect in agglutination was found in the exomer-defective cfr1Δ mutant. In both the spm1Δ and the cfr1Δ mutants, this slow agglutination was due to a significant defect in Map4p localization at the cell surface. Thus, Spm1p and Cfr1p must be regulating the h- agglutinin Mam3p and/or other protein(s) that might be required for agglutination.

In *S. pombe*, the exocyst is necessary for the correct localization of the glucanases required for cell separation during cytokinesis (Martin-Cuadrado et al., 2005). Here, we have shown that exocyst is also required for mating. When we analyzed the role of the exocyst in agglutination, we found that in the sec8-1 mutant, agglutination did not take place and that this defect was correlated with a low level of Map4p, although some Map4p could be detected by microscopic observation and by Western blot, suggesting that Sec8p could also regulate other protein(s) that might be required for agglutination. About half of the sec8-1 asci exhibited abnormal spores, indicating that Sec8p also plays a role in spore development. Surprisingly, in the absence of the Exo70p exocyst subunit Map4p was detected in the cell wall of the mating cells and agglutination was as efficient as in the WT control. These results showed that Sec8p and Exo70p are differentially required for agglutination. A role for some exocyst subunits in the trafficking of adhesion molecules required for synaptic partner choice has been suggested in *Drosophila* (Mehta et al., 2005). Thus, the participation of exocyst in the regulation of adhesion molecules seems to be a process that is not species-specific.

The defect in sporulation exhibited by the exo70Δ mutant was more dramatic than that of the sec8-1 mutant. Although the possibility that Exo70p might be more necessary for sporulation than Sec8p cannot be ruled out, it is important to take into account that the sec8-1 mutant carries a point mutation while the exo70Δ strain is a null mutant. The function of Sec8p in sporulation seems to be restricted to the last steps of the spore cell wall synthesis because a significant number of asci carry immature spores. In the absence of Exo70p, FSM development was severely impaired and the spore cell wall could not be synthesized. As a consequence, almost no spores could be detected in the exo70Δ mating mixtures. In mammalian cells, exocyst components coprecipitate with the plasma membrane t-SNARE syntaxin (Hsu et al., 1996), and in *S. pombe*, the syntaxin-like protein Psy1p is essential for FSM development (Shimoda, 2004; Shimoda & Nakamura, 2004; Nakamura et al., 2008). Thus, it is possible that the exocyst–Psy1p interaction is required for the incorporation of new membrane material and/or certain proteins into the developing FSM during sporulation. Additionally, the LEP Meu14p was abnormally distributed in the exo70Δ asci. It will be interesting to determine whether the exocyst is required for the proper assembly of the LEP complex and, consequently, for FSM development or whether in the absence of the exocyst, new membrane material cannot be incorporated into the developing FSM and, as a consequence, the LEP complex cannot develop properly and cannot encircle the nuclei.

In the meu14Δ mutant, the SPBs are unstable and appear to be fragmented, which indicates that Meu14p plays a role in SPB stability (Okuzaki et al., 2003). In the exo70Δ mutant, a significant percentage of SPBs were fragmented, even though these cells carried Meu14p. In mammalian cells, Exo70p associates with microtubules, microtubule-organizing centers, and centrosomes (Xu et al., 2005). Thus, it is possible that in yeast, the exocyst might play a direct role in SPB stability during sporulation. However, the fact that in the exo70Δ mutant the defect in the FSM development was stronger than the defect in the SPBs suggests that the main function of Exo70p is to contribute to FSM development. These results suggest that FSM development has an influence on the stability of the SPBs and that the different steps in spore development are inter-regulated.

In *S. cerevisiae*, the exocyst localizes specifically to the sites of active secretion and cell growth, where it mediates...
the secretion of certain proteins (He et al., 2007). Additionally, the Sec8p exocyst subunit is required for sporulation at a postmeiotic step (Neiman, 1998), although the specific role of Sec8p in this process is not known. Our data show that the exocyst plays a role in sexual development in both yeasts. In *S. pombe*, Sec8p and Exo70p localize to the septal area during vegetative growth (Wang et al., 2002). However, deletion of sec8+ is lethal while deletion of exo70+ is not (Wang et al., 2002, 2003), which indicates a different requirement for these exocyst subunits during vegetative growth. We have found that agglutination requires Sec8p, but not Exo70p, Exo70p, but not Sec8p, is essential for FSM development, and that both Sec8p and Exo70p are required for the proper synthesis of the spore cell wall. Additionally, we have found that Sec8p and Exo70p colocalize at the spore surface, but not at the cell–cell contact area in the prezygotes. These results strongly suggest that Sec8p and Exo70p are present in different subcomplexes; one of them (required for agglutination) would lack Exo70p. The fact that Exo70p is also observed at the tip of the contacting shmoos suggests that, although under our experimental conditions we have not observed a mating defect in the exo70Δ mutant, this protein might also play some role during the initial steps of mating. A different exocyst subcomplex, carrying Sec8p and Exo70p, would be required for sporulation. In this subcomplex, the presence of Exo70p seems to be more relevant than the presence of Sec8p for the FSM development.

There is increasing evidence suggesting that different exocyst components play different roles and that there are subcomplexes in the exocyst. Thus, in *Drosophila*, it has been shown that exocyst function is divisible and so different components play distinct roles. Additionally, different GTPases regulate the activity of this multiprotein complex by interacting with different subunits (Wu et al., 2008), and the localization of different subunits to the sites of active secretion has different requirements (Zajac et al., 2005). Thus, exocytosis of specific proteins by the exocyst is subject to a complex regulation. Our results support the notion of different exocyst subunits playing distinct roles in some developmental processes in a variety of organisms, from unicellular eukaryotes to metazoa.

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Authors’ contribution

N.d.L., M.H., and M.-Á.C. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the
online version of this article:

Table S1. Strains used in this work.

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