Sequential and Distinct Roles of the Cadherin Domain-containing Protein Axl2p in Cell Polarization in Yeast Cell Cycle

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Polarization of cell growth along a defined axis is essential for the generation of cell and tissue polarity. In the budding yeast *Saccharomyces cerevisiae*, Axl2p plays an essential role in polarity-axis determination, or more specifically, axial budding in *MATa* or α cells. Axl2p is a type I membrane glycoprotein containing four cadherin-like motifs in its extracellular domain. However, it is not known when and how Axl2p functions together with other components of the axial landmark, such as Bud3p and Bud4p, to direct axial budding. Here, we show that the recruitment of Axl2p to the bud neck after S/G2 phase of the cell cycle depends on Bud3p and Bud4p. This recruitment is mediated via an interaction between Bud4p and the central region of the Axl2p cytoplasmic tail. This region of Axl2p, together with its N-terminal region and its transmembrane domain, is sufficient for axial budding. In addition, our work demonstrates a previously unappreciated role for Axl2p. Axl2p interacts with Cdc42p and other polarity-establishment proteins, and it regulates septin organization in late G1 independently of its role in polarity-axis determination. Together, these results suggest that Axl2p plays sequential and distinct roles in the regulation of cellular morphogenesis in yeast cell cycle.

INTRODUCTION

Development of cell polarity is essential for the formation of diverse cell types in multicellular organisms, and it is critical for carrying out a number of cellular functions, including neuronal transmission, nutrient transport across epithelia, chemotaxis, and asymmetric cell division. Studies from various model systems indicate that the overall design and the core mechanisms underlying polarity establishment are conserved from yeast to humans, which involves a hierarchy of temporally and spatially coordinated events, including selection of a polarity-axis, cytoskeletal polarization, and cytoskeleton-guided protein trafficking (Drubin and Nelson, 1996; Nelson, 2003). In the budding yeast *Saccharomyces cerevisiae*, polarity-axis determination or bud-site selection differs in different cell types (Hicks *et al.*, 1977; Chant and Pringle, 1995). Haploid *MATa* or α cells bud in an axial pattern, i.e., new buds are formed adjacent to the site of previous cell division. Diploid *MATa*/ α cells bud in a bipolar pattern, i.e., daughter cells bud at the distal pole, which is opposite to the site of cell division, whereas mother cells bud at either the distal or the proximal pole. The current thought on bud-site selection and polarized growth in *S. cerevisiae* is that an axial or a bipolar landmark in the cell

cortex, which is assembled in previous cell cycle(s), is recognized by the Rsr1p (also known as Bud1p) GTPase module, and the positional information is relayed to polarity-establishment machinery, which is centered on the conserved Cdc42p GTPase module, resulting in the recruitment and the activation of Cdc42p at the chosen site (Chant, 1999; Casamayor and Snyder, 2002; Park and Bi, 2007). Activated Cdc42p polarizes the actin cytoskeleton, including actin patches and actin cables, which are involved in endocytosis and exocytosis, respectively; and polarized exocytosis results in bud formation (Moseley and Goode, 2006; Park and Bi, 2007).

Genetic analysis indicates that the axial landmark consists of Axl1p, Axl2p (also known as Bud10p), Bud3p, Bud4p, and the septins (Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p/Sep7p) (Flescher *et al.*, 1993; Fujita *et al.*, 1994; Adames *et al.*, 1995; Chant *et al.*, 1995; Halme *et al.*, 1996; Roemer *et al.*, 1996; Sanders and Herskowitz, 1996). Null or conditional-lethal mutations in each of these genes result in bipolar budding in *MATa* or α cells, but they have no effect on the budding pattern of *MATa*/ α cells. The bipolar landmark consists of at least Bud8p, Bud9p, Rax1p, and Rax2p (Chen *et al.*, 2000; Harkins *et al.*, 2001; Fujita *et al.*, 2004; Kang *et al.*, 2004a). Null mutations in any of these genes result in unipolar (for *BUD8* and *BUD9*) or nonrandom (*RAX1* and *RAX2*) budding in *MATa*/ α cells, but they have no effect on the axial budding of *MATa* or α cells. The axial- or bipolar-landmark proteins are thought to recruit Bud5p, the guanine nucleotide-exchange factor (GEF) for Rsr1p, and also Bud2p, the GTPase-activating protein (GAP) for Rsr1p, to the cortical site, which activates the GTPase cycling of Rsr1p

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(Park *et al.*, 1999; Kang *et al.*, 2001, 2004b; Marston *et al.*, 2001). Null mutations in *RSR1*, *BUD2*, or *BUD5* result in random budding in all cell types. Rsr1p-GTP binds to Cdc24p, the GEF for Cdc42p, whereas Rsr1p-GDP binds to Bem1p, a polarity-establishment protein that also binds to Cdc24p and Cdc42p-GTP (Zheng *et al.*, 1995; Park *et al.*, 1997; Gulli *et al.*, 2000; Bose *et al.*, 2001; Kozminski *et al.*, 2003). These molecular interactions constitute positive-feedback loops to localize, activate and amplify Cdc42p at the chosen site. As a consequence, a new bud site is assembled and is marked by polarized actin cytoskeleton and a septin ring.

Axl2p plays a key role in axial budding. First, the budding pattern of *axl1Δ*, *bud3Δ*, or *bud4Δ* cells is reverted to axial budding by *rax1Δ* (Lord *et al.*, 2002; Fujita *et al.*, 2004); in contrast, *axl2Δ rax1Δ* cells bud randomly (Fujita *et al.*, 2004), suggesting that Axl2p is the true axial landmark whereas the functions of Axl1p, Bud3p, and Bud4p in axial budding may be more regulatory in nature. Second, Axl2p is thought to recruit Bud5p to the axial landmark (Kang *et al.*, 2001; Marston *et al.*, 2001), which, via Rsr1p and Cdc24p as described above, causes accumulation of Cdc42p at the chosen site. Thus, understanding the role of Axl2p in axial budding is essential for understanding how a new growth site is selected and assembled in *MATa* or α cells. Axl2p is a type I membrane glycoprotein with its bulk N-terminal region sticking out into the extracellular space, a single transmembrane domain spanning the plasma membrane, and a C-terminal tail staying in the cytoplasm (Roemer *et al.*, 1996). The extracellular domain of Axl2p consists of four putative cadherin-like motifs (Dickens *et al.*, 2002), whose function remains unknown. The expression of Axl2p peaks in late G1, unlike other components of the axial landmark such as Bud3p and Bud4p, whose expression peaks at S/G2 and M phase, respectively (Cho *et al.*, 1998; Spellman *et al.*, 1998; Lord *et al.*, 2000). In addition, Bud3p and Bud4p localize exclusively to the bud neck in a septin-dependent manner in medium- and large-budded cells (Chant *et al.*, 1995; Sanders and Herskowitz, 1996), whereas Axl2p localizes to the sites of polarized growth early in the cell cycle, which depends on an intact secretory pathway (Powers and Barlowe, 1998, 2002). It is not known whether Axl2p plays an additional role in polarized growth independently of its role in bud-site selection, as suggested by its early cortical localization. Axl2p also localizes to the bud neck some time after bud emergence (Halme *et al.*, 1996; Roemer *et al.*, 1996), but it is not clear whether Bud3p and Bud4p are required for this neck localization (Halme *et al.*, 1996; Roemer *et al.*, 1996). It is also not known how Axl2p interacts with Axl1p, Bud3p, and Bud4p to form a functional axial landmark.

The septins are a family of GTP-binding proteins that form heteromeric complexes and filaments *in vivo* and *in vitro* (Gladfelter *et al.*, 2001b; Longtine and Bi, 2003; Hall and Russell, 2004; Joo *et al.*, 2005; Versele and Thorner, 2005; Kinoshita, 2006). Septins play important roles in cytokinesis, mitosis, protein trafficking, and apoptosis. Overexpression of septins has been associated with variety of different human tumors (Hall and Russell, 2004; Hall *et al.*, 2005). In *S. cerevisiae*, septins localize to the bud neck throughout the cell cycle, and they function as a scaffold, upon which proteins involved in axial budding such as Bud3p and Bud4p, chitin synthesis, and cytokinesis are anchored (Gladfelter *et al.*, 2001b; Longtine and Bi, 2003; Versele and Thorner, 2005). Septins also function as a diffusion barrier at the bud neck to prevent the free movement of membrane or membrane-

associated proteins between cellular compartments (Barral *et al.*, 2000; Takizawa *et al.*, 2000; Dobbelaere and Barral, 2004). Septins undergo dynamic structural changes in the cell cycle (Caviston *et al.*, 2003; Dobbelaere *et al.*, 2003). At the beginning of the cell cycle, septins are dynamic and freely exchangeable with cytosolic septins at the presumptive bud site. After bud emergence, septin collar or hourglass at the bud neck becomes very stable. After the septin collar is split into two distinct rings in anaphase, septins become dynamic again. These organizational changes presumably underlie the involvements of the septins in different cellular processes such as bud morphogenesis and other functions described above.

The initial formation of the septin collar involves at least three steps: the recruitment of the septins to the presumptive bud site, which depends on Cdc42p and, at least in part, its effectors Gic1p and Gic2p (Caviston *et al.*, 2003; Longtine and Bi, 2003; Iwase *et al.*, 2006); the septin-ring assembly, which depends on Cdc42p GTP hydrolysis (Gladfelter *et al.*, 2002; Smith *et al.*, 2002; Caviston *et al.*, 2003) and its effector, the PAK Cla4p (Cvrckova *et al.*, 1995; Weiss *et al.*, 2000; Goehring *et al.*, 2003; Gladfelter *et al.*, 2004; Kadota *et al.*, 2004; Versele and Thorner, 2004); and the septin-ring maturation into a collar or hourglass, which is regulated by a number of proteins, such as Bni5p (Lee *et al.*, 2002), Nap1p (Mortensen *et al.*, 2002; Gladfelter *et al.*, 2004; Iwase and Toh-e, 2004), and the protein kinases Cla4p (Dobbelaere *et al.*, 2003; Versele and Thorner, 2004), Gin4p (Longtine *et al.*, 1998a) and Elm1p (Bouquin *et al.*, 2000). Many more septin regulators are likely to be identified if more sensitive approaches are used. For example, overexpression of the Swe1p kinase, which causes a G2 delay and elongated bud morphology by inhibiting Cdc28p kinase activity, is known to exacerbate a septin defect (Gladfelter *et al.*, 2005). By using this tool, a number of proteins, including Bud3p, Bud4p, and Bud5p, are also found to play a role in septin organization, although the timing and the mechanisms underlying the functions of these proteins in septin organization remain unknown.

Many mutations in the effector-loop region of *CDC42* cause septin defects to some extent (Richman *et al.*, 1999; Kozminski *et al.*, 2000; Richman and Johnson, 2000; Gladfelter *et al.*, 2001a). Two such mutations, *cdc42^{V36T}* and *cdc42^{V36A}*, were found to cause a decrease in the intrinsic GTPase activity of Cdc42p, elongated bud morphology, and septin-organization defects (Gladfelter *et al.*, 2001a, 2002). A similar mutation, *cdc42^{V36G}*, was identified from our screen for temperature-sensitive mutations by a polymerase chain reaction (PCR)-based random mutagenesis approach and was found to display a highly penetrant defect in septin organization even at the permissive temperature (Caviston *et al.*, 2003). We used this allele to perform gene dosage-suppressor screens in the hope of identifying genes that would help us elucidate how Cdc42p regulates septin organization. *AXL2* and several other functionally relevant genes were identified from such screens. Detailed structure-function analysis indicates that Axl2p is recruited to the bud neck in a Bud3p- and Bud4p-dependent manner via an interaction between the cytoplasmic tail of Axl2p and Bud4p. The role of Axl2p in the sequential assembly of the axial landmark and in the selection of the bud site does not require its expression in late G1. In addition, we found that Axl2p interacts with Cdc42p and other polarity-establishment proteins and that it regulates septin organization early in the cell cycle independently of its role in bud-site selection.

