CELL CYCLE
2016, VOL. 15, NO. 14, 1898–1907
http://dx.doi.org/10.1080/15384101.2016.1189043

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REPORT

Cellular localization of Sun4p and its interaction with proteins in the yeast birth scar

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ABSTRACT

Yeast harbor several proteins with predicted glucanase activity that are potentially involved in cell wall remodeling during different processes, including mitosis. Here, we showed that 2 of these putative glucanases, Sun4p and Dse2p, co-localize to the yeast birth scar, dependently on presence of the third glucanase, Egt2p. The absence of these glucanases results in inefficient mother-daughter cell separation. The Sun4p, Dse2p and Egt2p localize to the daughter side of the bud neck, possibly forming a complex, and are involved in the separation of the virgin daughter from the mother cell during mitosis. The formation of properly assembled birth scars that delimitate cell wall area restricted in the next budding is dependent on the presence of Aim44p and its transcriptional regulator, Swi5p. *AIM44* or *SWI5* deletion caused the "budding within the birth scar" phenotype, together with altered localization of the birth scar proteins Sun4p and Dse2p, indicating the impairment of birth scar protein complexes.

ARTICLE HISTORY
Received 9 March 2016
Revised 5 May 2016
Accepted 6 May 2016

KEYWORDS
cell wall; glucanases; SUN family of proteins; yeast birth scar

Introduction

Sun4p protein is a member of the SUN family of proteins that are homologous to β-glucosidases and exhibit different localization and functions. Sun4p has been identified in the cell wall and possibly also in mitochondria.[1] This protein is also efficiently released from cells growing either in liquid medium or within yeast colonies.[2] Although little is known about the function of Sun4p and other SUN family proteins, Sun4p may be involved in cell separation, which is delayed in the *sun4*Δ strain; this strain often possesses more than one bud, and daughter cells often remain attached to the mother cell.[3] Sun4p homolog from *Candida albicans*, Sun41p, plays a role in virulence and biofilm formation, cell attachment to a substrate, and cell separation and hyphae formation, leading to the hypothesis that the SUN family proteins in *C. albicans* are involved in cell integrity maintenance during cell division, accompanied by cell wall remodeling.[4] The Sun4p homolog from *Aspergillus fumigatus* is also functionally related to the cell wall, being involved in hyphae growth.[5]

The number of cell divisions of a particular yeast cell is often reflected by the number of bud scars (chitin-rich rings stainable by Calcofluor dye) remaining on the cell surface after daughter cell separation. These bud scars are relatively small in diameter (1.8–2.4 μm) and are different in structure and composition from the so-called birth scar, i.e., the ring structure that remains on the surface of the daughter cell after its separation from the mother.[6-8] In contrast to bud scars, each cell has only one birth scar, which is not stainable by Calcofluor white, as it contains much less chitin than bud scars, but it binds wheat-germ agglutinin that can be used for birth scar visualization when labeled with fluorescent dye. The birth scar is a zone restricted for budding, it is larger than bud scars (3–3.7 μm), and its size increases with cell age.[8] Although birth scar composition and function are rather unknown, mutants (such as *isw2*Δ or cells with Dse1p overproduction) exhibiting the "budding within the birth scar" phenotype have been isolated.[9] Daughter cell separation includes a step in which the septum is degraded from the daughter cell side by different hydrolytic enzymes[10-13] encoded by genes regulated by the Ace2p transcription factor that accumulates in daughter cell nuclei during cell division.[12]

Here, we identified Sun4p, Dse2p and Dse4p as new proteins that localize to the yeast birth scar. Dse2p birth scar localization depends on Egt2p, and Sun4p localization depends on both Dse2p and Egt2p. Co-localization studies indicate that Egt2p, Dse2p and Sun4p form a complex in the birth scar, while Dse4p is excluded from this complex. Ace2p does not regulate *SUN4* expression, but it is essential for proper Sun4p localization to the birth scar via expression of Sun4p docking proteins, Dse2p and Egt2p. Proper birth scar localization of Sun4p and Dse2p is disrupted in a strain with *AIM44* deletion, displaying a budding within the birth scar phenotype.

Results

Sun4p localizes to the birth scar of yeast cells in an Ace2p transcription factor-dependent manner

With the aim to obtain information on Sun4p cell wall localization, we used the yeast strain BY-Sun4p-HA with an HA tag on the C-terminus of Sun4p [2] and analyzed Sun4p-HA localization in living cells by direct immunofluorescence (IF) using anti-HA-Alexa Fluor 488 antibodies. To decrease the possibility that

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Supplemental data for this article can be accessed on the publisher's website.

C-terminal tagging affects Sun4p localization, we also prepared the strain with N-terminally tagged Sun4p (BY-HA-Sun4p). Figures 1A and B show that both Sun4p-HA and HA-Sun4p are visible at the same precisely defined position in each cell. Co-staining with Calcofluor white, the dye that stains yeast bud scars with the exception of the birth scar, together with staining with Alexa-588 labeled Wheat Germ Agglutinin (WGA-588), which stains all scars including the birth scar, showed that Sun4p localizes to the birth scar of yeast cells. Immunofluorescence was assayed in non-fixed and thus non-permeabilized cells; Sun4p thus localize to the birth scar with both C- and N-termini exposed on the outside of the cell.

A comparison of the expression profile of the *SUN4* gene in various genome wide expression experiments using SPELL (http://spell.yeastgenome.org/) revealed similarities in *SUN4* expression with a group of other genes that localize to the cell wall (Figure S1). The expression of some of these genes (e.g., *EGT2, DSE1, DSE2, DSE4*) is positively regulated by the transcription factors Ace2p and Swi5p.[12,13] We therefore prepared strains derived from BY-Sun4p-HA (wt) with individual deletions of *SWI5* and *ACE2*. Western blot (WB) analysis showed that Sun4p-HA is synthesized at comparable levels in wt and knockout (KO) strains, but this protein is not released extracellularly in the absence of Ace2p (Fig. 1H). IF revealed that Sun4p-HA disappears from birth scars in the absence of Ace2p (Fig. 1C). The deletion of the *SWI5* gene caused a clear "budding within the birth scar phenotype," described previously for the *isw2*Δ strain,[9] and a more diffuse localization of Sun4p-HA in the region of the birth scar compared to wt (Figs. 1D and S2).

