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ADP-ribosylation factor arf6p may function as a molecular switch of new end take off in fission yeast

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Abstract

Small GTPases act as molecular switches in a wide variety of cellular processes. In fission yeast *Schizosaccharomyces pombe*, the directions of cell growth change from a monopolar manner to a bipolar manner, which is known as 'New End Take Off' (NETO). Here I report the identification of a gene, *arf6*$^+$, encoding an ADP-ribosylation factor small GTPase, that may be essential for NETO. *arf6Δ* cells completely fail to undergo NETO. arf6p localizes at both cell ends and presumptive septa in a cell-cycle dependent manner. And its polarized localization is not dependent on microtubules, actin cytoskeletons and some NETO factors (bud6p, for3p, tea1p, tea3p, and tea4p). Notably, overexpression of a fast GDP/GTP-cycling mutant of arf6p can advance the timing of NETO. These findings suggest that arf6p functions as a molecular switch for the activation of NETO in fission yeast.

Keywords: ARF; Cell polarity; NETO; *Schizosaccharomyces pombe*; Small GTPase

The fission yeast *Schizosaccharomyces pombe* has been used as a model system to define the molecular mechanism responsible for the establishment of cell polarity [1,2]. In fission yeast, newly born cells initially grow in a monopolar fashion at the site that preexisted before cell division (old end), and later they initiate growth at the site of previous cell division (new end) in a bipolar fashion. The transition is known as NETO [3].

The superfamily of small GTPases has been implicated in the regulation of diverse biological processes [4]. ADP-ribosylation factor GTPases (Arfs) comprise a conserved family of proteins in the Ras superfamily of small GTPases [5,6]. The Arf proteins were originally identified as cofactors required for cholera-toxin-catalyzed ADP-ribosylation of α-subunit of heterotrimeric G proteins, Gs [7]. Mammalian Arfs are categorized into three classes. Human ARF1 and ARF3 belong to class I. ARF4 and ARF5 belong to class II and ARF6 is the sole class III member [5,8]. ARF1 and ARF6 are the most well studied members of the Arf family [9]. ARF1 is required for the binding of coatomer protein to Golgi complex integrity and transport along the secretory pathway [10]. ARF6 has been implicated in both endocytosis and cytoskeletal organization at the cell periphery [11–13]. In *S. pombe*, Arfs (two members) had not been well characterized [14]. In the present study, I demonstrate that one of the two members may function as a molecular switch for NETO in fission yeast.

Materials and methods

Yeast genetic methods, medium and strains. Growth media and genetic manipulation of yeast were as described in detail [15]. Yeast strains used in this study are listed in Table 1.

Schizosaccharomyces pombe cDNA library, and cloning and disruption of the arf6$^+$ *gene.* The *S. pombe* cDNA library was kindly provided by P. Nurse [16,17]. A plasmid (pMH1), which was incidentally isolated from this cDNA library, contained the complete *arf6*$^+$ cDNA. Genomic locus of *arf6*$^+$ was amplified by PCR and was cloned into pUC13 (pUC-*arf6*G).

Abbreviations: Arf, ADP-ribosylation factor; GFP, green fluoresent protein; HA, hemagglutinin; NETO, new end take off.

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Table 1
Schizosaccharomyces pombe strains used in this study

Strains	Genotype	Source
TN214	*h⁻ leu1-32 ura4-D18 his3-d3*	C. Shimoda
AJY86	*h⁻ leu1-32 ura4-D18 his3-d3 arf6Δ::his3⁺*	This work
AJY50	*h⁻ leu1-32 ura4-D18 his3-d3 tea1Δ::his3⁺*	This work
AJY64	*h⁻ leu1-32 ura4-D18 his3-d3 tea3Δ::his3⁺*	This work
AJY69	*h⁻ leu1-32 ura4-D18 his3-d3 tea4Δ::his3⁺*	This work
AJY83	*h⁻ leu1-32 ura4-D18 his3-d3 bud6Δ::his3⁺*	This work
AJY65	*h⁻ leu1-32 ura4-D18 his3-d3 for3Δ::his3⁺*	This work

Gene disruption of arf6⁺. I made a construct containing a *his3⁺* marker instead of the ORF (pUC-*arf6*DH) and used it for gene disruption. Gene disruption was confirmed by PCR. Initial disruptions were generated in diploid strains and then sporulated to produce *arf6Δ* spores. His⁺ and His⁻ spores were germinated equally, indicating that *arf6⁺* is not required for vegetative growth.