Table 1. *Saccharomyces cerevisiae* strains used in this study

Name	Genotype	Source
YEF473A	a <i>his3 leu2 lys2 trp1 ura3</i>	Bi and Pringle (1996)
YEF2921	a <i>his3 leu2 lys2 trp1 ura3 cdc42^{V36G}</i>	Caviston <i>et al.</i> (2003)
YEF3490	α <i>his3Δ leu2Δ lys2Δ ura3Δ axl2Δ::KanMX</i>	Invitrogen
YEF3491	α <i>his3Δ leu2Δ lys2Δ ura3Δ axl1Δ::KanMX</i>	Invitrogen
YEF3492	α <i>his3Δ leu2Δ lys2Δ ura3Δ bud3Δ::KanMX</i>	Invitrogen
YEF3493	α <i>his3Δ leu2Δ lys2Δ ura3Δ bud4Δ::KanMX</i>	Invitrogen
YEF4889	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX bem1Δ::HIS3, pRS425-AXL2-3HA</i>	This study ^a
YEF4894	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX gin4Δ::TRP1 BUD3-AXL2-C::GFP-URA3 CDC3::mCherry-LEU2</i>	This study ^b
YEF4895	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX gin4Δ::TRP1 GIC2-AXL2-C::GFP-URA3 CDC3::mCherry-LEU2</i>	This study ^b
YEF4896	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX gin4Δ::TRP1 CDC3::mCherry-LEU2</i>	This study ^b
YEF4897	a <i>his3 leu2 lys2 trp1 ura3 bud5Δ::HIS3 gin4Δ::TRP1 CDC3::mCherry-LEU2</i>	This study ^b
Y5563	α <i>leu2-Δ0 his3-Δ1 ura3-Δ0 met15-Δ0 lyp1Δ can1Δ::MFA1pro-HIS3</i>	Tong <i>et al.</i> (2001)
LSY134	α <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX</i>	See text
LSY136	α <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX</i>	See text
LSY143	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX gin4Δ::TRP1</i>	This study ^c
LSY220	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX BUD3::GFP-TRP1</i>	See text
LSY222	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX BUD4::GFP-TRP1</i>	See text
LSY295	α <i>leu2-Δ0 his3-Δ1 ura3-Δ0 met15-Δ0 lyp1Δ can1Δ::MFA1pro-HIS3 axl2Δ::NatMX</i>	See text
M-267	a <i>his3 leu2 lys2 trp1 ura3 gin4Δ::TRP1</i>	Longtine <i>et al.</i> (1998a)
M-1075	a <i>his3 leu2 lys2 trp1 ura3 swe1Δ::LEU2</i>	Caviston <i>et al.</i> (2003)
M-1077	α <i>his3 leu2 lys2 trp1 ura3 swe1Δ::LEU2</i>	Longtine <i>et al.</i> (2000)
AM101	α <i>his3 leu2 lys2 trp1 ura3 bud3Δ::HIS3</i>	J. Pringle
BY4742	α <i>his3Δ leu2Δ lys2Δ ura3Δ</i>	Invitrogen
KNY118	α <i>his3 leu2 lys2 trp1 ura3 bud4Δ::HIS3</i>	J. Pringle
JGY806	a <i>his3 leu2 lys2 trp1 ura3 bud5Δ::HIS3 gin4Δ::TRP1</i>	This study ^d
JGY824	a <i>his3 leu2 lys2 trp1 ura3 rsr1Δ::TRP1 gin4Δ::TRP1</i>	This study ^d
JGY826	a <i>his3 leu2 lys2 trp1 ura3 bud2Δ::TRP1 gin4Δ::TRP1</i>	This study ^d
JGY841	α <i>his3 leu2 lys2 trp1 ura3 axl1Δ::KanMX</i>	See text
JGY860	a <i>his3 leu2 lys2 trp1 ura3 cdc42^{V36G} swe1Δ::LEU2</i>	See text
JGY864	a <i>his3 leu2 lys2 trp1 ura3 cdc42^{V36G} axl2Δ::KanMX swe1Δ::LEU2</i>	See text
JGY969	a <i>his3 leu2 lys2 trp1 ura3 CDC3::GFP-LEU2</i>	This study ^b
JGY1061	a <i>his3 leu2 lys2 trp1 ura3 cdc42^{V36G} axl1Δ::KanMX</i>	See text
JGY1063	a <i>his3 leu2 lys2 trp1 ura3 cdc42^{V36G} bud3Δ::HIS3</i>	See text
JGY1065	a <i>his3 leu2 lys2 trp1 ura3 cdc42^{V36G} bud4Δ::HIS3</i>	See text
JGY1382	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX BUD3::3HA-HIS3MX BUD4::13MYC-TRP1</i>	See text
JGY1437	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX BUD3::GFP-TRP1 bud4Δ::HIS3</i>	See text
JGY1439	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX bud3Δ::HIS3 BUD4::GFP-TRP1</i>	See text
JGY1498	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX BUD3::GFP-URA3</i>	See text
JGY1499	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX BUD3-AXL2-C::GFP-URA3</i>	See text
JGY1612	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX gin4Δ::TRP1 BUD3::GFP-URA3</i>	See text
JGY1613	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX gin4Δ::TRP1 BUD3-AXL2-C::GFP-URA3</i>	See text
JGY1624	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX GIC2::GFP-URA3</i>	See text
JGY1625	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX GIC2-AXL2-C::GFP-URA3</i>	See text
JGY1626	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX gin4Δ::TRP1 GIC2::GFP-URA3</i>	See text
JGY1627	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX gin4Δ::TRP1 GIC2-AXL2-C::GFP-URA3</i>	See text
JGY1570	a <i>his3 leu2 lys2 trp1 ura3 axl2Δ::KanMX, pRS425-AXL2-3HA</i>	This study ^e

^a First, a *bem1 Δ ::HIS3* was introduced in diploid strain YEF473 (Bi and Pringle, 1996) by using a PCR-based method (Baudin *et al.*, 1993) with the plasmid pRS303 (*HIS3*, integrative plasmid) as the template, yielding the strain YEF1453 (**a**/ α *BEM1/bem1 Δ ::HIS3*). Chromosomal DNA isolated from YEF1455 (**a** *bem1 Δ ::HIS3*), a segregant of YEF1453, was used as template to amplify the *bem1 Δ* locus by PCR and introduced into JGY1570, yielding strain YEF4889. The correctness of the *bem1 Δ* strains was confirmed by PCR and mutant phenotypes.

^b YIp128-CDC3-mCherry and YIp128-CDC3-GFP were linearized by BglII digestion and introduced into the strains JGY1613, JGY1627, LSY143, JGY806, and YEF473A.

^c A segregant from the cross of LSY136 and M-267.

^d Strains LRSy378 (**a** *bud5 Δ ::HIS3*), JGY787 (**a** *rsr1 Δ ::TRP1*), which is a segregant of LRSy389 (**a**/ α *rsr1 Δ ::TRP1/rsr1 Δ ::TRP1*), and JGY789 (**a** *bud2 Δ ::TRP1*), which is a segregant of LRSy391 (**a**/ α *bud2 Δ ::TRP1/bud2 Δ ::TRP1*), were crossed to M-267 (α *gin4 Δ ::TRP1*). The resulting diploids were sporulated and tetrads were dissected to generate the desired strains. All LRSy strains were supplied by the Pringle laboratory.

^e Plasmid pRS425-AXL2-3HA was introduced into the strain LSY134.

MATERIALS AND METHODS

Strains and Genetic Methods

Yeast strains used in this study are listed in Table 1. Standard culture media and genetic techniques were used except where noted (Guthrie and Fink, 1991). *Escherichia coli* strains DH12S (Invitrogen, Carlsbad, CA) and BL21 (Invitrogen) were used as hosts for plasmid manipulation and recombinant

protein expression, respectively. Oligonucleotide primers for PCR were purchased from Integrated DNA Technologies (Coralville, IA).

Construction of Plasmids and Yeast Strains

Common plasmid vectors used in this study include YEp24 (2 μ , *URA3*), YEp352 (2 μ , *URA3*), pRS426 (2 μ , *URA3*), pRS316 (*CEN*, *URA3*), pRS306 (integrative, *URA3*), and YIp128 (integrative, *LEU2*). Other plasmids include

pUG23 (*CEN*, *HIS3*, *pMET25-C-yEGFP3* for C-terminal green fluorescent protein [GFP] tagging), pUG35 (*CEN*, *URA3*, *pMET25-C-yEGFP3* for C-terminal GFP tagging), pUG36 (*CEN*, *URA3*, *pMET25-N-yEGFP3* for N-terminal GFP tagging) (supplied by J. H. Hegemann, Heinrich-Heine-Universität Duesseldorf, Germany); pOAD (*CEN*, *LEU2*, *GAL4-AD*), pGBDU-C1 (2μ , *URA3*, *GAL4-BD*) (James *et al.*, 1996), YIp211-CD3C-GFP (Caviston *et al.*, 2003), pRS316-SWE1-12MYC (pJM1091) (McMillan *et al.*, 2002), YEp352-AXL1 (p129) (Adames *et al.*, 1995), and YEp24-STE23 (p261; isolated from a YEp24-based genomic DNA library). Plasmids YEp24-CD42, YEp24-BEM1, YEp24-AXL2, YEp24-ELM1, and YEp24-CLA4 used in Figure 1 of this study were all isolated from the multicopy-suppressor screen with *cdc42*^{V36G} mutant.

YEp352-BUD3 was constructed by inserting an 8.2-kb PstI fragment carrying *BUD3* from p35-1 (Chant *et al.*, 1995) into PstI-digested YEp352. YEp352-BUD4 was constructed by inserting a 7.3-kb SacI fragment carrying *BUD4* from pJC109 (kindly provided by M. Lord, University of Vermont) into SmaI-digested YEp352. Ylp128-CDC3-GFP was constructed by inserting a 5.3-kb SalI-EcoRI fragment carrying *CDC3-GFP* from Ylp211-CDC3-GFP into Ylp128-C. Ylp128-CDC3-mCherry was constructed by PCR-amplifying a NotI fragment carrying *mCherry* by using a pair of primers with NotI site and the plasmid pKT355 (supplied by K. Thorn, University of California at San Francisco) as the template, the resulting NotI-*mCherry* fragment was used to replace the NotI-*GFP* fragment in Ylp128-CDC3-GFP. Ylp211-CDC3-GFP, Ylp128-CDC3-GFP, and Ylp128-CDC3-mCherry were linearized with BglII for integration at the *CDC3* locus in yeast. Plasmids pUG23-AXL2, pUG23-AXL2-N (residues 1–503), pUG23-AXL2-N+TM (residues 1–535), and pUG23-AXL2-C (residues 530–823) were constructed via gap repair of PCR-amplified *AXL2* fragments onto EcoRI-digested pUG23 vector in yeast. Yeast chromosomal DNA carrying wild-type *AXL2* was used as the template for the PCRs, and the *AXL2* fragments were inserted between the PstI and EcoRV sites of the pUG23 vector.

AXL2-C and various regions of AXL2-C in Figure 4B of this study were first amplified by PCR as EcoRI-BamHI fragments, and they were inserted into EcoRI- and BamHI-digested pGBDU-C1 vector. These fragments in pGBDU-C1 were further digested with EcoRI and SalI and ligated into the pUG36 vector to generate GFP fusions to various regions of Axl2p-C. To generate a series of strains each containing a modified allele of AXL2 at its chromosomal locus, several strategies were used. To generate chromosomal *axl2*¹⁻⁸²³, *axl2*¹⁻⁷²⁵, *axl2*¹⁻⁶⁸⁵, *axl2*¹⁻⁶⁴⁶, and *axl2*¹⁻⁶²⁷ constructs (1-823 stands for residues 1-823 of Axl2p) in yeast, AXL2-C⁵³⁰⁻⁸²³, AXL2-C⁵³⁰⁻⁷²⁵, AXL2-C⁵³⁰⁻⁶⁸⁵, AXL2-C⁵³⁰⁻⁶⁴⁶, and AXL2-C⁵³⁰⁻⁶²⁷ were isolated as EcoRI-SalI fragments from pGBDU-based constructs and ligated into EcoRI- and SalI-digested pRS306-T_{CYC1} vector, which was constructed by inserting a 297-base pair EcoRI-KpnI fragment containing the 3'-UTR region of CYC1 gene from the pUG36 vector into the pRS306 vector. The resulting plasmids were linearized with SphI for integration at the AXL2 locus in yeast. After integration, wild-type AXL2 on the chromosome will be replaced by these mutant constructs. To generate chromosomal *axl2*¹⁻⁵⁴⁴, 726-823, *axl2*¹⁻⁵⁴⁴, 686-823, *axl2*¹⁻⁵⁴⁴, 628-823, *axl2*¹⁻⁵⁴⁴, 641-725, *axl2*¹⁻⁵⁴⁴, 641-685, and *axl2*¹⁻⁵⁴⁴, 686-725 constructs in yeast, AXL2-C⁷²⁶⁻⁸²³, AXL2-C⁶⁸⁶⁻⁸²³, AXL2-C⁶²⁸⁻⁸²³, AXL2-C⁶⁴¹⁻⁷²⁵, AXL2-C⁶⁴¹⁻⁶⁸⁵, and AXL2-C⁶⁸⁶⁻⁷²⁵ were isolated as EcoRI-SalI fragments from pGBDU-based constructs and ligated into EcoRI- and SalI-digested pRS306-AXL2-MidT_{CYC1} vector, which was constructed by inserting a PCR-amplified BamHI-EcoRI AXL2 fragment encoding residues 253-544 into the pRS306-T_{CYC1} vector. The resulting plasmids were linearized with HindIII for integration at the AXL2 locus in yeast. After integration, wild-type AXL2 on the chromosome will be replaced by these mutant constructs. To generate the chromosomal *axl2*¹⁻⁵⁴⁴ construct in yeast, a PCR-amplified SphI-BamHI fragment containing a stop codon and the 3'-untranslated region (UTR) (407 base pairs) of AXL2 right after the SphI site was inserted into SphI- and BamHI-digested pRS426-AXL2 vector to generate pRS426-AXL2¹⁻⁵⁴⁴. The purpose of this step is to introduce a stop codon and the 3'-UTR after the internal SphI site of AXL2. The AXL2 fragment encoding residues 239-544 with the stop codon and the 3'-UTR was isolated as a 1.4-kb EcoRI-BamHI fragment from pRS426-AXL2¹⁻⁵⁴⁴ and was inserted into the pRS306 vector to generate pRS306-AXL2-MS1, which was then linearized with HpaI to integrate at the AXL2 locus in yeast.