These data revealed that some proteins encoded by genes, the expression of which is positively regulated by Ace2p, are indispensable for Sun4p birth scar localization and its release from cells. Thus, Ace2p effect on Sun4p localization is not direct but most likely mediated by some mediator/docking protein whose expression is controlled by Ace2p. In addition, the protein products of some genes regulated by Swi5p are predicted to participate in the proper selection of a new budding site or in the prevention of budding within the birth scar.

Dse2p, Dse4p and Egt2p proteins are involved in proper localization of Sun4p-HA to the birth scar and affect the levels of Sun4p-HA that are released extracellularly

With the aim to identify the cell wall proteins involved in Sun4p-HA localization, we first prepared a set of the strains with *SUN4*-HA derived from EUROSCARF strains knocked out in genes with a similar expression pattern in SPELL (Figure S1) and in other genes potentially localized to the birth scar (Table S1). The Sun4p-HA-knockout strains were then screened for Sun4p-HA production and cellular localization by WB and IF (data not shown). Four of the 14 analyzed strains (*dse2*Δ, *dse4*Δ, *egt2*Δ and *aim44*Δ) showed significantly changed pattern of Sun4p-HA production and/or localization. New KO strains were then prepared, derived from parental BY-Sun4-HA and BY-HA-Sun4p strains (Table S1). In the absence of Dse2p or Egt2p, Sun4p-HA disappeared completely from the birth scar, similar to the situation in the *ace2*Δ strain (Figs. 1F, G and C), and its amount (determined by WB) in the extracellular space (*dse2*Δ) or in the cell wall (*egt2*Δ) was significantly reduced (Fig. 1H). In the BY-*dse4*-Sun4p-HA strain, the amount of Sun4p-HA in the cell wall was reduced (Fig. 1H), but the Sun4p-HA was present in the birth scar normally, as in the wt (Figure S2). High Sun4p levels identified by WB in cell wall extracts of the BY-*dse2*-Sun4p-HA strain (with protein levels similar to wt) in parallel with the absence of Sun4p-HA in the birth scars (as observed by IF) indicate that Sun4p is also present in the cell wall, with its C- and N-termini facing inside the cells, which is the direction opposite to that observed in the birth scar. Cell wall localization of Sun4p is also supported by the fact that the *sun4*Δ strain significantly decreases sensitivity to zymolyase,[2] implicating Sun4p role in cell wall remodeling. The dual localization of Sun4p may signify a dual function, with one related to birth scar localization in the separation of daughter cells from mother cells (together with other glucanases probably involved in secondary septum destruction) [14] and the other function linked to the cell wall localization of proteins in cell wall organization during bud growth.[3,14] Presented data indicate that Dse2p and Egt2p proteins are involved in the proper localization of Sun4p-HA to the birth scar. Both *DSE2* and *EGT2* genes are regulated by Ace2p, which is in agreement with the observed Sun4p-HA localization.

Dse2p-HA localizes to the birth scar dependently of Egt2p and independently of Sun4p

We prepared a series of strains with Dse2p labeled on its C-terminus (Dse2p-HA) or N-terminus (HA-Dse2p), together with other cell wall/birth scar genes (*SUN4, EGT2, DSE4*) individually deleted. In the wt strain, similar to Sun4p-HA, Dse2p-HA localized to the birth scar (Fig. 2A) and was present in the cell lysate, cell wall and extracellular space (Fig. 2F). Co-staining with WGA-588 indicated that similar to Sun4p-HA, Dse2p-HA localizes to the rim of the birth scar. However, N-terminally-labeled HA-Dse2p localized mostly to the cell wall in a punctuate pattern (Fig. 2B), and fluorescence in the birth scar was detectable only in a few cells. In addition, the distribution of cell wall-localized fluorescence was asymmetric in some cells, forming a gradient in the cell wall with higher HA-Dse2p concentration closer to the birth scar. Cell wall fluorescence was not typically present in the cell wall of small buds, but it was visible in those that were already enlarged. These data indicated that either N- or C-terminal tagging affects Dse2p localization or both termini of Dse2p are differently accessible for the antibody in different locations on cell surfaces. The latter would suggest that Dse2p could be present in different complexes with other proteins and/or polysaccharides in the cell wall and the birth scar. To distinguish between these 2 possibilities, we prepared the strain containing double tagged Dse2p with a HA tag on the N-terminus and a Myc tag on the C-terminus. We then detected both tagged tails using anti HA-Alexa488 antibody in parallel with anti-Myc-Alexa647 antibody. The results (Fig. 2C) showed red fluorescence (C-terminus) exclusively in the birth scar and green fluorescence (N-terminus) largely distributed in the cell wall (and rarely in the birth scar), thus proving that N- and C-terminal tails of Dse2p are differently exposed in these 2 regions of the cell surface. Such dual localization of tagged Dse2p variants disproved the potential