Microscopy. Yeast cells were photographed with Leitz DMRD microscope or Nikon Eclipse E600 microscope.

Construction of the arf6p mutants. Three different arf6p mutants (arf6p^{T35N}, arf6p^{Q75L}, and arf6p^{L165N}) were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Results

Disruption of arf6⁺ caused a complete defect in NETO

A database search revealed that there are two ORFs with significant homology to the human Arfs in *S. pombe*: SPBC4F6.18 (*arf1⁺*) [14] and SPBC1539.08. I focused on the characterization of SPBC1539.08 encoding a protein of 20.7 kDa (184 amino acid in length). Alignments of fission yeast and human Arfs are shown in Fig. 1A. Percentage identities between two Arfs are shown in Fig. 1B. The phylogenetic relationships of them are designated in Fig. 1C. The amino acid sequence of SPBC1539.08 is most closely related to human ARF6 belonging to class III Arf (75% identical). And I found only 65–66% identities with human ARF1 and ARF3 (class I) and 65% identities with human ARF4 and ARF5 (class II). Thus I named this gene as *arf6⁺* as the *S. pombe* ARF6 ortholog.

The *arf6⁺* cDNA and the genomic locus of *arf6⁺* were cloned (Fig. 1D). And cells with a deletion of *arf6⁺* were generated by replacing the coding region of *arf6⁺* with *his3⁺* (Fig. 1D). *arf6Δ* cells were viable and grew at wild-type rates and exhibited normal rod shapes (Fig. 1E). The positioning of septa was also normal in *arf6Δ* cells. But I found that their patterns of cell growth were not normal: *arf6Δ* cells seemed to have a defect in NETO (Fig. 1F). Growth patterns of *arf6Δ* cells were assayed by the staining with FITC-conjugated lectin (Fig. 1G, see legend). In wild-type cells, since cell growth occurred at both cell ends, cell ends were not stained with lectin (Fig. 1G). On the other hand, only one end (old end) was not stained in *arf6Δ* cells (Fig. 1G), suggesting that *arf6Δ* cells grow only at old ends. Moreover, I did the quantitative analysis for the frequency of NETO. Region of previous site of cell division (birth scar) is not stained by Calcofluor and appears as a dark band [2] (Fig. 1H left: arrow head). New end growth is represented by a bright region beyond this dark band. The relative length of new end growth for individual cell was quantified by *P* value as an index for new end growth (Fig. 1H right, see legend). In wild-type cells, there was a broad distribution of *P* values, indicating that NETO occurred normally (Fig. 1I). On the other hand, in *arf6Δ* cells, *P* values of over 99% of cells were below 0.15 (*P* values < 0.15) (Fig. 1I), indicating that new end growth does not occur in *arf6Δ* cells. Thus, I concluded that *arf6Δ* cells have a complete defect in NETO. In the absence of *arf6⁺*, cells can grow only at old ends, revealing that the mechanisms of new end growth and old end growth are different, and that arf6p is dispensable for growth at old ends.

Distributions of microtubules and actin cytoskeletons in arf6Δ cells

I examined whether *arf6Δ* cells have defects in the organization of microtubules and actin cytoskeletons. Wild-type cells often had a bipolar distribution of actin patches (Fig. 1J, left). On the other hand, *arf6Δ* cells had a monopolar distribution of actin patches (Fig. 1J, right). Their patterns of actin distributions coincide with their active growth zones.