Plasmid pRS426-AXL2 was constructed by inserting a PCR-amplified SacI-BamHI fragment carrying AXL2, including 970 base pairs of upstream and 407 base pairs of downstream sequences, into the SacI- and BamHI-digested pRS426 vector. Plasmid pRS426-AXL2¹⁻⁶⁴⁶ was constructed by replacing the SphI-KpnI insert of pRS426-AXL2 with the SphI-KpnI fragment from pRS306-AXL2³³⁰⁻⁶⁴⁶. Plasmids pRS426-AXL2¹⁻⁵⁴⁴, ⁶⁴¹⁻⁷²⁵, pRS426-AXL2¹⁻⁵⁴⁴, ⁶⁴¹⁻⁶⁸⁵, and pRS426-AXL2¹⁻⁵⁴⁴, ⁷²⁶⁻⁸²³ were constructed by replacing the HindIII-KpnI insert of pRS426-AXL2 with the HindIII-KpnI fragments from pRS306-AXL2²⁵³⁻⁵⁴⁴, ⁶⁴¹⁻⁷²⁵, pRS306-AXL2²⁵³⁻⁵⁴⁴, ⁶⁴¹⁻⁶⁸⁵, and pRS306-AXL2²⁵³⁻⁵⁴⁴, ⁷²⁶⁻⁸²³, respectively. Plasmid pRS425-AXL2-3HA was constructed by inserting a 4.9-kb SpeI fragment of AXL2-3HA from pJC246 (Lord *et al.*, 2000) into SpeI-digested pRS425 vector (2 μ , LEU2).

The generation of *BUD3-C-GFP* and *BUD3-AXL2-C-GFP* fusion constructs involves several steps. First, a PCR-amplified BamHI–EcoRI fragment encoding residues 1477–1636 of Bud3p without the stop codon was inserted into the pRS306-*T_{CYC1}* vector to generate pRS306-BUD3-C-*T_{CYC1}*. Second, an EcoRI–SalI fragment carrying *AXL2-C*, encoding residues 530–823 of Axl2p, from pGBDU-AXL2-C was inserted into pRS306-BUD3-C-*T_{CYC1}* to generate pRS306-BUD3-AXL2-C. Third, a unique MluI site within *T_{CYC1}* of pUG35 and

pUG23-AXL2-C was destroyed by MluI digestion followed by filling-in with the Klenow fragment of DNA polymerase I to generate pUG35-ΔMluI and pUG23-AXL2-C-ΔMluI. Fourth, the EcoRI-KpnI fragment of yEGFP3-T_{CYC1} from pUG35-ΔMluI was inserted into EcoRI- and KpnI-digested pRS306-BUD3-C-T_{CYC1} to generate pRS306-BUD3-C-GFP. Similarly, the SphI-KpnI fragment of AXL2-C-yEGFP3-T_{CYC1} from pUG23-AXL2-C-ΔMluI was inserted into SphI- and KpnI-digested pRS306-BUD3-AXL2-C vector to generate pRS306-BUD3-AXL2-C-GFP. Plasmids pRS306-BUD3-C-GFP and pRS306-BUD3-AXL2-C-GFP were linearized with MluI and integrated at the *BUD3* locus of LSY134 (*a axl2Δ::KanMX*) to generate strains JGY1498 and JGY1499, respectively. Similarly, these digested plasmids were integrated at the *BUD3* locus of LSY143 (*a axl2Δ::KanMX gin4Δ::TRP1*) to generate strains JGY1612 and JGY1613, respectively.

To generate chromosomal *GIC2-C-GFP* and *GIC2-AXL2-C-GFP* fusion, a 0.93-kb *SpeI* and *EcoRI* fragment carrying *GIC2-C*, which encodes residues 75–383 of *Gic2p*, was amplified by PCR, and it was ligated into *SpeI*- and *EcoRI*-digested pRS306-BUD3-C-GFP and pRS306-BUD3-AXL2-C-GFP vectors to replace *BUD3-C* with *GIC2-C* fragment. The resulting plasmids, pRS306-GIC2-C-GFP and pRS306-GIC2-AXL2-C-GFP, were digested with *AflII* and integrated at the *GIC2* locus of LSY134 (**a** *axl2Δ::KanMX*) to generate strains JGY1624 and JGY1625, respectively. Similarly, these digested plasmids were integrated at the *GIC2* locus of LSY143 (**a** *axl2Δ::KanMX gin4Δ::TRP1*) to generate strains JGY1626 and JGY1627, respectively.

Plasmids pEGKT-CDC42, pEGKT-BEM1, pEGKT-CDC24, pEGKT-RHO1, and pEGKT-GAB1 expressed N-terminal glutathione S-transferase (GST) fusion proteins under the control of the *GAL1* promoter of pEGKT (2 μ , URA3) (Mitchell *et al.*, 1993). To generate pEGKT-CDC42^{Q61L}, pEGKT-CDC42^{T17N} and pEGKT-CDC42^{D57Y}, a 0.6-kb BamHI-HindIII fragment carrying CDC42^{Q61L}, CDC42^{T17N}, and CDC42^{D57Y}, respectively, from pGEX-KG-CDC42^{Q61L}, pGEX-KG-CDC42^{T17N}, and pGEX-KG-CDC42^{D57Y} (supplied by D. Lew, Duke University Medical Center) (Gladfelter *et al.*, 2002), was inserted into pEGKT vector. To generate pGEX-KG-CDC42^{D57Y}, a 0.6-kb EcoRI-SacI fragment carrying CDC42^{D57Y} from pHB2-GST-CDC42^{D57Y} (Moskow *et al.*, 2000) was used to replace the EcoRI-SacI fragment carrying CDC42 in pGEX-KG-CDC42 (Gladfelter *et al.*, 2002). Plasmid pEGKT-AXL2-C was constructed by inserting the BamHI-Sall fragment carrying AXL2-C from pUG36-AXL2-C into pEGKT vector. To construct the pEGKT306 vector (integrative, URA3), an ~2.0-kb NcoI-BamHI fragment containing the *GALI* promoter and *GST* gene from pEGKT-AXL2-C was ligated into the NcoI and BamHI sites of pRS306-T_{CYC1} vector. Various regions of AXL2-C in the EcoRI-Sall fragments from the pGBDU-based constructs were inserted into pEGKT306 to generate pEGKT306-AXL2-C and its derivatives carrying different regions of AXL2-C. The resulting constructs were linearized with NcoI to integrate at the *ura3* locus in yeast.

Complete deletion of *AXL1* was constructed in diploid strain YEF473 (Bi and Pringle, 1996) by using the PCR-based method with pFA6a-KanMX6 template (Longtine *et al.*, 1998b). The heterozygous diploid strain was sporulated, and tetrads were dissected to generate strain JGY841. To construct strains LSY134 (α *axl2Δ::KanMX*) and LSY136 (α *axl2Δ::KanMX*), the *axl2Δ::KanMX* locus in strain YEF3490 (Invitrogen) was amplified by PCR and transformed into YEF473. The resulting heterozygote was sporulated, and the tetrads were dissected to generate LSY134 and LSY136. Strains LSY220 and LSY222 were constructed by crosses of LSY134 with KNY311 (α *BUD3::GFP-TRP1*) and KNY313 (α *BUD4::GFP-TRP1*) strains (supplied by J. Pringle, Stanford University Medical Center), followed by tetrad dissection. Similarly, JGY1437 and JGY1439 were constructed by crosses of LSY220 and LSY222 with AM101 and KNY118, followed by tetrad dissection. Strains JGY860, JGY1061, JGY1063, and JGY1065 were constructed by crosses of YEF2921 carrying plasmid pRS316-CDC42 with strains M-1077 (Longtine *et al.*, 2000), JGY841, AM101, and KNY118, respectively, and followed by loss of pRS316-CDC42 and tetrad dissection. To generate JGY864, strains M-1075 and LSY136 were crossed, and tetrads of heterozygous diploid were dissected to generate JGY808 (α *axl2Δ::KanMX swe1Δ::LEU2*), which was then crossed with YEF2921 carrying pRS316-CDC42, and tetrads of heterozygous diploid were dissected after the loss of pRS316-CDC42 to generate JGY864. To construct LSY295 (α *axl2Δ::NatMX*) for synthetic genetic array (SGA) analysis, the *axl2Δ::KanMX* locus in strain LSY134 was first switched to *axl2Δ::NatMX* as described previously (Tong *et al.*, 2001), which was then PCR amplified and transformed into the strain Y5663 to yield LSY295. LSY295 was crossed to ordered arrays of yeast deletion strains, which include deletions of all non-essential genes in *S. cerevisiae*, to identify genes that display synthetic-lethal or synthetic-sick relationship with *AXL2* (Tong *et al.*, 2001).

To construct JGY1382, chromosomal *BUD3* in the strain ML550 (**a** *BUD4::13MYC-TRP1*) (Lord *et al.*, 2002) was first tagged with 3HA epitope by the PCR-based method with pFA6a-3HA-His3MX template (Longtine *et al.*, 1998b) to generate JGY1332 (**a** *BUD3::3HA-HIS3MX BUD4::13MYC-TRP1*), which was then crossed with LSY136, and tetrads of the heterozygous diploid were dissected to generate JGY1382.

Genetic Suppressor Screens

Genetic suppressor screens were carried out in the yeast strain YEF2921 (a *cdc42^{V36G}*). Transformants were grown on SC-Ura plates at 35°C. In the first screen, a YEp24 (2 μ , *URA3*)-based genomic DNA library (Carlson and Botstein,

1982) was used. From ~6600 transformants screened, 52 suppressors were isolated; 41 of them were determined to carry *CDC42* (7×), *BEM1* (12×), *CLA4* (6×), *ELM1* (9×), and *AXL2* (7×). The rest suppressor plasmids each carry multiple open reading frames (ORFs) without clearly defined roles in cellular morphogenesis. Among the seven *AXL2*-containing suppressor plasmids, four carry full-length *AXL2*, which encodes a protein of 823 amino acids, whereas the other three plasmids carry two different 3'-end-truncated versions of *AXL2*, encoding residues 1–621 and 1–685 of Axl2p, respectively. The truncations occur at the two *Sau3AI* restriction sites near the 3' end of the *AXL2* ORF. Subcloning analysis confirmed that *AXL2* was solely responsible for the suppression. In the second screen, a p366 (*CEN, LEU2*)-based genomic DNA library was used (Bi and Pringle, 1996). From ~6000 transformants, 33 suppressors were isolated, and 20 of them were determined to carry *CDC42* (11×), *BEM1* (1×), *CLA4* (2×), and *AXL2* (6×).

Microscopy

A computer-controlled Eclipse 800 microscope (Nikon, Tokyo, Japan) and a high-resolution charge-coupled device camera (model C4742-95; Hamamatsu Photonics, Bridgewater, NJ) were used to visualize cell morphology and GFP-tagged proteins by differential interference contrast (DIC) and fluorescence microscopy, respectively. The images were acquired using Phase 3 Imaging Systems (Glen Mills, PA). To determine the budding pattern of a yeast strain, bud scars were visualized by staining with calcofluor as described previously (Pringle, 1991).