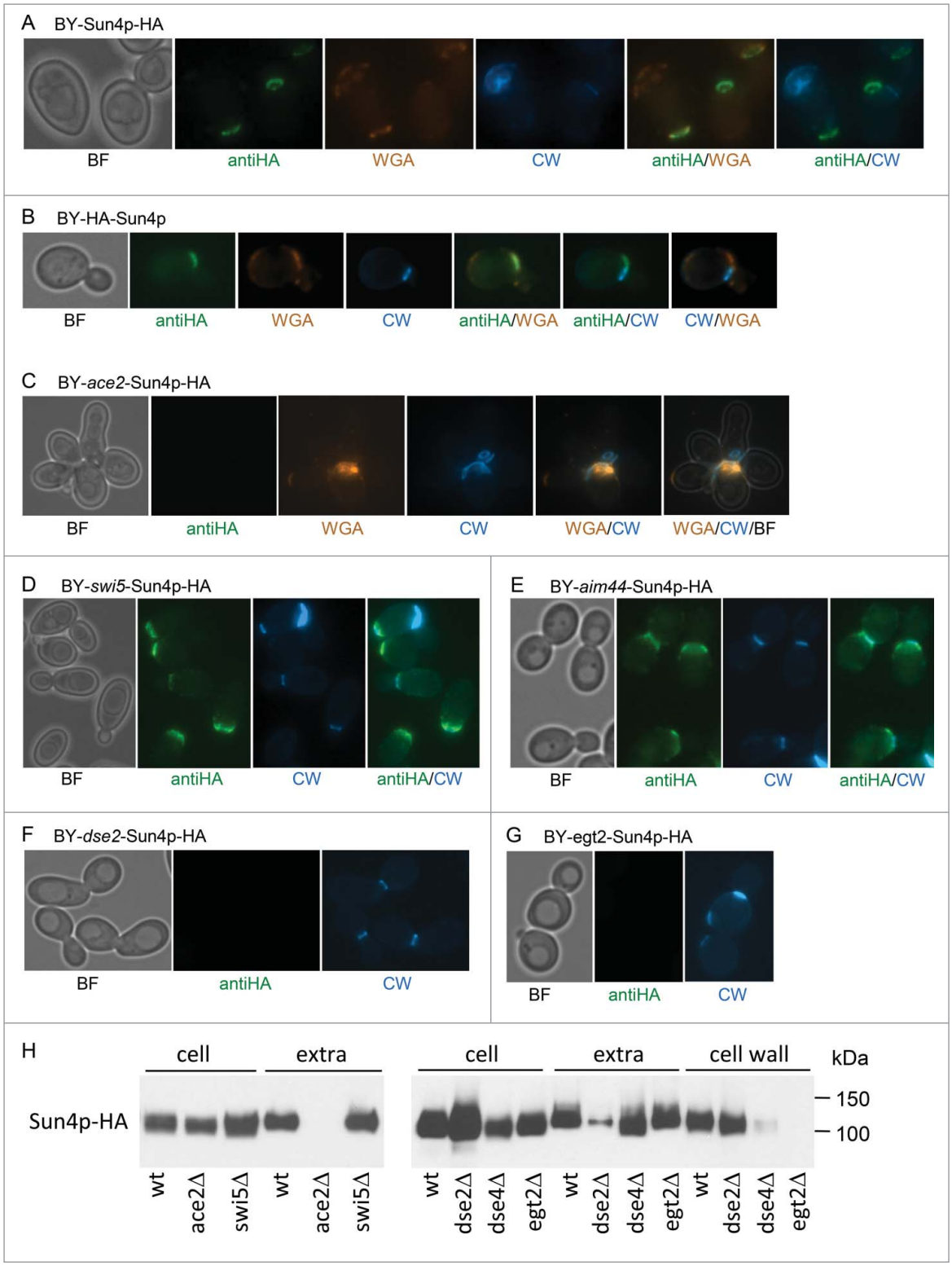

Figure 1. Sun4p localization in wt and KO cells. Sun4p-HA and HA-Sun4p localization to the birth scar in the wt strain (A, B); the absence of Sun4p-HA in the birth scar of *ace2*Δ (C), *egt2*Δ (G) and *dse2*Δ (F) strains; and diffuse Sun4-HA localization and budding within the birth scar phenotype of the *swi5*Δ strain (D), similar to the phenotype of *aim44*Δ (E). Sun4p-HA cellular, extracellular and cell wall localization estimated in wt and KO strains by WB (H). Loading controls for WB are in Figure S5 A. Projections of Z-stacks are used to show Sun4p-HA localization to the birth scar ring, A, C and D. Transversal optical sections of cells are shown in B, E, F and G. BF, bright field; anti-HA, antibody against HA-tag; WGA, WGA-588; CW, Calcofluor white. A-F, Representative cells from at least 10 fields (>1000 cells per strain) are shown; H, representative results of 4 independent biological experiments are shown.

attachment of this protein to the cell surface by a GPI anchor, which was predicted by a genome-wide approach.[15]

We then analyzed Dse2p localization in different KO strains. In the absence of Egt2p, Dse2p-HA was not visible in the birth scar (similar to Sun4p-HA in *egt2*Δ) (Fig. 2D). Dse2p-HA amounts in the cell wall disappeared as shown by WB, but this protein was still present in cells and released to the extracellular space (Fig. 2F). In BY-*egt2*-HA-