I introduced a GFP-α2-tubulin plasmid (pDQ105) [18] into wild-type and *arf6Δ* cells. Although the *arf6Δ* cells have a defect in NETO, they had a normal microtubule distribution (Fig. 1K). Microtubules, which are thought to have a central role in eukaryotic cell polarity, may not be affected by the absence of arf6p.

Localization of arf6p

Next, I determined the subcellular localization of arf6p. I constructed a hemagglutinin-tagged (HA-tagged) fusion of arf6p on the expression vector, pREP3X [19] (pREP3X-*arf6*-HA). And I replaced its *nmt1⁺*-promoter with the native promoter of the *arf6⁺* gene (pARF6-HA). Since the distribution of *P* values in *arf6Δ* cells containing pARF6-HA was same as in wild-type cells, arf6p-HA was functional (Fig. 1I). Immunofluorescence staining of wild-type cells containing pARF6-HA revealed that signals of arf6p-HA were present in the regions of both cell ends and presumptive septa (Fig. 2A). After cell division, newly born cells had arf6p-HA signals at their previous division sites (new ends) (Fig. 2A, panel 1). Later, arf6p-HA signals at the preexisted sites (old ends) increased gradually prior to NETO, and then the signals at both cell ends were equally detected (Fig. 2A, panels 2–6). When the cells were at the cytokinesis, arf6p-HA signals enriched at the presumptive septa (Fig. 2A, panel 7). And arf6p-HA signals at old ends were progressively diminished and were absent by anaphase (Fig. 2A, panels 8 and 9).