GST Pull-Down Assay

Yeast strains carrying pEGKT vector or pEGKT-*AXL2*-C were grown at 30°C to A_{600} of 0.4–0.5 in SC-Ura medium containing 2% raffinose. Galactose was added to a final concentration of 2%, and the cultures were grown for another 4 h to induce the expression of GST fusion proteins. Cells were then harvested and washed with phosphate-buffered saline (PBS) buffer. About 60 A_{600} units of cells were resuspended in 0.5 ml of cell lysis buffer (PBS buffer, 1 mM EDTA, 0.1% NP-40, plus a cocktail of protease inhibitors), and they were disrupted by vortexing in the presence of glass beads at 4°C. Cell debris was removed by centrifugation at $10,000 \times g$ for 15 min. The supernatant was incubated with 50 μ l of glutathione-Sepharose 4B beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with gentle rocking at 4°C for ~16 h. The beads were washed three times with cell lysis buffer, and the bound proteins were eluted by boiling in 100 μ l of 2X SDS sample buffer for 5 min. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by standard immunoblotting procedure by using enhanced chemiluminescence reagents. Primary antibodies used were mouse monoclonal antibodies against GST, MYC, hemagglutinin (HA), and GFP (Covance Research Products, Richmond, CA). Secondary antibody was horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA). GST fusion proteins in the pull-down precipitates were analyzed by 12.5% SDS-PAGE and immunoblotted with an anti-GST antibody.

For detecting the interactions of Axl2p with Cdc42p, Bem1p, and Cdc24p, strain JGY1570 carrying pEGKT-based constructs was grown in liquid SC-Leu-Ura media containing 2% raffinose. Cells were harvested after galactose induction for 4–8 h and lysed in 0.5 ml of GPLB buffer (PBS buffer, 5 mM $MgCl_2$, 1 mM dithiothreitol, plus a cocktail of protease inhibitors). Triton X-100 was added to a final concentration of 0.5% to the cell lysates. Cell lysates were gently rocked at 4°C for 60 min to extract membrane-bound proteins, such as Axl2p and Cdc42p. After centrifugation at $10,000 \times g$ for 15 min, the supernatants were used to perform the pull-down assay.

RESULTS

Identification of *AXL2* as a Dosage Suppressor of a *cdc42^{V36G}* Mutant

Previously, we have shown that a temperature-sensitive (Ts) *cdc42^{V36G}* mutant displayed severe defects in septin organization and cell morphology, including the formation of elongated buds and of cell chains at the permissive temperature of 23°C (Caviston *et al.*, 2003). The mutated residue valine 36 sits in the “effector loop” region of Cdc42p, which is thought to mediate the interactions of Cdc42p with its downstream effectors (Wittinghofer and Nassar, 1996) and its regulators such as GEFs, GAPs, and GDI (DerMardirossian and Bokoch, 2005). Because *cdc42^{V36G}* cells display highly penetrant septin-organization defects (Caviston *et al.*, 2003) (Figure 1B), we thought that by isolating dosage suppressors of *cdc42^{V36G}*, we might be able to gain some new insight into how Cdc42p is involved in septin organization. We performed two genetic screens to search for genes that, when

Figure 1. Multicopy *AXL2* suppresses *cdc42^{V36G}* in the absence of *SWE1*. (A) Suppression of *cdc42^{V36G}* by multicopy *BEM1*, *AXL2*, *ELM1*, and *CLA4*. Strain YEF2921 (a *cdc42^{V36G}*) carrying the YEp24 vector, YEp24-*CDC42*, YEp24-*BEM1*, YEp24-*AXL2*, YEp24-*ELM1*, or YEp24-*CLA4* was streaked onto SC-Ura plates and incubated for 3 d at the indicated temperatures. (B) Deletion of *SWE1* suppresses the morphological, and, to some extent, the septin-organization defects of *cdc42^{V36G}* cells. Strains YEF2921 (a *cdc42^{V36G}*) and JGY860 (a *cdc42^{V36G} swe1Δ*) carrying Ylp211-*CDC3*-GFP were grown at 24°C. Cell morphology and septin organization, indicated by Cdc3p-GFP, were visualized by DIC and fluorescence microscopy. (C) Differential suppression of *cdc42^{V36G} swe1Δ* mutant by multicopy *BEM1*, *AXL2*, *ELM1*, and *CLA4*. Strain JGY860 (a *cdc42^{V36G} swe1Δ*) carrying the YEp24 vector, YEp24-*CDC42*, YEp24-*BEM1*, YEp24-*AXL2*, YEp24-*ELM1*, or YEp24-*CLA4* was streaked onto SC-Ura plates and incubated at 24°C or 35°C for 3 d. (D) *SWE1* modulates the genetic interaction between *CDC42* and *AXL2*. Strains M-1075 (a *swe1Δ*), JGY860 (a *cdc42^{V36G} swe1Δ*), and JGY864 (a *cdc42^{V36G} axl2Δ swe1Δ*) were transformed with equal amounts of plasmid pRS316 or pRS316-*SWE1*-12MYC and grown on SC-Ura plate at 24°C for 3 d.

overexpressed, could rescue the growth defect of *cdc42^{V36G}* mutant at 35°C (see details of the screens in *Materials and*

Methods). *BEM1*, *CLA4*, and *AXL2* were isolated from both the multicopy-suppressor and the low copy-suppressor screens, whereas *ELM1* was isolated only from the multicopy-suppressor screen (Figure 1A; data not shown). Among these suppressors, *BEM1* showed the strongest suppression for the growth defect. *CLA4* and *ELM1* were weaker than *AXL2*. The morphological defects of the cells at 24°C also seemed to be suppressed to some extent by these multicopy suppressors with *CLA4* and *BEM1* showing the best suppression (data not shown).

Bem1p and Cla4p form a complex with Cdc42p and Cdc24p. This complex regulates Cdc24p by Cla4p-mediated phosphorylation, although the function of this phosphorylation remains controversial (Gulli *et al.*, 2000; Bose *et al.*, 2001). It is possible that the phosphorylation of Cdc24p by Cla4p is involved in a positive feedback to establish and/or maintain Cdc42p polarization at the incipient bud site (Bose *et al.*, 2001; Butty *et al.*, 2002; Irazoqui *et al.*, 2003). Cla4p is also directly involved in the assembly of the septin ring (Cvrckova *et al.*, 1995; Weiss *et al.*, 2000; Versele and Thorner, 2004). In addition, *BEM1* and *CLA4* are known multicopy suppressors for a number of *cdc42* effector-loop mutants (Richman *et al.*, 1999; Kozminski *et al.*, 2000; Richman and Johnson, 2000; Gladfelter *et al.*, 2001a; Gladfelter *et al.*, 2002). In particular, overexpression of either *CLA4* or *BEM1* suppressed the morphological and septin-organization defects of the *cdc42^{V36T}* and *cdc42^{V36A}* cells (Gladfelter *et al.*, 2001a). Together, these data suggest that the suppression of *cdc42^{V36G}* mutant by *BEM1* and *CLA4* could result from a general enhancement of Cdc42p downstream signaling and/or of Cdc42p polarization state at the presumptive bud site.

Unlike *BEM1* and *CLA4*, previous studies had not identified a genetic interaction of *CDC42* with either *AXL2* or *ELM1*. Neither *AXL2* nor *ELM1* was isolated from our previous multicopy-suppressor screens by using the *cdc42-201* and *cdc42^{G60D}* mutants (Gao *et al.*, 2004). *AXL2* encodes a type I transmembrane glycoprotein that participates in axial bud-site selection in haploid *MATa* or α cells (Halme *et al.*, 1996; Roemer *et al.*, 1996). *ELM1* encodes a protein kinase that is involved in septin organization and entry into mitosis (Blacketer *et al.*, 1993; Sreenivasan and Kellogg, 1999; Bouquin *et al.*, 2000). Because Elm1p plays an important role in the phosphorylation of Cla4p and of a Nim1-related protein kinase, Gin4p (Sreenivasan and Kellogg, 1999; Bouquin *et al.*, 2000) and because Gin4p is directly involved in the assembly of the septin ring (Longtine *et al.*, 1998a; Mortensen *et al.*, 2002), we examined whether overexpression of *GIN4* suppresses the *cdc42^{V36G}* mutant. We found that a low-copy plasmid carrying *GIN4* indeed suppressed the *cdc42^{V36G}* mutant at 35°C (data not shown). The suppression of the *cdc42^{V36G}* mutant by *ELM1*, *CLA4*, and *GIN4*, which encode protein kinases that function coordinately to control septin organization (Tjandra *et al.*, 1998; Bouquin *et al.*, 2000), is consistent with the fact that the most prominent defect of the *cdc42^{V36G}* mutant is in the assembly of the septin ring (Caviston *et al.*, 2003) (Figure 1B).

AXL2* Suppresses the *cdc42^{V36G}* Mutant Independently of *SWE1

Defects in bud formation, particularly in septin organization, in a number of mutants, including *elm1Δ*, *cla4Δ*, and *gin4Δ* mutants, trigger a Swe1p-dependent G2 delay that inhibits the apical-to-isotropic switch of bud growth, leading to an elongated bud morphology (Barral *et al.*, 1999; Bouquin *et al.*, 2000; Longtine *et al.*, 2000; Weiss *et al.*, 2000; Gladfelter *et al.*, 2005). To investigate whether the elongated bud mor-

phology of *cdc42^{V36G}* cells depends on Swe1p, we constructed a *cdc42^{V36G} swe1Δ* strain and found that deletion of *SWE1* dramatically improved the morphology of *cdc42^{V36G}* cells. The elongated bud morphology disappeared, and the septin defects, in particular, the localization of septins to the bud cortex, was greatly reduced (Figure 1B). However, septins at the bud neck were still misorganized. Cells still formed clusters and displayed temperature-sensitive growth at 35°C (Figure 1C). These results indicate that Swe1p plays a role in the exacerbation of the septin defects of the *cdc42^{V36G}* cells, supporting a conclusion reached in a recent study (Gladfelter *et al.*, 2005). However, it is noteworthy that the septin defects at the bud neck of the *cdc42^{V36G}* cells are largely independent of Swe1p.

The phenotypes of the *cdc42^{V36G} swe1Δ* mutant, including its temperature-sensitive growth and its improved cell morphology and septin organization, offered a potential way to classify our isolated suppressors into distinct functional groups. Indeed, we found that multicopy *BEM1* or *AXL2* suppressed the *cdc42^{V36G} swe1Δ* mutant at 35°C, whereas multicopy *CLA4* or *ELM1* failed to do so (Figure 1C). These results suggest that the suppression by *CLA4* or *ELM1* may be mediated through septin organization and/or Swe1p. In contrast, the suppression by *AXL2* and *BEM1* may occur via other unknown mechanisms.

To probe the functional interaction between *CDC42* and *AXL2* further, we found that *cdc42^{V36G}* and *axl2Δ* were synthetically sick at 24°C. Tetrad analysis showed that the majority of the *cdc42^{V36G} axl2Δ* double mutants failed to form colonies and that the few viable double mutants grew much more slowly than the *cdc42^{V36G}* single mutants did at 24°C. In contrast, the *cdc42^{V36G} axl2Δ swe1Δ* triple mutants from the same tetrad analysis grew very well (Figure 1D). Because of the heterogeneous behavior by the different *cdc42^{V36G} axl2Δ* segregants in their abilities to form colonies, it was difficult to determine the nature of the synthetic-sickness observed between *cdc42^{V36G}* and *axl2Δ*. This problem was circumvented by introducing a centromere-based plasmid carrying *SWE1* into the relatively healthy *cdc42^{V36G} axl2Δ swe1Δ* triple mutant. The *SWE1* plasmid inhibited the growth of the triple mutant as well as the *cdc42^{V36G} swe1Δ* double mutant, albeit to a lesser extent (Figure 1D). These results suggest that the synthetic-sickness between *cdc42^{V36G}* and *axl2Δ* is likely due to an exacerbated septin-organization defect that triggers Swe1p-dependent cell cycle delay (it is nearly impossible to distinguish the septin defects quantitatively in *cdc42^{V36G}* cells versus in *cdc42^{V36G} axl2Δ* cells, due to the already severe and diverse septin defects in the single mutant). These results also implicate a role of Axl2p in septin organization, a point that is strongly supported by other genetic studies described below.