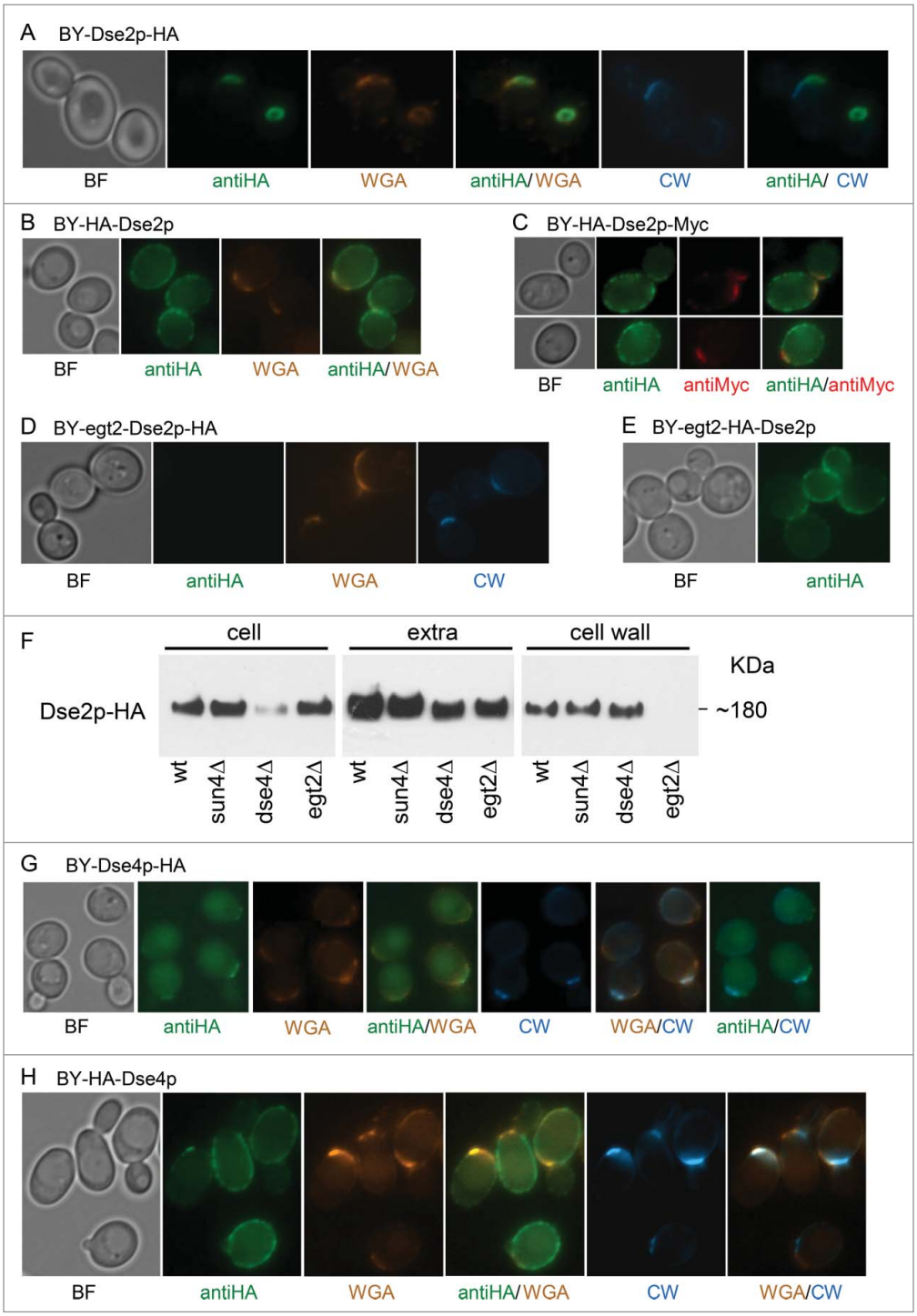

Figure 2. Dse2p and Dse4p localization in wt and KO cells. Dse2p-HA and Dse4p-HA localization to the birth scar (A, G) and HA-Dse2p and HA-Dse4p localization to the cell wall (B, H) of the wt strain; the absence of Dse2p-HA in the birth scar (D) and the presence of HA-Dse2p in the cell wall (E) of the *egt2*Δ strain; and the presence of HA-Dse2p-Myc both in the birth scar and cell wall (C). Dse2p-HA cellular, extracellular and cell wall localization estimated in wt and KO strains by WB (F). Loading controls for WB are in Figure S5 B. Projections of Z-stacks are used to show Dse2p-HA localization to the birth scar ring (A). Transversal optical sections of cells are shown in B, C, D, E, G and H. BF, bright field; anti-HA, antibody against HA-tag; anti-Myc, antibody against Myc tag; WGA, WGA-588; CW, Calcofluor white. A-E, G and H, Representative cells from at least 10 fields (>1000 cells per strain) are shown; F, representative result of 4 independent biological experiments is shown.

Dse2p, however, HA-Dse2p was still detectable by IF in the cell wall, as in the wt strain (Fig. 2E). These data indicate that Egt2p is important for proper Dse2p localization to the birth scar, but it is not important for Dse2p targeting to the cell wall. However, in the absence of Egt2p, Dse2p (similarly to Sun4p) disappeared from cell wall extracts as determined by WB. This observation indicates that Egt2p also plays a role in the cell wall, possibly in stabilizing Dse2p and Sun4p attachment to the cell wall. In the absence of Egt2p, Dse2p and Sun4p are more easily released and thus can disappear from the cell wall during its purification. Unfortunately, we did not succeed in analyzing the localization of Egt2p because neither GFP nor HA tagging allowed us to visualize this protein in the cells (not shown).

In the absence of Dse4p, Dse2p-HA localized to the birth scar and HA-Dse2p to the cell wall in a similar manner as in the wt strain (Figure S3 A). Additionally, in the absence of Sun4p, the localization of both Dse2p-HA and HA-Dse2p was also not affected (Figure S3 A), which indicates that Sun4p is not required for correct Dse2p localization, while Dse2p is indispensable for the proper localization of Sun4p to the birth scar. Egt2p is crucial for the birth scar localization of both Sun4p-HA and Dse2p-HA.

Similar to Dse2p, Dse4p localizes to the birth scar and the cell wall

To analyze the localization of Dse4p, we prepared strains with N- and C-terminally tagged Dse4p and analyzed Dse4p localization by direct IF. In wt, Dse4p-HA was visible in the birth scars of some cells, although the fluorescence was very weak and often visible only in small part of the birth scar (Fig. 2G). The stronger fluorescence of HA-Dse4p presented as a punctate pattern was detectable in the cell wall of the BY-HA-Dse4p strain. These data showed that Dse2p and Dse4p exhibit similar dual localization in the birth scar and the cell wall with the same protein orientation. In contrast to the asymmetric cell wall localization of HA-Dse2p close to the birth scar, HA-Dse4p was almost evenly distributed in the cell wall of mother cells (Fig. 2H). However, HA-Dse4p cell wall fluorescence was in some cases weaker or absent in the cell wall of the buds (Figure S4 B). Asymmetric HA-Dse4p localization was not affected by deletion of genes *CDC11* and *SHS1* coding for septins (Figure S4 B).