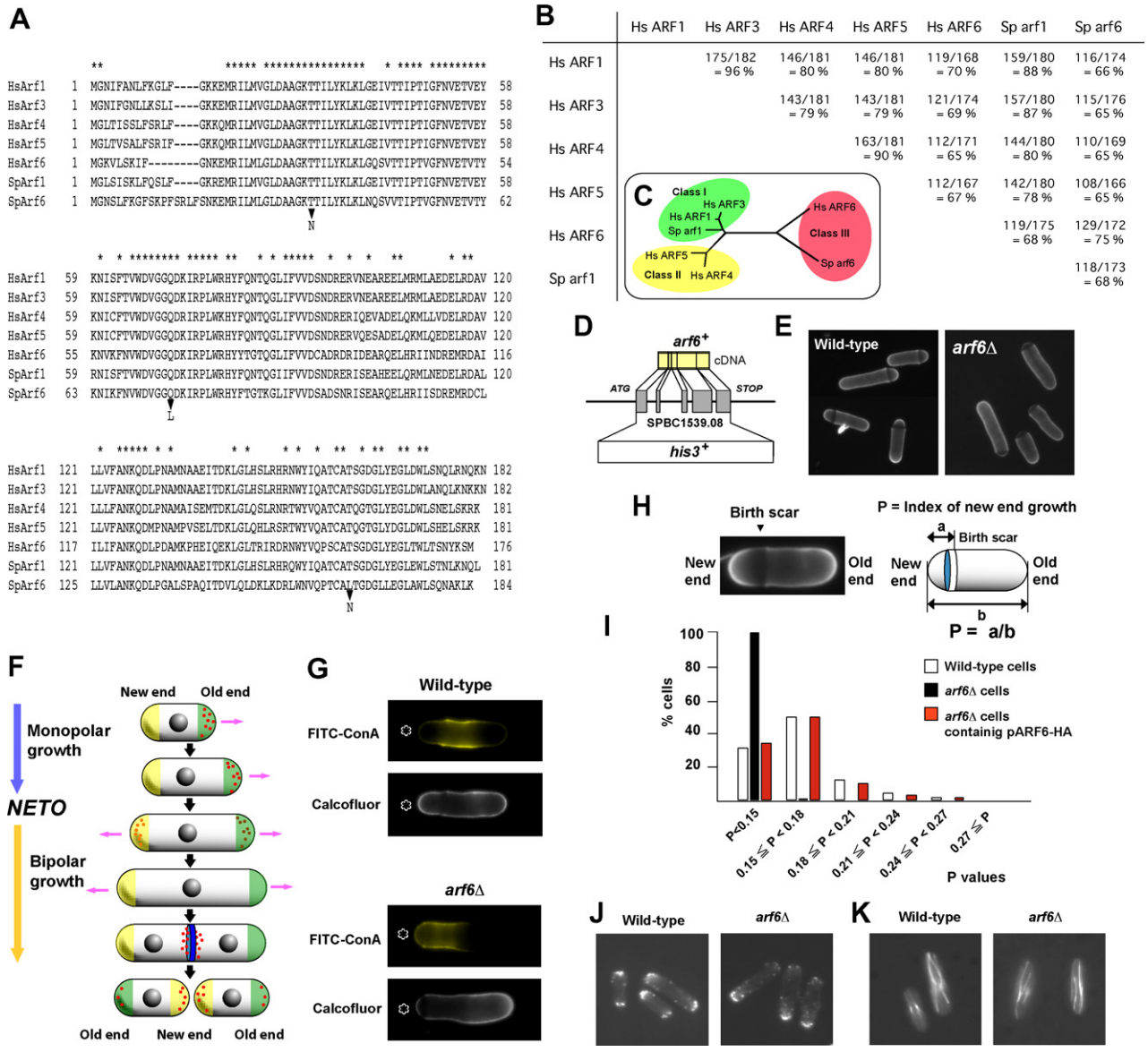

Fig. 1. Characterization of the *S. pombe arf6*$^{+}$ gene and *arf6Δ* cells. (A) Alignments of human Arfs and fission yeast Arfs (Hs, *H. sapiens*; Sp, *S. pombe*). Asterisks indicate the identical residues among all sequences. Positions of amino acid residues of three arf6p mutants (arf6p^{T35N}, arf6p^{Q75L}, and arf6p^{L165N}) are indicated by arrow heads. (B) Comparisons of percentage identities between two Arfs. Percentage identities are calculated using NCBI Blast2 sequences. (C) Phylogenetic relationships among human and fission yeast Arfs. Green shading indicates class I, yellow shading indicates class II, and red shading indicates class III. Phylogenetic relationships were calculated using the ClustalW program. (D) Structure of the *arf6*$^{+}$ gene. Exons (shaded boxes) and cDNA (top) of *arf6*$^{+}$ were indicated. The coding region of *arf6*$^{+}$ was replaced with *his3*$^{+}$. (E) Cell morphology of wild-type and *arf6Δ* strains stained with Calcofluor. (F) Cell growth and NETO in *S. pombe*. A newborn cell has two cell ends: new end (yellow) and old end (green). Red circles indicate actin patches. Blue ring indicates septum. (G) *arf6Δ* cell exhibited monopolar growth at its old end. Wild-type and *arf6Δ* cells were treated with FITC-conjugated lectin (FITC-ConA) to stain their cell walls. Then cells were washed and were incubated in medium for 45 min. And cells were stained with Calcofluor. Unlabeled zones with FITC-ConA indicate the growing ends. New ends were indicated by asterisks. (H) (left) A wild-type cell stained with Calcofluor. An area of previous cell division (birth scar) is marked as dark band. New end growth is measured as the length between the cell tip and the birth scar. (right) Schematic representation of the left panel. The length *a* indicates the distance between the cell tip and birth scar. The length *b* indicates the cell length. *P* value as an index of new end growth equals *a*/*b*. (I) Wild-type and *arf6Δ* cells were stained with Calcofluor. Distributions of *P* values in wild-type (white bars) and *arf6Δ* (black bars) cells were presented in the histogram ($n = 200$ cells). Distribution of *P* values in *arf6Δ* cells containing pARF6-HA (red bars) was also presented ($n = 200$ cells). (J) Actin organization in *arf6Δ* cells. Wild-type (left) and *arf6Δ* (right) cells were fixed and stained with AlexaFluor phalloidin. (K) Microtubule organization in *arf6Δ* cells. Wild-type (left) and *arf6Δ* (right) cells were transformed with pDQ105.