***Axl2p* Interacts with *Cdc42p*, *Bem1p*, and *Cdc24p* In Vivo**

How did the multicopy *AXL2* suppress the *cdc42^{V36G}* mutant? One possibility is that Axl2p directly interacts with Cdc42p and/or its regulators to regulate Cdc42p functions. To examine this possibility, we performed GST pull-down assays. GST-Cdc42p, GST-Bem1p, or GST-Cdc24p, along with controls, were expressed in *axl2Δ* cells carrying *AXL2-3HA* on a high-copy plasmid, and they were pulled down with glutathione-Sepharose beads. The presence of Axl2p-3HA was detected by immunoblotting with an anti-HA antibody. Axl2p-3HA was pulled down with GST-Cdc42p, GST-Bem1p, and also weakly with GST-Cdc24p, but not with the two controls, GST-Rho1p and an endoplasmic reticulum protein GST-Gab1p (Grimme *et al.*, 2004) (Figure 2A), indicating that a pool of Axl2p forms complexes with

Figure 2. Genetic and biochemical interactions between Axl2p and Cdc42p. (A) Axl2p forms complex with Cdc42p, Bem1p, and Cdc24p in vivo. Strain JGY1570 (a *axl2Δ*, pRS425-AXL2-3HA) carrying the pEGKT vector (GST), pEGKT-CDC42 (GST-Cdc42p), pEGKT-BEM1 (GST-Bem1p), pEGKT-CDC24 (GST-Cdc24p), pEGKT-RHO1 (GST-Rho1p), or pEGKT-GAB1 (GST-Gab1p) was grown to the exponential phase in liquid SC-Leu-Ura medium containing 2% raffinose at 30°C. Galactose was added to a final concentration of 2%, and the cultures were grown for another 4 h to induce the expression of GST fusion proteins. GST-tagged proteins were pulled down with glutathione-Sepharose beads from equal amounts of Triton X-100-solubilized cell lysates. The amount of soluble Axl2p-HA in the cell lysates (Input) and in the pull-down precipitates (Bound) was analyzed by SDS-PAGE and immunoblotted with an anti-HA antibody. The amount of GST fusion proteins in the pull-down precipitates (Bound) was immunoblotted with an anti-GST antibody. (B) Axl2p interacts with Cdc42p independently of Bem1p. The GST-pull down experiment was performed as described in A except that the induction time for the expression of GST or GST-Cdc42p by galactose in host strain YEF4889 (*bem1Δ*) was 8 h instead of 4 h for all other strains, due to the slow growth of the *bem1Δ* strain. (C) Axl2p preferentially interacts with GDP-bound form of Cdc42p in vivo. A similar experiment, as described in A, was performed in strain JGY1570 (a *axl2Δ*, pRS425-AXL2-3HA) carrying pEGKT-CDC42 (WT), pEGKT-CDC42^{Q61L}, pEGKT-CDC42^{T17N}, or pEGKT-CDC42^{D57Y}. (D) The suppression of the *cdc42^{V36G}* mutant depends on the first one third of Axl2p-C. Strain YEF2921 (a *cdc42^{V36G}*) carrying the pRS426 vector, pRS426-AXL2 (1-823), pRS426-AXL2¹⁻⁵⁴⁴, pRS426-AXL2¹⁻⁶⁴⁶, pRS426-AXL2^{1-544, 641-725}, pRS426-AXL2^{1-544, 641-685}, and pRS426-AXL2^{1-544, 726-823} was streaked onto SC-Ura plates and incubated at 24 or 35°C for 3 d.

Cdc42p, Bem1p, and Cdc24p in vivo. Because Cdc42p and Bem1p are known to interact with each other (Gulli *et al.*, 2000; Bose *et al.*, 2001), we examined whether Axl2p could still interact with Cdc42p in the absence of Bem1p. As shown in Figure 2B, GST-Cdc42p still pulled down Axl2p-3HA, suggesting that Axl2p-Cdc42p interaction can occur independently of Bem1p.

To understand the nature of the in vivo biochemical interaction of Axl2p with Cdc42p, we examined whether Axl2p forms a complex with Cdc42p in different nucleotide-bound states, and we found that Axl2p preferentially formed a complex with Cdc42p^{T17N} and Cdc42p^{D57Y}, two mutants locked in the nucleotide-free or GDP-bound form (Moskow *et al.*, 2000; Nevins and Thurmond, 2003). A small fraction of Axl2p also formed complex with Cdc42p^{Q61L}, which is locked in the GTP-bound form (Figure 2C). In vitro protein-binding assays with recombinant proteins purified from bacteria failed to detect an interaction between MBP-Axl2p-C and GST-Cdc42p^{Q61L}, GST-Cdc42p^{T17N}, GST-Cdc42p^{D57Y}, or GST-Bem1p, although a strong and consis-

tent interaction between the positive control MBP-Gic2p, an effector of Cdc42p, and GST-Cdc42p^{Q61L} was detected (data not shown). These results suggest that the interaction between Axl2p and Cdc42p may not be direct. However, bacterially expressed Axl2p-C would lack yeast-specific modifications of the Axl2p tail, which contains several potential phosphorylation sites. This caveat may explain our failure to detect an interaction between Axl2p and Cdc42p in vitro.

In addition to the full-length gene, three truncated versions of AXL2 were isolated from the multicopy-suppressor screen, which are predicted to encode two C-terminally truncated proteins Axl2p¹⁻⁶²¹ and Axl2p¹⁻⁶⁸⁵. Axl2p¹⁻⁶²¹ carries only the first one third of its intracellular domain. To characterize further the region of Axl2p-C (the intracellular domain of Axl2p) required for the suppression, a series of constructs carrying different regions of Axl2p-C fused to the N-terminal extracellular domain plus the transmembrane domain of Axl2p were generated and their ability to suppress the *cdc42^{V36G}* mutant was examined. Consistently, Axl2p¹⁻⁶⁴⁶, a fusion construct carrying the first one third of

Axl2p-C (530-646 amino acids), was sufficient to suppress the *cdc42^{V36G}* mutant at 35°C (Figure 2D). In contrast, two other fusion constructs carrying either the middle portion (1-544 plus 641-725 amino acids) or the last one third (1-544 plus 726-823 amino acids) of Axl2p-C were unable to suppress. These results, together with our finding that Axl2p¹⁻⁶⁴⁶ lacks the Bud4p-interacting region and likely localizes to the cortex of a small bud, but not to the bud neck of middle- and large-budded cells (Figure 4, A and B), indicate that the first one third of Axl2p-C is involved in an interaction with Cdc42p and that this interaction likely occurs early in the cell cycle.

The Suppression of the *cdc42^{V36G}* Mutant by AXL2 Is Independent of Its Role in Bud-Site Selection

Before this study, Axl2p is known only for its role in the selection of an axial-budding site, a function that requires cooperation with three other components of the axial land-

mark, Bud3p, Bud4p, and Axl1p. To determine whether Axl2p shares its function in the suppression of *cdc42^{V36G}* mutant with these landmark proteins, we examined the effect of overexpression of Bud3p, Bud4p, and Axl1p on *cdc42^{V36G}* mutant and found that multicopy *BUD3*, *BUD4*, *AXL1*, and *STE23* (*STE23* encodes a functional homologue of Axl1p in the processing of mating pheromone α -factor; Adames *et al.*, 1995) did not suppress the *cdc42^{V36G}* mutant at 35°C (Figure 3A). In addition, *BUD3*, *BUD4*, and *AXL1* were all dispensable for the suppression of *cdc42^{V36G}* mutant by *AXL2*, as multicopy *AXL2* suppressed *cdc42^{V36G} bud3 Δ* , *cdc42^{V36G} bud4 Δ* and *cdc42^{V36G} axl1 Δ* mutant at 35°C (Figure 3B). These results indicate that *AXL2* does not share the function in the suppression of *cdc42^{V36G}* mutant with other components of the axial landmark.

To determine which region of the cytoplasmic tail of Axl2p is required for its role in bud-site selection, an expanded panel of *axl2* constructs used for suppression of

Figure 3. Axl2p displays separable functions in *cdc42^{V36G}* suppression and bud-site selection. (A) Multicopy *BUD3*, *BUD4*, *AXL1*, and *STE23* does not suppress *cdc42^{V36G}* mutant. Strain YEF2921 (a *cdc42^{V36G}*) carrying the YEp24 vector, YEp24-*AXL1*, YEp24-*STE23*, YEp24-*AXL2*, YEp24-*BUD3*, or YEp24-*BUD4* was streaked onto SC-Ura plates and incubated at 24 or 35°C for 3 d. (B) *BUD3*, *BUD4*, and *AXL1* are dispensable for the suppression of *cdc42^{V36G}* mutant by multicopy *AXL2*. Strains JGY1061 (a *cdc42^{V36G} axl1 Δ*), JGY1063 (a *cdc42^{V36G} bud3 Δ*), and JGY1065 (a *cdc42^{V36G} bud4 Δ*) were transformed with the YEp24 vector or YEp24-*AXL2* and the transformants were streaked onto SC-Ura plates and incubated at 24 or 35°C for 3 d. (C) The middle portion of Axl2p-C is required for the role of Axl2p in bud-site selection. A series of *AXL2* constructs carrying different regions of its tail were transformed into strain LSY134 (a *axl2 Δ*). All these constructs contain sequences encoding the N-terminal portion and the transmembrane domain of Axl2p. The budding pattern of each strain was determined by staining of bud scars with Calcofluor. One hundred cells with four or more bud scars were counted for each strain.

cdc42^{V36G} mutants was integrated into *axl2Δ* cells (Figures 2D and 3C), which bud in a bipolar pattern (Halme *et al.*, 1996; Roemer *et al.*, 1996). We found that a construct carrying the middle portion of Axl2p-C, 1-544 plus 641-725 amino acids, was fully functional in directing axial budding, whereas the two other fusion constructs carrying either the first half (1-685 amino acids) or the last one third of the tail (1-544 plus 726-823 amino acids) were unable to direct axial budding (Figure 3C), indicating that the middle portion of Axl2p-C, residues 641-725, plays an essential role in axial bud-site selection. These results, together with the *cdc42^{V36G}* suppression data described in the previous section, indicate that Axl2p plays at least two roles in polarized cell growth, one requiring interactions with Cdc42p and other polarity proteins, and the other being in the bud-site-selection process. These two roles are mediated via distinct regions of the cytoplasmic tail of Axl2p.

Roles of the Middle Portion of Axl2p-C in Axial Bud-Site Selection: Interaction With Other Components of the Axial Landmark

Among the components of the axial landmark, Axl2p displays a unique localization pattern in the cell cycle, an early bud-cortex localization similar to that of some polarity-establishment proteins, and a late bud-neck localization similar to that of other axial-landmark proteins. To determine which region of Axl2p is required for its targeting to the bud neck, the location where other axial-landmark proteins reside, a series of GFP fusion constructs containing different *AXL2* fragments under the control of a methionine-regulated promoter were made and introduced into *axl2Δ* cells for localization studies. We observed that the N-terminal region of Axl2p localized primarily in the vacuole and occasionally was also observed faintly decorating the plasma membrane (Figure 4A). In contrast, the N-terminal region plus the transmembrane domain localized to the bud cortex in small- to medium-budded cells, and to the bud neck region in large-budded cells. However, the latter localization was often seen more spread out at the bud-neck region. Interestingly, we found that the cytoplasmic tail of Axl2p localized to the bud neck as a tight double ring in medium- to large-budded cells, indicating that Axl2p-C plays a role in anchoring Axl2p to the bud neck. In addition, a fraction of Axl2p-C also localized to the nucleus. Further analysis with a constellation of methionine promoter-controlled GFP-*AXL2* C-terminal fragments, which expressed the fusion proteins at comparable levels (Figure 4B), indicates that the minimal bud neck-targeting region consists of amino acids 641-685, which is located in the middle portion of Axl2p-C (Figure 4, A and B, Axl2p-C9).

The bud-neck localization of Axl2p-C resembles that of Bud3p, Bud4p, and Axl1p (Chant *et al.*, 1995; Sanders and Herskowitz, 1996; Lord *et al.*, 2002). We found that the neck localization of Bud3p-GFP, Bud4p-GFP, or Axl1p-GFP did not depend on Axl2p (data not shown). In contrast, the neck localization of Axl2p-C depended on Bud3p and Bud4p, but not Axl1p (Figure 4C), raising the possibility that Axl2p-C might interact with Bud3p and Bud4p *in vivo*. Indeed, we found that GST-Axl2p-C pulled down Bud3p and Bud4p (Figure 4D), and the formation of this protein complex seems to be largely mediated by the middle region, residues 641-725, of Axl2p-C (Figure 4E), consistent with the requirement of this region in bud-neck targeting. In addition, the association of Axl2p-C with Bud3p seems to be mediated by Bud4p, because in the absence of Bud4p, GST-Axl2p-C no longer pulled down Bud3p (Figure 4F). In contrast, GST-Axl2p-C still efficiently pulled down Bud4p in the absence of

Bud3p (Figure 4F). These results demonstrate that Axl2p is recruited to the axial landmark through an interaction between the middle portion of its cytoplasmic tail with Bud4p. Because Bud4p hardly localizes to the bud neck in *bud3Δ* cells (Sanders and Herskowitz, 1996) yet still interacts with Axl2p-C, our results also suggest that the Axl2p-Bud4p interaction can occur in locations other than the bud neck and that the failure of Axl2p-C to localize in *bud3Δ* cells presumably reflects a decreased targeting of Bud4p to the bud neck in the mutant cells.