In absence of Sun4p, HA-Dse4p localized to the cell wall as in wt (Figure S4 A). Unfortunately, construction of strains with HA-Dse4p derived from *dse2*Δ and *egt2*Δ KO strains failed, which may indicate that tagged Dse4p protein is not fully functional and its combination with a deletion of *DSE2* or *EGT2* is lethal to cells. Similarly, we were not able to prepare the strain with the 2 tags (C and N-terminal) of Dse4p. The identification of Dse4p localization to the daughter site of the septum in fixed yeast cells and to the whole daughter cell after partial cell wall digestion[16] supports our finding of dual Dse4p localization. However, in our experiment performed with living non-fixed cells, Dse4p was detected in addition to cell walls of mother cells also in cell walls of some of the buds (Figure S4 B).

Sun4p co-localizes with Dse2p but not with Dse4p to the birth scar

The above data show a relationship among Sun4p, Dse2p and Dse4p localization. To examine the mutual localization of these proteins in more detail, we prepared strains with different combinations of tagged versions of these proteins. Strain BY-HA-Sun4p-Dse2p-Myc showed the clear co-localization of Dse2p and Sun4p to the birth scar (Fig. 3A), indicating that these 2 proteins could form a complex. As the birth scar localization of both proteins is dependent on the presence of functional Egt2p (Figs. 1 and 2), and in addition, Dse2p is essential for Sun4p localization, we assume that Egt2p keeps Sun4p and Dse2p at the birth scar through interaction with Dse2p.

Analysis of potential co-localization of Sun4p with Dse4p in the birth scar using the BY-HA-Sun4p-Dse4p-Myc strain indicates partially different localization of Sun4p and Dse4p in the birth scar (Fig. 3B). In this strain, HA-Sun4p exhibited standard birth scar localization. Weak Dse4p-Myc fluorescence predominantly localized also to the birth scar, but to other positions compared to HA-Sun4p. The analyses were complicated by rather weak Dse4p-Myc fluorescence, but the results indicated that Dse4p does not form a complex with Sun4p (and thus probably not with Dse2p) in the birth scar. This conclusion is in agreement with the observation that the deletion of the *DSE4* gene does not affect the birth scar localization of Sun4p and Dse2p. In the BY-HA-Dse4p-Dse2p-Myc and BY-HA-Dse4p-Sun4p-Myc strains (Figs. 4A and 3C), HA-Dse4p present in the cell wall did not co-localize with any of the other proteins present in the birth scar. As assumed, complementary fluorescence of N-terminal-tagged Dse4p in the cell wall and of both Sun4p and C-terminal-tagged Dse2p in the birth scars was clearly visible.

The question remained whether Dse4p co-localizes with Dse2p in the cell wall. Analysis of the BY-HA-Dse4p-Myc-Dse2p strain, however, showed mostly complementary fluorescence of HA-Dse4p and Myc-Dse2p in the cell wall (Fig. 4B). Both proteins exhibited a typical punctate pattern, which was generally more polarized for HA-Dse2p and more evenly distributed for HA-Dse4p. These data indicate that these proteins localize to different positions in the cell wall and therefore most likely do not interact.

The absence of Aim44p causes changes in the localization of birth scar proteins Sun4p and Dse2p and the "budding within the birth scar" phenotype

Deletion of another Swi5p-regulated gene, *AIM44*, in the BY-Sun4p-HA and BY-Dse2p-HA strains significantly changed the budding pattern and birth scar localization of Sun4p-HA and Dse2p-HA. The BY-*aim44*-Sun4p-HA and BY-*aim44*-Dse2p-HA strains exhibited a "budding within the birth scar" phenotype, similar to the strain deleted in the *SWI5* gene that codes for Swi5p, the transcriptional regulator of *AIM44*. Both Sun4p-HA and Dse2p-HA exhibited diffuse birth scar localization (Figs. 1E and S3 B), similar to Sun4p-HA localization in the *swi5*Δ strain (shown in Fig. 1D). These findings indicate that Aim44p is involved in assembly of the birth scar proteins Sun4p, Dse2p and Egt2p.

Discussion

We identified novel proteins in the yeast birth scar, the structure of largely unknown composition. Dse2p, Dse4p and most likely Sun4p showed dual localization on the yeast cell surface, being present within the birth scar and in the rest of the cell wall. In both locations, these proteins expose different termini of their structures (Fig. 5), indicating either different orientations of these proteins or the presence of additional factors (proteins and/or carbohydrates) that cover specific domains of Sun4p and/or Dse2p and/or Dse4p. According to our model-scheme, GPI-anchored Egt2p keeps the complex of Dse2p and Sun4p in the birth scar or helps to expose parts of these

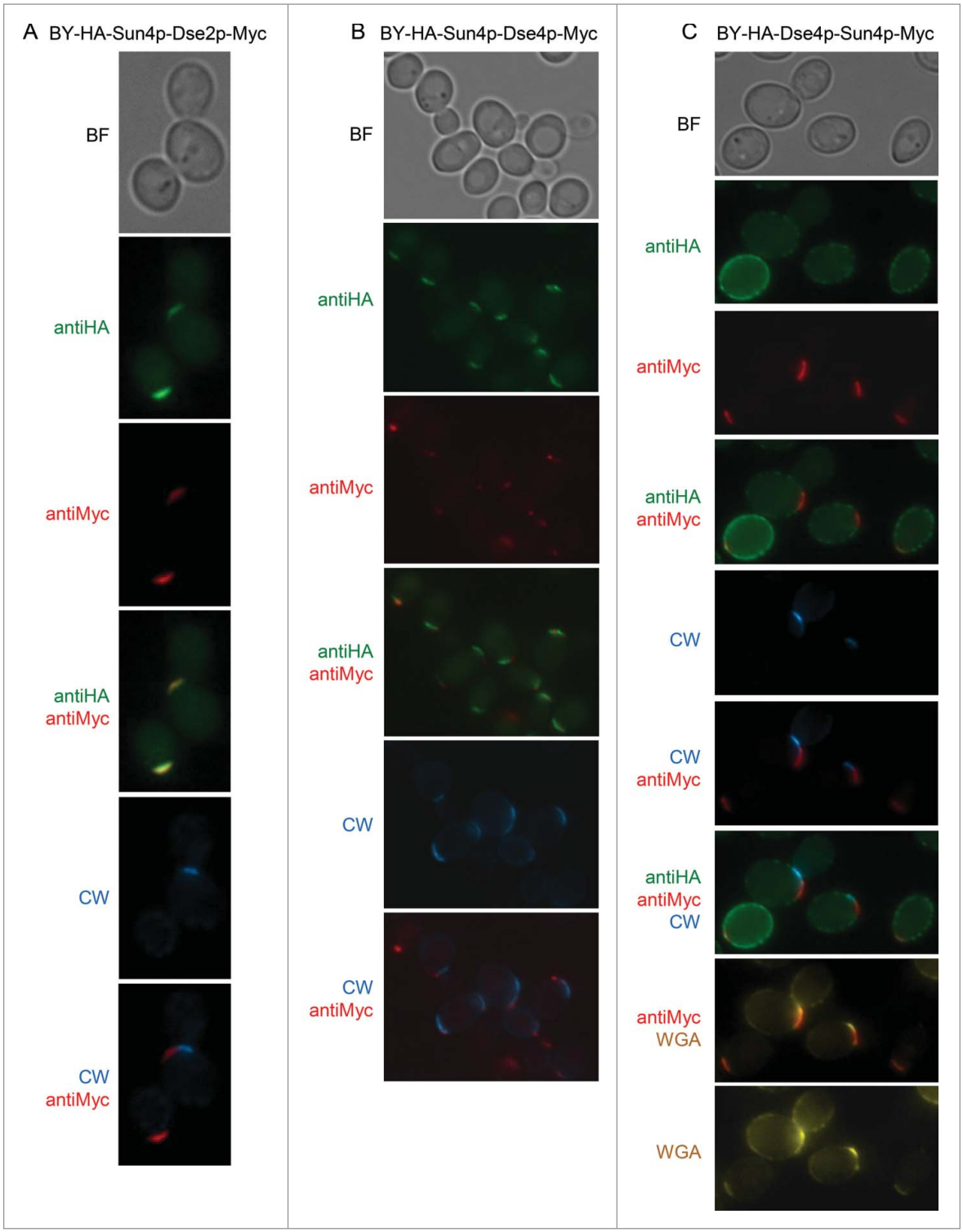

Figure 3. Co-localization of Sun4p with Dse2p and Dse4p in wt cells. Transversal optical sections of cells (A, B, C). BF, bright field; anti-HA, antibody against HA-tag; anti-Myc, antibody against Myc tag; WGA, WGA-588; CW, Calcofluor white. Representative cells from at least 10 fields (>1000 cells per strain) are shown.

proteins to the external space. According to WB, Egt2p also helps to stabilize Dse2p and Sun4p binding to the cell wall. The Ace2p transcriptional regulator is indispensable for Sun4p localization to the cell wall because it induces the expression of Egt2p and Dse2p. *SUN4* expression is not affected by Ace2p.

Our results provide direct evidence of Egt2p-dependent Dse2p and Sun4p birth scar localization that can explain the decreased cell separation efficiency in strains with deletions of *DSE2*, *EGT2* or *SUN4* encoding proteins with predicted glucanase activity.[11,12,17] Hydrolytic activity against β-(1,3)-glucan of Sun4p homologues, *Af*Sun1p of *Aspergillus fumigatus* and *Ca*Sun41p of *Candida albicans*, was confirmed.[5] We therefore hypothesize that Egt2p, Dse2p and Sun4p proteins form complexes that first localize to the daughter side of the bud neck during mitosis, similar to Dse4p,[16] and later to the birth scar of the daughter cell.