We also found that Axl2p-C9, the minimal region for bud-neck targeting in the Axl2p cytoplasmic tail, was unable to direct axial budding (Figure 3C). However, this region plus additional C-terminal sequence (residues 641-725), representing the middle portion of Axl2p-C, was able to confer axial budding to *axl2Δ* cells (Figure 3C). Thus, the middle portion of Axl2p-C may play an additional role in axial budding, in addition to the recruitment of Axl2p to the bud neck. For example, the cytoplasmic tail of Axl2p has been suggested to recruit and/or activate Bud5p, the GEF for Rsr1p (Kang *et al.*, 2001). It is noteworthy that even though the minimal targeting region, Axl2p-C9, localized to the bud neck, the signal at the neck was relatively weaker than the full-length Axl2p-C (Figure 4A). GST-Axl2p-C9 did not pull down detectable amount of Bud3p (Figure 4E). Even a larger fragment of Axl2p-C (residues 641-725), which is sufficient for axial budding, pulled down less Bud3p than the full-length Axl2p-C did. These results suggest that the minimal targeting domain alone cannot target to the bud neck as efficiently as the full-length tail and/or cannot maintain a stable association with Bud3p, presumably via Bud4p.

Axl2p Plays a Role in Septin Organization

The suppression of *cdc42^{V36G}* cells, which display pronounced defects in septin organization, by multicopy *AXL2*, *ELM1*, and *CLA4* suggests that *AXL2* may play a role in septin organization. This conclusion is further supported by the fact that a SGA analysis (Tong *et al.*, 2001) with an *axl2Δ* mutant against ordered arrays of >4000 yeast deletion strains identified 38 potential synthetic-sick interactions, and further tetrad analysis on these mutants confirmed a synthetic-sick interaction of *axl2Δ* with *elm1Δ*, but not with the rest of these candidates. To explore the potential role of Axl2p in septin organization further, we examined the synthetic effect of *axl2Δ* with *gin4Δ* and *cla4Δ* mutants, which are, primarily if not exclusively, defective in the assembly of the septin ring (Cvrckova *et al.*, 1995; Longtine *et al.*, 1998a; Weiss *et al.*, 2000; Versele and Thorner, 2004). We found that *axl2Δ gin4Δ* and *axl2Δ cla4Δ* mutants displayed synthetic morphological defects, including elongated bud morphology and cell chains, as well as septin-organization defects (Figure 5A; data not shown). The septins, indicated by Cdc3p-GFP, were often mislocalized to the bud cortex and misorganized at the bud neck (Figure 5A), indicating that Axl2p indeed plays a role in septin organization. The phenotype of *axl2Δ gin4Δ* mutant seemed more penetrant than *axl2 cla4Δ* mutant (data not shown), but less so than *axl2Δ elm1Δ* mutant (data not shown). *axl2Δ gin4Δ* cells also grew slightly slower than *gin4Δ* cells at 30°C.

We also found that overexpression of GST-Axl2p-C fusion protein induced elongated bud morphology in ~20% of the cells, whereas overexpression of GST alone caused a similar phenotype in only 1% of the cells (Figure 5B). In those GST-Axl2p-C-overexpressing cells with an elongated bud, septin organization seems to be defective (Figure 5B). Septins were often mislocalized to the bud-tip region, and they also seemed to be misorganized or less concentrated at the

Figure 4. The targeting of Axl2p to the bud neck is mediated by an interaction of Axl2p-C with Bud4p. (A) The pUG23 vector carrying full-length Axl2p (residues 1–823), N-terminal portion (Axl2p-N, residues 1–503), N-terminal portion plus transmembrane domain (Axl2p-N+TM, residues 1–535), the C-terminal tail (Axl2p-C, residues 530–823), and the pUG36 vector carrying the C9 fragment (Axl2p-C9, residues 641–685), the minimal bud neck-targeting region of Axl2p, was transformed into LSY134 (a *axl2Δ*) strain. The localization of GFP fusion proteins was visualized. (B) The expression levels and the targeting capacities of different Axl2p fragments. The pUG36 vector carrying different regions of Axl2p-C was transformed into LSY134 (a *axl2Δ*) strain. The resulting strains were grown to exponential phase in SC-Ura media at 25°C, and the localizations of different GFP fusion proteins were determined. The same strains were grown in SC-Ura-Met media at 30°C for 17 h to induce the expression of different GFP-Axl2p fusions. The levels of these fusion proteins were determined by Western blot with an antibody against GFP. (C) The plasmid pUG23-AXL2-C was transformed into BY4742 (WT), YEF3491 (*α axl1Δ*), YEF3492 (*α bud3Δ*), and YEF3493 (*α bud4Δ*) strains. The localization of Axl2p-C-GFP was visualized and quantified. For each strain, 50 cells with nuclei positioning at the extreme ends of the mother-daughter axis were counted. One hundred percent of such cells for the wild-type and *axl1Δ* strains displayed bud-neck localization, in comparison with 0 and 2% for the *bud3Δ* and *bud4Δ* strains, respectively. (D) Axl2p interacts with Bud3p and Bud4p. Strain JGY1382 (a *axl2Δ BUD3-3HA BUD4-13MYC*) was transformed with pEGKT or pEGKT-AXL2-C. GST and GST-Axl2p-C were pulled down from cell lysates by glutathione-Sepharose beads. Cell lysates (Input) and bound fractions were resolved by 6% SDS-PAGE (for Bud3p-HA and Bud4p-MYC) or 12.5% SDS-PAGE (for GST-fusions) and immunoblotted with antibodies against HA, MYC, and GST. (E) Axl2p-Bud3p interaction is mediated mainly via the middle one third of Axl2p-C. Strain JGY1464 (*α axl2Δ BUD3-3HA KCC4-13MYC*) was transformed with pEGKT306 (GST only), or pEGKT306 carrying various fragments of Axl2p-C. GST and GST fusion proteins were pulled down from cell lysates by glutathione-Sepharose beads. Cell lysates (Input) and bound fractions were resolved by 6% SDS-PAGE (for Bud3p-HA) or 12.5% SDS-PAGE (for GST fusions) and immunoblotted with antibodies against HA and GST. Note that GST-Axl2p-C (641–685) fusion failed to pull down Bud3p-HA, even though this fragment of Axl2p was able to localize to the bud neck (see Axl2p-C9 in A). This presumably reflects differences of various Axl2p fragments in neck-targeting efficiency and/or their differential abilities to form stable Axl2p-Bud3p complexes. (F) Axl2p-Bud3p interaction is mediated by Bud4p. Strains LSY220 (a *axl2Δ BUD3-GFP*), JGY1437 (a *axl2Δ BUD3-GFP bud4Δ*), LSY222 (a *axl2Δ BUD4-GFP*), and JGY1439 (a *axl2Δ bud3Δ BUD4-GFP*) were transformed with pEGKT-AXL2-C. Glutathione-Sepharose beads were used to pull down GST-Axl2p-C from cell lysates. GST-Axl2p-C, Bud3p-GFP, and Bud4p-GFP in the bound fraction (Bound) as well as Bud3p-GFP and Bud4p-GFP in the cell lysates (Input) were resolved by SDS-PAGE and detected by immunoblotting with anti-GST and anti-GFP antibodies.

Figure 5. Axl2p is involved in septin organization. (A) Synthetic defects in cell morphology and septin organization between *axl2Δ* and *gin4Δ*. Strains YEF473A (a WT), LSY136 (α *axl2Δ*), M-267 (a *gin4Δ*), and LSY143 (a *axl2Δ gin4Δ*) carrying YIp128-CDC3-GFP were grown on SC-Leu plates at 24°C for overnight. Cell morphology and Cdc3p-GFP localization were visualized. (B) Overexpression of the cytoplasmic tail of Axl2p causes septin defects. Strain JGY969 (a CDC3-GFP) carrying pEGKT or pEGKT-AXL2-C was streaked onto SC-Ura plate containing 2% galactose and 1% raffinose and grown at 30°C for ~16 h. Then, they were visualized for cell morphology and Cdc3p-GFP. (C) A set of AXL2 constructs as indicated was transformed into LSY143 (a *axl2Δ gin4Δ*) strain, and the transformants were grown on SC-Trp plates at 30°C overnight and then visualized for cell morphology. Top, rescue of morphological defect. Bottom, representative cell morphology. The strength of rescue was scored from strong (+++++) to no rescue (-).

bud neck. These results further support our notion that Axl2p is involved in septin organization.

The Role of Axl2p in Septin Organization Is Mediated Largely by the Middle Portion of Axl2p-C

Because the *axl2Δ gin4Δ* cells display severe defects in cell morphology and septin organization, this provides an excellent opportunity to address the requirement of Axl2p-C in septin organization. A series of constructs with different

regions of Axl2p-C fused to the N-terminal region plus the transmembrane domain of Axl2p were introduced into *axl2Δ gin4Δ* cells. The construct carrying the middle portion (residues 641–725) of Axl2p-C rescued the morphological defect of *axl2Δ gin4Δ* cells, whereas the construct carrying the last one-third (residues 726–823) of Axl2p-C did not rescue the morphology at all (Figure 5C). Interestingly, the construct carrying the first one third (residues 530–627) of Axl2p-C also seemed to rescue the morphological defect to

Figure 6. Late G1 expression of Axl2p is required for septin organization, but not for bud-site selection. (A) The GFP-tagged *BUD3*, *BUD3-AXL2-C*, *GIC2*, and *GIC2-AXL2-C* constructs were introduced into strain LSY134 (a *axl2Δ*) and LSY143 (a *axl2Δ gin4Δ*) strains. The budding pattern of strain LSY134 (a *axl2Δ*) carrying each of the four GFP fusion constructs (strains JGY1498, JGY1499, JGY1624, and JGY1625) was determined by staining of bud scars with calcofluor. The morphology of strain LSY143 (a *axl2Δ gin4Δ*) carrying these constructs (JGY1612, JGY1613, and JGY1626, and JGY1627) was also examined. The asterisk indicates that cells carrying the *GIC2-AXL2-C* construct still bud largely in a bipolar pattern, although there is a reduction in the percentage of cells budding bipolarly and a corresponding increase in the percentage of cells budding axially as indicated in the text. (B) The localization of Bud3p-Axl2p-C-GFP and Gic2p-Axl2p-C-GFP in strain LSY134 (a *axl2Δ*) (JGY1499 and JGY1625) was visualized. Please note that cells 1 and 2 are from the same field, reflecting the expression level and/or bud neck-targeting capacity of the fusion protein Bud3p-Axl2p-C-GFP. Images of cells 3 and 4 are taken and processed with the same settings. (C) A representative field of cell morphology of strain LSY143 (a *axl2Δ gin4Δ*) carrying GFP-tagged *BUD3*, *BUD3-AXL2-C*, *GIC2*, or *GIC2-AXL2-C* was shown. (D) A representative field of cells from strain LSY143 (a *axl2Δ gin4Δ CDC3::mCherry-LEU2*) carrying *BUD3-AXL2-C* and *GIC2-AXL2-C* (YEF4894 and YEF4895) was shown. (E) Expression levels of Bud3p-GFP and Bud3p-Axl2p-C-GFP in LSY134 (a *axl2Δ*) and LSY143 (a *axl2Δ gin4Δ*) strains. Total proteins were extracted and analyzed by immunoblotting using antibodies to GFP and porin (as a loading control). (F) Expression levels of Gic2p-GFP and Gic2p-Axl2p-C-GFP in LSY134 (a *axl2Δ*) and LSY143 (a *axl2Δ gin4Δ*) strains. Immunoblotting was performed as described in E.

some extent (Figure 5C). The morphological rescue by Axl2p-C constructs was correlated with improved septin organization (data not shown). Thus, this study identified a major role for the middle portion of Axl2p-C and a minor role for the first one third of Axl2p-C in septin organization.

Late G1 Expression of Axl2p Is Not Required for Bud-Site Selection, but It Is Required for Septin Organization

The expression of *AXL2*, *BUD3*, and *BUD4* is cell cycle regulated with their expression peaked in late G1, S/G2, and M phase, respectively (Cho *et al.*, 1998; Spellman *et al.*, 1998; Lord *et al.*, 2000). As a transmembrane protein, Axl2p is thought to rely on the highly polarized secretory machinery in late G1 to be delivered to the correct location (Powers and Barlowe, 1998), and the early expression of *AXL2* is thought to play a key role in bud-site selection, as constitutive or delayed expression affects the localization and function of Axl2p (Lord *et al.*, 2000). We reasoned that the observed loss of function could result from the mislocalization of Axl2p alone, and thus, the requirement of the early expression of Axl2p for its role in bud-site selection is still an open question. To examine this possibility, we generated a Bud3p-Axl2p-C chimera protein by fusing *AXL2-C* to the 3' end of

the *BUD3* ORF and integrated it into the chromosomal *BUD3* locus in *axl2Δ* cells. Bud3p-Axl2p-C was expressed under the control of the endogenous *BUD3* promoter and served as the sole source of Bud3p and Axl2p in the cell. By this approach, the expression of Axl2p-C was delayed to S/G2 phase without affecting its bud-neck localization. We found that the full-length Axl2p-C (residues 530–823) or the middle portion (residues 641–725) of the Axl2p tail, when fused to Bud3p, was completely functional in directing axial bud-site selection in *axl2Δ* cells (Figure 6A). 92% of axial budding for the middle portion of Axl2p tail fused to Bud3p). The chimeric Bud3p-Axl2p-C was expressed at a comparable level with Bud3p (Figure 6E) and displayed a similar localization profile in the cell cycle as Bud3p did. 100% of tiny-budded cells (Figure 6B, cell 2) displayed no or extremely faint signal of Bud3p-C-GFP (n = 12) or Bud3p-Axl2p-C-GFP (n = 11). In contrast, 100% of medium- to large-budded cells (Figure 6B, cell 1) expressing Bud3p-C-GFP (n = 32) or Bud3p-Axl2p-C-GFP (n = 47) displayed bud-neck localization. Interestingly, the expression of Bud3p-Axl2p-C completely failed to rescue the morphological and septin-organization defects of *axl2Δ gin4Δ* cells (Figure 6, C and D).

Using a similar approach, we fused *AXL2*-C to the 3' end of *GIC2* ORF, which encodes a Cdc42p effector (Brown *et al.*, 1997; Chen *et al.*, 1997). Gic2p was chosen because its expression level peaks around late G1/S and drops after bud emergence due to ubiquitin-mediated protein degradation (Jaquenoud *et al.*, 1998), which allows us to examine the effect of Gic2p-Axl2p-C expression in late G1. The *GIC2*-*AXL2*-C fusion construct was found to be largely unable to direct axial bud-site selection in *axl2Δ* cells (Figure 6A), although there was an increase of axial-budding cells in the population, from 18% for cells carrying *GIC2* alone to 30% for cells carrying *GIC2*-*AXL2*-C. However, when introduced into *axl2Δ gin4Δ* cells, the *GIC2*-*AXL2*-C construct fully rescued the morphological and the septin-organization defects (Figure 6, C and D). Gic2p-Axl2p-C was expressed (Figure 6F) and localized to the bud neck in 100% of tiny- ($n = 25$) and large-budded ($n = 58$) cells (Figure 6B, cells 3 and 4), consistent with an expected early expression and also with the observed partial suppression of the bud-site-selection defect. For unknown reasons, this fusion protein did not localize to the tip of a tiny bud as reported previously (Brown *et al.*, 1997; Iwase *et al.*, 2006). One possible explanation is that the Gic2p-Axl2p-C fusion protein is brought to and concentrated at the presumptive bud site by Gic2p. Once arriving at that site, the Axl2p-C portion of the fusion protein interacts with the septins or a septin-associated protein, resulting in the bud-neck localization. In addition, the endogenous level of Gic2p displayed very faint signal at the presumptive bud site, a phenomenon that has been observed previously (Jaquenoud *et al.*, 1998; Jaquenoud and Peter, 2000). Together, the results with the Bud3p- and the Gic2p-Axl2p-C fusion proteins indicate that late G1 expression of Axl2p is not required for bud-site selection, but it is required for septin organization.

The Role of Axl2p in Septin Organization Is Independent of Its Role in Bud-Site Selection

Like Axl2p, the components of the Rsr1 GTPase module, including the GEF Bud5p, the GAP Bud2p, and the Rsr1 GTPase itself, also localize to the bud cortex or bud neck in late G1 (Park *et al.*, 1999; Kang *et al.*, 2001; Marston *et al.*, 2001; Park *et al.*, 2002). To determine whether the role of Axl2p in septin organization depends on the Rsr1p GTPase module, we constructed double mutants, *bud5Δ gin4Δ*, *bud2Δ gin4Δ*, and *rsr1Δ gin4Δ*, and we compared them to the *axl2Δ gin4Δ* mutant in terms of cell morphologies and septin organization. We found that deletion of each of the components of the Rsr1 GTPase module did not appreciably affect the cell morphologies (Figure 7A) or septin organization (Figure 7B; data not shown), demonstrating that the role of Axl2p in septin organization is independent of the Rsr1p GTPase module.

DISCUSSION

Before this study, Axl2p was known only for its role in bud-site selection (Halme *et al.*, 1996; Roemer *et al.*, 1996). Based mainly on mutant analysis and localization studies, Axl2p, along with the septins, Bud3p, Bud4p, and Axl1p, were thought to be the components of the axial landmark (Chant, 1999; Casamayor and Snyder, 2002). However, it was not known how these proteins were assembled into a functional complex that participates in axial budding. In this study, we showed that Axl2p is targeted to the bud neck in a Bud3p- and Bud4p-dependent manner. In addition, we showed that late G1 expression of Axl2p is not required for its role in bud-site selection. Our study also uncovers two

Figure 7. The role of Axl2p in cell morphogenesis and septin organization is independent of the Rsr1 GTPase module. (A) Cells of strains LSY143 (*axl2Δ gin4Δ*), JGY806 (*bud5Δ gin4Δ*), JGY826 (*bud2Δ gin4Δ*), and JGY824 (*rsr1Δ gin4Δ*) were grown on YPD plates at 25°C for overnight, and cell morphologies were visualized by DIC microscopy. (B) Cells of strains LSY143 and JGY806 carrying *CDC3-mCherry* (Cdc3p-RFP) (strains YEF4896 and YEF4897) were grown on SC-Leu at 25°C for overnight, and the Cdc3p-RFP signal was visualized by fluorescence microscopy.

novel functions of Axl2p, an interaction with Cdc42p and other polarity proteins and a role in septin organization, both of which occur early in the cell cycle independently of its role in bud-site selection. These novel findings lay the foundation for understanding the key role of Axl2p in the selection of a polarity-axis and in linking the axis to polarity-establishment function.

Role of Axl2p in the Sequential Assembly of the Axial Landmark

Axl2p seems to be the key component of the axial landmark for two major reasons. First, deletion of *AXL1*, *AXL2*, *BUD3*, or *BUD4* all causes bipolar-budding pattern in haploid cells. But all these deletion strains except *axl2Δ* are reverted to axial budding when *RAX1*, which encodes a component of the bipolar landmark (Fujita *et al.*, 2004; Kang *et al.*, 2004a), is deleted (Lord *et al.*, 2002; Fujita *et al.*, 2004). In contrast, *axl2Δ rax1Δ* strains bud randomly (Fujita *et al.*, 2004). These data suggest that Axl2p is the true "physical" landmark for axial budding, whereas Bud3p, Bud4p, and Axl1p may "regulate" the functionality of Axl2p, including its localization and/or its activity. Second, Axl2p is thought to play a key role in communicating the positional information from the axial landmark in the cell cortex to Cdc42p-based polarity-establishment machinery via Bud5p, the GEF for Rsr1p GTPase (Kang *et al.*, 2001; Marston *et al.*, 2001). Thus, a thorough

molecular analysis of Axl2p is essential for understanding how polarity-axis is selected and how the axis selection is coordinated with polarized-growth machinery.

Previous studies indicate that the septins are the core components of the axial landmark (Flescher *et al.*, 1993), to which two other components, Bud3p and Bud4p, are anchored (Chant *et al.*, 1995; Sanders and Herskowitz, 1996). Axl1p localizes to the bud neck in a Bud3p- and, particularly, Bud4p-dependent manner (Lord *et al.*, 2002). Axl1p localization at the bud neck is also slightly decreased in *axl2Δ* cells (Lord *et al.*, 2002). Thus, the order of assembly of the axial landmark is from the septins to Bud3p and Bud4p to Axl1p, although whether and how these proteins interact with each other at the molecular level is not known. The bud-neck localization of Axl2p depends on the septins but not Axl1p (Halme *et al.*, 1996; Roemer *et al.*, 1996). It is not clear whether the neck localization of Axl2p depends on Bud3p and Bud4p. One study indicates that the neck localization of Axl2p does not depend on Bud4p (Roemer *et al.*, 1996), whereas another study suggests that Bud3p is required for refining the neck localization of Axl2p (Halme *et al.*, 1996). This discrepancy may arise from the complex and continuous localization of Axl2p in the cell cycle. Axl2p, a type I membrane glycoprotein, is delivered to the sites of polarized growth, including the presumptive bud site, the cortex of a small bud, and the neck of a large-budded cell (in late anaphase or telophase), via the secretory machinery (Halme *et al.*, 1996; Roemer *et al.*, 1996; Powers and Barlowe, 1998). This localization pattern of Axl2p in the cell cycle makes it difficult to conclude unambiguously whether there is a Bud3p- and Bud4p-dependent neck localization of Axl2p from onset of mitosis to late anaphase. Our study here resolves this issue. The N-terminal region plus the transmembrane domain of Axl2p localizes to the sites of polarized growth, presumably due to the delivery via secretory pathway (Figure 4A). Surprisingly, the intracellular domain of Axl2p (Axl2p-C) localizes to the bud neck on its own and this localization completely depends on Bud3p or Bud4p, but not Axl1p (Figure 4, A and C). In addition, Axl2p-C interacts with Bud4p regardless of the Bud3p presence, whereas Axl2p-C interacts with Bud3p only in the presence of Bud4p. This new finding, together with previously published data, indicates that the axial landmark is assembled at the bud neck sequentially with the following order, the septins→Bud3p and Bud4p→Axl2p and Axl1p. The detailed interactions among these components are largely unknown, which should be a focus of future studies.

Our analysis on the cytoplasmic tail of Axl2p (Axl2p-C) in bud-site selection also leads to two additional findings. First, by expressing a Bud3p-Axl2p-C chimeric protein under the control of the *BUD3* promoter, we demonstrated that late G1 expression of Axl2p is not required for bud-site selection. Second, we found that the role of Axl2p in bud-site selection is mediated by the middle one third of its tail, residues 641–725, a region that is sufficient for bud-neck localization and for interacting with Bud3p and Bud4p. This small region, when fused to the C terminus of Bud3p, is completely functional in directing axial budding. A key question in the future is to determine how this region of the Axl2p tail interacts with the downstream Rsr1p GTPase module to fulfill its role in axial budding. In particular, because Axl2p is required for the bud-neck localization of Bud5, the GEF for Rsr1p, and it is shown to associate with Bud5p via its cytoplasmic tail (Kang *et al.*, 2001; Marston *et al.*, 2001), it will be important to determine whether the central region of the Axl2p tail interacts with Bud5p.

Interactions of Axl2p With Cdc42p and Other Polarity-Establishment Proteins

Axl2p localizes first to the presumptive bud site, then to the cortex of a small bud, and finally to the bud neck of a large-budded cell (Halme *et al.*, 1996; Roemer *et al.*, 1996). As described above, the Bud3p- and Bud4p-dependent neck localization of Axl2p is sufficient for its role in axial budding. Then, what is the early function of Axl2p in the cell cycle as manifested by its localization at the sites of polarized growth? Our study here suggests that Axl2p is involved in polarized cell growth early in the cell cycle by interacting with Cdc42p, Bem1p, and Cdc24p independently of its role in bud-site selection.