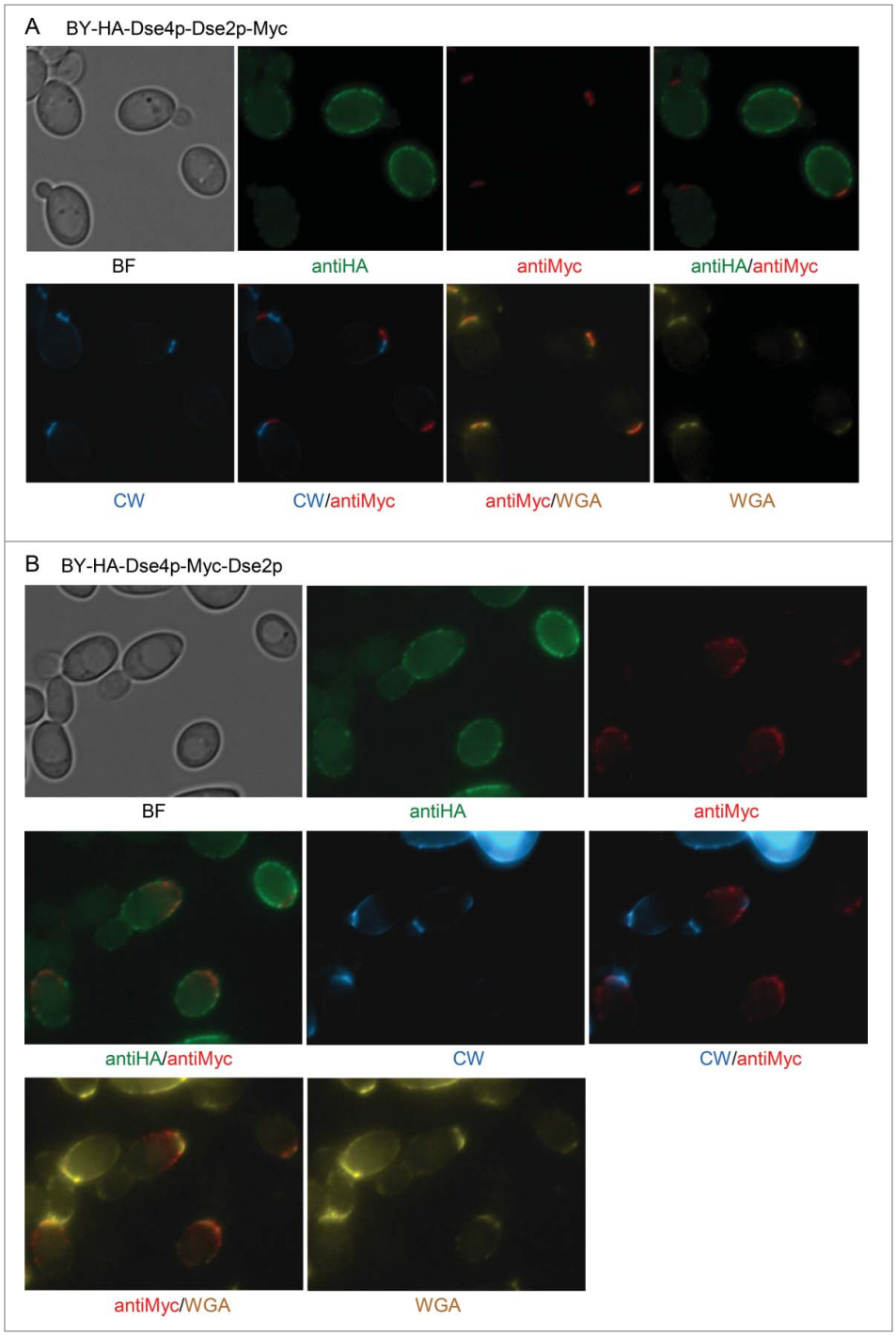

Figure 4. Co-localization of Dse2p and Dse4p in wt cells. Transversal optical sections of cells (A, B). BF, bright field; anti-HA, antibody against HA-tag; anti-Myc, antibody against Myc tag; WGA, WGA-588; CW, Calcofluor white. Representative cells from at least 10 fields (>1000 cells per strain) are shown.

In late mitosis, this complex presumably participates in mother-daughter cell separation. Sun4p exhibits asymmetric localization to the birth scar (not to bud scars), although it is not regulated by the daughter-cell-specific Ace2p transcription factor, which only activates genes in daughter cell nuclei.[12,18] Sun4p asymmetric localization is likely ensured by forming a complex with daughter-cell-specific Egt2p and Dse2p. In addition, fractions of Dse4p, Dse2p and Sun4p, which localize to the cell wall, may function in cell wall remodeling, which is important for proper bud growth.[14]

The disruption of *AIM44* and *SWI5* lead to altered Sun4p and Dse2p localization, together with a "budding within the birth scar" phenotype, where new buds form within the birth scar, i.e., within the zone that is restricted for budding in the wt strain. This phenotypic similarity of *aim44*Δ and *swi5*Δ can be explained by the fact that Swi5p is a transcriptional regulator of *AIM44*.[19] These findings implicate Aim44p in the formation of correctly assembled birth scars and consequently, in the selection of new bud-sites outside of the birth scar.

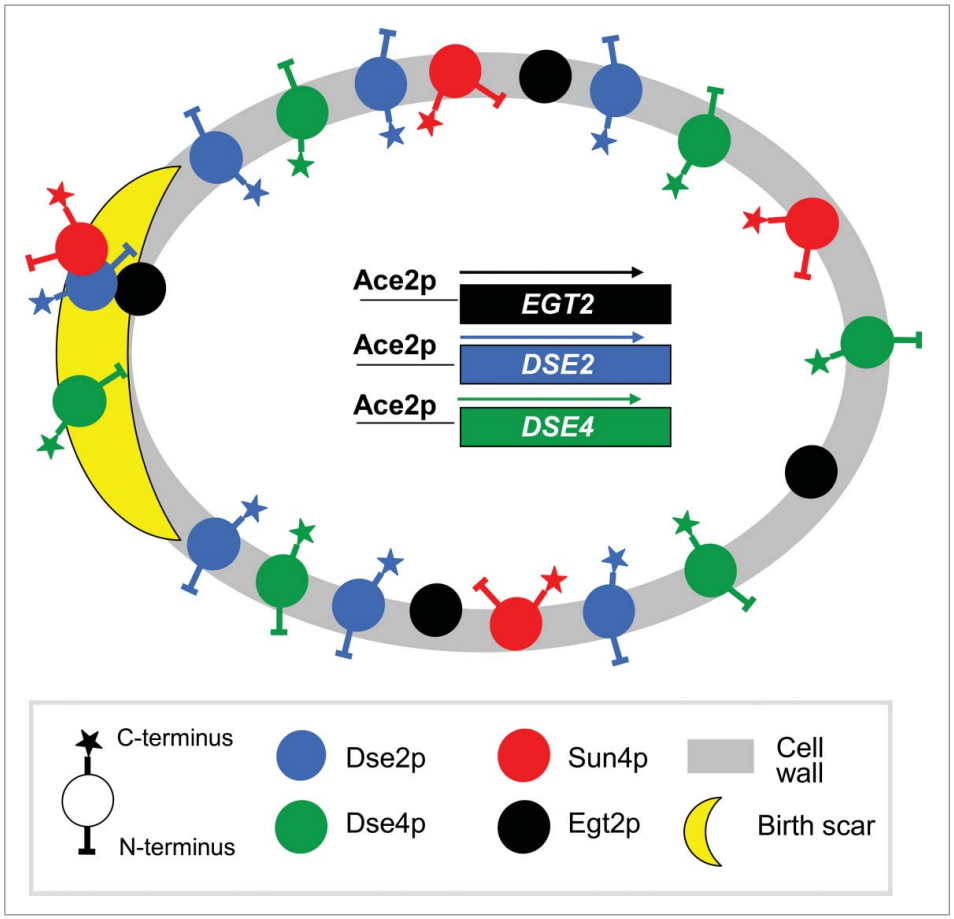

Figure 5. Schematic model of cell localization and interaction of Sun4p, Dse2p, Dse4p and Egt2p proteins.

Materials and methods

Yeast strains and media

The strains used in this study (Table S1) were all derived from *Saccharomyces cerevisiae* BY4742 (MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0). The strains were either obtained from the EUROSCARF collection or prepared in this study. Yeast cells were grown at 28°C in liquid YD medium (1% yeast extract, 2% glucose) or GM medium (1% yeast extract, 3% glycerol, pH 5) or in giant colonies (6 per plate) on GMA agar (GM, 2% agar, 30 mM $CaCl_2$). YPDA agar (YD, 1% peptone, 2% agar) with antibiotic supplements (G-418, 400 mg/l; nourseothricin, 200 mg/l or hygromycin B, 400 mg/l) or SDA agar (2% glucose, 100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 0.15% Wickerham's yeast nitrogen base supplemented with 150 mg/ml of uracil, leucine and lysine, 2% agar) were used for strain construction; when needed, SDA plates were supplemented with 1 mg/ml 5-fluoroorotic acid (5-FOA).

Strain construction

Deletions and both C-and N-tagged (HA or Myc) strains were made by PCR amplification of marked cassettes using appropriate plasmids and by subsequent transformation[20] of the particular strain. For deletions, primers with homology overhangs flanking the open reading frame were used to amplify the cassette. For C-tagged strains, primers flanking the stop codon of the target genes were used. N-tagging was performed according to.[21] Cassette with N-terminal HA-tag was inserted after the sequence encoding signal peptide as predicted in SGD (http://www.yeastgenome.org/); Terminal amino acids of the signal peptides are Ala (GCC) for Dse2p, Ala (GCT) for Sun4p and Cys (TGT) for Dse4p. Correct genomic integration of cassettes was verified by PCR using specific primers and by sequencing.

Isolation of fractions of extracellular, cellular and cell wall proteins

Cell lysates and extracellular material from colonies were collected as follows. The biomass of 3-day old whole colonies was collected and washed with 10 mM MES buffer, pH 6 supplemented with protease inhibitors (Complete, EDTA-free protease inhibitor mixture (Roche Applied Science), 100 mM PMSF (phenylmethylsulfonyl fluoride, Sigma) and 1 mM AEBSF (4-(2- aminoethyl) benzenesulfonyl fluoride, Sigma) with a volume equal to the biomass. Wash buffer (supernatant) containing extracellular material and cells were collected separately and stored at −75°C. The cells were broken with glass beads in the same buffer as above using FastPrep FP120 (Thermo Savant, NY, USA). The supernatant obtained by centrifugation at 1000 g for 3 min and subsequently 2000 g for 5 min was used as the cell lysate; the sediments of both centrifugations were combined and used for cell wall isolation according to.[22] In brief, the sediments were washed with ice-cold 1 mM PMSF

followed by solutions of 1 mM PMSF containing 5%, 2% and 1% of NaCl stepwise; each of the washings was repeated 4 times. All steps were performed at 4°C.

Determination of particular protein amounts

The proteins within the cell lysates (20 μg of proteins per slot, determined using a protein detection kit, Bio-Rad), purified cell walls, and extracellular proteins in amounts equivalent to the amount of cells that produced 20 μg of cell proteins were separated by SDS-PAGE using 9% gels, transferred to a PVDF membrane (Immobilon-P, Millipore) and stained with Coomassie blue (loading control). The HA-tagged and Myc-tagged proteins were detected using mouse monoclonal anti-HA (Cell Signaling Technology, #2367) and anti-Myc antibodies (Santa Cruz Biotechnology, sc-40) in combination with goat anti-mouse IgG-HRP as the secondary antibody (Santa Cruz Biotechnology, sc-2005). The peroxidase signal was visualized with Super Signal West Pico (Pierce) on Super RX medical X-ray film (Fuji).

Fluorescence microscopy

Cells grown in liquid GM medium overnight were washed in PEM buffer (0.1 M PIPES, 5 mM EGTA, 5 mM $MgCl_2$; pH 6.9 (KOH)) and treated with 2% Bovine serum albumin for 30 min at room temperature. Alexa Fluor 488-conjugated mouse IgG1 antibody against HA-tag (Cell Signaling Technology, #2350) or antibody against Myc-tag conjugated with Alexa Fluor 647 (Santa Cruz Biotechnology, sc-40 AF647) were added to final dilutions of 1:50 and incubated at room temperature for 1 hour. Cells were washed with dH_2O before Fluorescence/DIC/BF microscopy, which was performed using a Carl Zeiss AxioObserver.Z1 fluorescence microscope equipped with Axiocam 506 and a Plan-Apochromat 63x/1.40 Oil DIC M27 objective. Filter sets for Alexa Fluor 488 (excitation 450–490 nm; emission 500–550 nm) or for Alexa Fluor 647 (excitation 625–655 nm; emission 665–715 nm) were used. For visualization of bud/birth scars stained with Calcofluor White (2 μg/ml, Sigma) and/or Wheat Germ Agglutinin, Alexa Fluor 594 conjugate (10 μg/ml, Invitrogen), filter set for DAPI (excitation 335–383 nm; emission 420–470 nm) and for DsRed (excitation 538–562 nm; emission 570–640 nm) were used, respectively. Image acquisition was processed with ZEN 2012 (blue edition) software (Zeiss).

Abbreviations

BF	bright field
IF	immunofluorescence
KO	knockout
WB	Western blot
WGA-588	Alexa-588 labeled Wheat Germ Agglutinin.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Tomáš Hendrych for the help with antibody testing.

Funding

This work was supported by the Czech Science Foundation (13-08605S); Charles University in Prague (UNCE 204013); RVO 61388971; GAUK 903313; the project 'BIOCEV – Biotechnology and Biomedicine Center of the Academy of Sciences and Charles University' (CZ.1.05/1.1.00/02.0109) from the European Regional Development Fund.

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