This conclusion is supported by several observations. First, *AXL2* carried on a high-copy or low-copy plasmid both suppressed the temperature-sensitive growth defect of the *cdc42^{V36G}* mutant independently of other components of the axial landmark such as Bud3p, Bud4p, and Axl1p. In addition, multicopy *BUD3*, *BUD4*, or *AXL1* fails to suppress the *cdc42^{V36G}* mutant. These data suggest that Axl2p, among the known components of the axial landmark, has a unique relationship to Cdc42p. Second, truncated alleles of *AXL2*, which encode proteins containing the first one third of the Axl2p tail (residues 530–646), are sufficient for suppression of the *cdc42^{V36G}* mutant. These truncated Axl2p proteins lack the Bud3p- and Bud4p-dependent neck-targeting signal (residues 641–725) and localize to the sites of polarized growth only (data not shown). These data suggest that Axl2p interacts with Cdc42p early in the cell cycle via a region in the Axl2p tail that is different from that involved in axial budding. These data also imply that Axl2p interacts with Cdc42p via at least two pathways, one pathway involving the middle one third of the Axl2p tail and Bud5p as suggested previously (Kang *et al.*, 2001; Marston *et al.*, 2001), and the other pathway involving the first one third of the Axl2p tail and Cdc42p. Finally, *AXL2* (*SRO4*), along with *CDC42* (*SRO2*), *BEM1* (*SRO1*), and *SEC4* (*SRO6*), was isolated as a multicopy suppressor of a yeast strain depleted for Rho3p and Rho4p (Matsui and Toh-e, 1992), whose only known function is to regulate protein trafficking and actin organization (Matsui and Toh-e, 1992; Adamo *et al.*, 1999; Robinson *et al.*, 1999). In contrast, none of the components of the axial landmark or of the Rsr1p GTPase module was isolated in this screen. These data suggest that Axl2p may play a role in polarized cell growth like Cdc42p and Bem1p do.

Consistent with the genetic evidence for an Axl2p–Cdc42p interaction, we found that a fraction of Axl2p is associated with Cdc42p, Bem1p, and Cdc24p *in vivo*. These protein–protein interactions may account, at least in part, for the suppression of *cdc42^{V36G}* by multicopy *AXL2* or *BEM1*. However, it remains unknown how Axl2p regulates Cdc42p-related functions at the molecular level. It is possible that Axl2p is involved in local activation and/or stabilization of polarity complexes involving Cdc42p at the properly selected growth sites, because Axl2p is a type I membrane protein and seems to pull down GDP-locked or nucleotide-free form of Cdc42p preferentially (Figure 2C). To date, we have failed to detect a direct binding of Axl2p-C to Cdc42p^{Q61L} (GTP-bound form), Cdc42p^{D57Y} (GDP-bound or nucleotide-free form), Cdc42p^{T17N} (GDP-bound or nucleotide-free form), or Bem1p using bacteria-expressed proteins. It is possible that Axl2p interacts only with cortically localized protein complexes such as the Bem1p-based complex consisting of Cdc42p, Cdc24p, and Cla4p (Gulli *et al.*, 2000; Bose *et al.*, 2001). Alternatively, the interaction of Axl2p with Cdc42p or Bem1p could be modulated by posttranslational modifica-

tion such as phosphorylation, which is lacking in bacteria-expressed proteins.

Role of Axl2p in Septin Organization

AXL2 was initially isolated as a multicopy suppressor of *spa2Δ cdc10-10* lethality and *axl2Δ* cells exhibited mild morphological defects, including “droopy” bud, elongated necks, and short cell chains (Roemer *et al.*, 1996). These data are consistent with a role of Axl2p in septin organization, a conclusion that is firmly established by our observations. First, multicopy AXL2 suppresses the growth defect of the *cdc42^{V36G}* mutant, which is mainly defective in septin organization (Caviston *et al.*, 2003), but not of other *cdc42*-Ts mutants, including *cdc42-1* (Adams *et al.*, 1990), *cdc42-201* (Zhang *et al.*, 2001), *cdc42-13* (Zhang *et al.*, 2001), or *cdc42^{G60D}* (Caviston *et al.*, 2002) (data not shown). Second, deletion of AXL2 in our laboratory strain does not produce any discernible morphological defects except an oval cell shape, which is likely due to the bipolar budding of the *axl2Δ* cells. However, *axl2Δ* cells displays strong synthetic defects in cell morphology and septin organization with *gin4Δ* and *cla4Δ* cells, which are primarily defective in the assembly of the septin ring (Cvrckova *et al.*, 1995; Longtine *et al.*, 1998a; Weiss *et al.*, 2000; Versele and Thorner, 2004). Third, SGA analysis reveals that *axl2Δ* cells displays strong synthetic growth and morphological defects with cells deleted for *ELM1*, which encodes a known septin regulator (Bouquin *et al.*, 2000). Finally, overexpression of GST-Axl2p-C results in increased number of cells with an elongated bud, which correlates with defective septin organization.

When and how does Axl2p regulate septin organization? A structure–function analysis indicates that the middle one third of the cytoplasmic tail of Axl2p plays a major role, whereas the first one third plays a minor, role in septin organization. When the full-length tail of Axl2p is fused to the C terminus of Bud3p, the fusion protein is expressed after S/G2 and is completely functional in bud-site selection, but it fails to rescue the septin-organization defects of *axl2Δ gin4Δ* cells. In contrast, when the full-length tail of Axl2p is fused to the C terminus of Gic2p, the fusion protein is expressed in late G1 and rescues the morphological and septin-organization defects of *axl2Δ gin4Δ* cells completely, whereas largely fails to correct the bud-site-selection defect of *axl2Δ* cells. Thus, it seems that the same tail of Axl2p is involved in distinct functions sequentially in the cell cycle, an early function in septin organization and a late function in bud-site selection.

The middle one third of the Axl2p tail is known to interact with Bud3p and Bud4p. Although these proteins are suggested to play a role in septin organization (Gladfelter *et al.*, 2005), they are unlikely to mediate the role of Axl2p in this process for the following reasons. First, Bud3p and Bud4p are expressed after S/G2, whereas the major role of Axl2p in septin organization occurs in late G1. Second, in contrast to *axl2Δ gin4Δ* mutant, *bud3Δ gin4Δ* and *bud4Δ gin4Δ* mutants displayed largely normal cell morphology when grown in synthetic complete media. Only a small fraction of cells from these two double mutants displayed elongated bud morphology and long cell chains (data not shown). Third, *axl2Δ bud4Δ gin4Δ* triple mutant displayed stronger morphological defect than *axl2Δ gin4Δ* mutant did at 30°C, and the triple mutant grew more slowly than *axl2Δ gin4Δ* mutant did (data not shown). These data suggest that the role of Axl2p in septin organization is at least partially distinct from that of Bud3p and Bud4p. The synthetically enhanced septin defects between *axl2Δ* and *bud3Δ* or *bud4Δ* can be explained if we assume that these proteins regulate different aspects of

septin organization. For example, Axl2p could be involved in the initial septin-ring assembly, whereas Bud3p and Bud4p could be involved in the disassembly of the septin ring at the end of the cell cycle.

Although our study identified a role for Axl2p in septin organization, it is not clear how Axl2p participates in this process. Our observations indicate that this function of Axl2p seems to partially overlap with those of Elm1p, Nap1p, Gin4p, and Cla4p, which participate in the assembly of the septin ring (Gladfelter *et al.*, 2001b; Longtine and Bi, 2003; Versele and Thorner, 2005). It is likely that Axl2p regulates the assembly of the septin ring via at least two pathways, one pathway involving the interactions with Cdc42p and Bem1p, which are required for septin recruitment and ring assembly (Gladfelter *et al.*, 2002; Iwase *et al.*, 2006), and the other pathway involving an interaction with the septins or septin-associated proteins to help anchor the septin filaments to the plasma membrane.

A Model for the Sequential and Distinct Roles of Axl2p in Cell Polarization in the Cell Cycle: A Common Design of a Signaling Module between Yeast and Mammals?

This study, along with previously published data, indicates that Axl2p plays multiple roles in cell polarization in the cell cycle, including bud-site selection, polarity establishment, and septin organization. These functions occur at different phases of the cell cycle and may be mediated via distinct domains in the Axl2p tail. Here, we propose a model that accounts for the diverse functions of Axl2p in the cell cycle (Figure 8). In late G1 and upon the activation of the START, the axial landmark (septins→Bud3p and Bud4p→Axl2p and Axl1p) associated with the old division site is recognized by the Rsr1p GTPase module, and the positional information is communicated to the Cdc42p GTPase module to assemble a new bud site at an adjacent position (stage I). At this stage, the middle one third of the Axl2p-C is involved in its interaction with Bud3p and Bud4p to be part of the landmark, and, presumably, it is also involved in an interaction with the Rsr1p GTPase module to determine the position for the new bud. Subsequently, Rsr1p interacts with Cdc24p and Cdc42p to recruit and/or activate the Cdc42p GTPase module at the chosen site (Zheng *et al.*, 1995; Park *et al.*, 1997; Kozminski *et al.*, 2003; Shimada *et al.*, 2004), resulting in polarized organization of the actin cytoskeleton, which guides secretion to form the new bud. As the new bud grows, more Axl2p is delivered to the growth sites via the secretory pathway (Powers and Barlowe, 1998, 2002) (stage II). At this stage, the first one third of the Axl2p tail interacts with Cdc42p, Bem1p, and Cdc24p to affect polarity establishment, whereas this region and the middle one third of the Axl2p tail are involved in septin organization by an undefined mechanism. Upon bud emergence (stage III), Axl2p localizes to the bud cortex, together with Bud5p, Rsr1p, and polarity-establishment proteins such as Cdc42p and Bem1p to continue its fine-tuning role in polarized growth. At this stage, Axl2p could interact with Cdc42p via two distinct domains. The first one third of the Axl2p tail could interact with Cdc42p and Bem1p independently of bud-site-selection proteins, and the middle one third of the Axl2p tail could interact with Cdc42p via Bud5p, Rsr1p, and Cdc24p (Kang *et al.*, 2001; Marston *et al.*, 2001). Around G2/M, Axl2p is recruited to the bud neck in a Bud3p- and Bud4p-dependent manner to assemble the axial landmark that will be used at the beginning of the next cell cycle (stage IV). At this stage, the middle one third of the Axl2p tail is involved in its interaction

Figure 8. Sequential and distinct roles of Axl2p in the cell cycle. See text for details. Briefly, at late G1, Axl2p is delivered to the new budding site through polarized secretion, where it interacts with Cdc42p and regulates septin organization. During G2/M, Axl2p is recruited to the bud neck via an interaction with Bud3p and Bud4p, two axial bud-site-selection determinants that themselves target to the bud neck in a septin-dependent manner. In the late G1 of the following cell cycle, components of the axial landmark associated with the leftover septin ring activate the GTPase cycling of Rsr1p, which leads to the selection of a new budding site next to the preceding division site. For illustration purpose, the objects in the diagram are not drawn in scale. Axl2p, Cdc42p, the septins, and Bud3p/Bud4p are colored in red, blue, yellow, and green, respectively.

with Bud3p and Bud4p. After cytokinesis and cell separation, the axial landmark is associated with the old division sites on both the mother and the daughter cells (stage V). This cyclic behavior of Axl2p repeats itself upon the entry of the cell into the next cell cycle.

The N-terminal region of Axl2p contains four cadherin-like domains (Dickens *et al.*, 2002), the exact function of which is not known. In epithelial cells, E-cadherin and the Rap1 GTPase, a close homologue of the *S. cerevisiae* Rsr1p (McCabe *et al.*, 1992; Ruggieri *et al.*, 1992), are involved in the establishment of adherens junctions (Takeichi, 1995; Knox and Brown, 2002; Price *et al.*, 2004). In addition, the cytoplasmic tail of E-cadherin interacts with C3G, a GEF for Rap1, and this interaction seems to play a positive role in E-cadherin-mediated cell-cell adhesion (Hogan *et al.*, 2004). Furthermore, Cdc42 seems to function downstream of Rap1 (Hogan *et al.*, 2004). Although Axl2p and E-cadherin do not

share any sequence homology in their cytoplasmic tails, the basic design in the signaling pathways involving these proteins in the polarity development of their respective cells seems similar, Axl2p→Bud5p→Rsr1p→Cdc42p for polarity-axis determination and polarity establishment in *S. cerevisiae*, and E-cadherin→C3G→Rap1→Cdc42 for adherens-junction positioning and establishment in epithelial cells. In addition, Cdc42-controlled polarized protein trafficking results in the delivery of more Axl2p or E-cadherin to their functional locations (Powers and Barlowe, 1998; Hogan *et al.*, 2004), which may constitute a positive feedback loop for polarity establishment in these very different cell types. Besides these apparent similarities, we also found that Axl2p genetically interacts with Cdc42p independently of the bud-site-selection proteins and regulates septin organization. It will be interesting to determine whether these novel aspects of Axl2p functions are conserved in epithelial cells in the future.

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