Localization of arf6p is independent on actin and microtubule cytoskeletons

To determine if the polarized localization of arf6p at both cell ends is dependent on microtubules or actin cytoskeletons, I examined the localization of arf6p-HA in cells treated with methyl 2-benzimidazolecarbamate (MBC, a microtubule-specific depolymerizing drug) [20] or Latrunculin A (LatA, a potent anti-actin drug) [21]. Wild-type cells containing pARF6-HA were grown at logarithmic

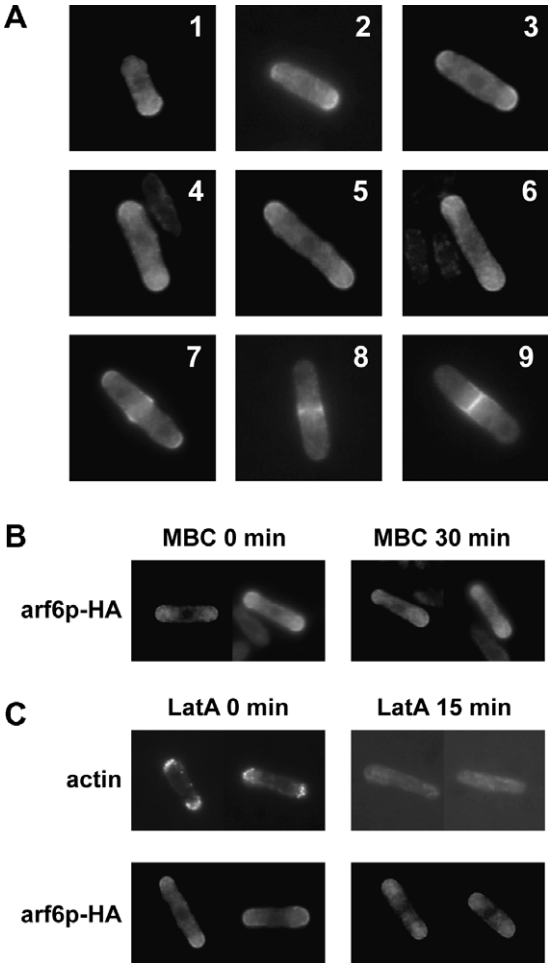

Fig. 2. Subcellular localization of HA-tagged arf6p, and its localization did not depend on microtubules and actin cytoskeletons. (A) Wild-type cells containing pARF6-HA were stained with anti-AlexaFluor-labeled HA antibody as described previously [31]. Cells at progressively later stages of the cell cycle are shown from numbers 1 to 9. (B) Wild-type cells containing pARF6-HA were treated with DMSO or with MBC dissolved in DMSO for 30 min. (C) Wild-type cells containing pARF6-HA were treated with DMSO or with LatA dissolved in DMSO for 15 min.

phase, and then added MBC to the final concentration of 25 μg/ml, or added LatA to that of 100 μM. In MBC-treated cells, no perturbation of cell-end localization of arf6p-HA was seen after 30 min (Fig. 2B). Treatment of the cells with LatA for 15 min resulted in a complete delocalization of actin (Fig. 2C); however, arf6p-HA signals were still localized at both cell ends (Fig. 2C). These results suggested that the polarized localization of arf6p was not dependent on both microtubules and the actin cytoskeletons.

Localization of arf6p in some mutants which have defects in NETO

Numerous genes involved in NETO have been identified previously [1,2]. I examined whether some NETO factors are required for the localization of arf6p. The Kelch-repeat protein tea1p is a key mediator between microtubules and actin cytoskeletons, and is known to have an important function in NETO [1,22]. tea1p resides on the growing microtubule plus end, and is localized at cell tips as a cell-end marker protein [23]. In *tea1Δ* cells, arf6p-HA signals were localized at cell ends and a presumptive septum as observed in wild-type cells (Fig. 3A). bud6p is an actin-binding protein [24]. In *bud6Δ* cells, arf6p-HA signals were localized at cell ends and a presumptive septum as in wild-type cells (Fig. 3B). for3p is an actin nucleator responsible for the assembly of actin cables [25]. Signals of arf6p-

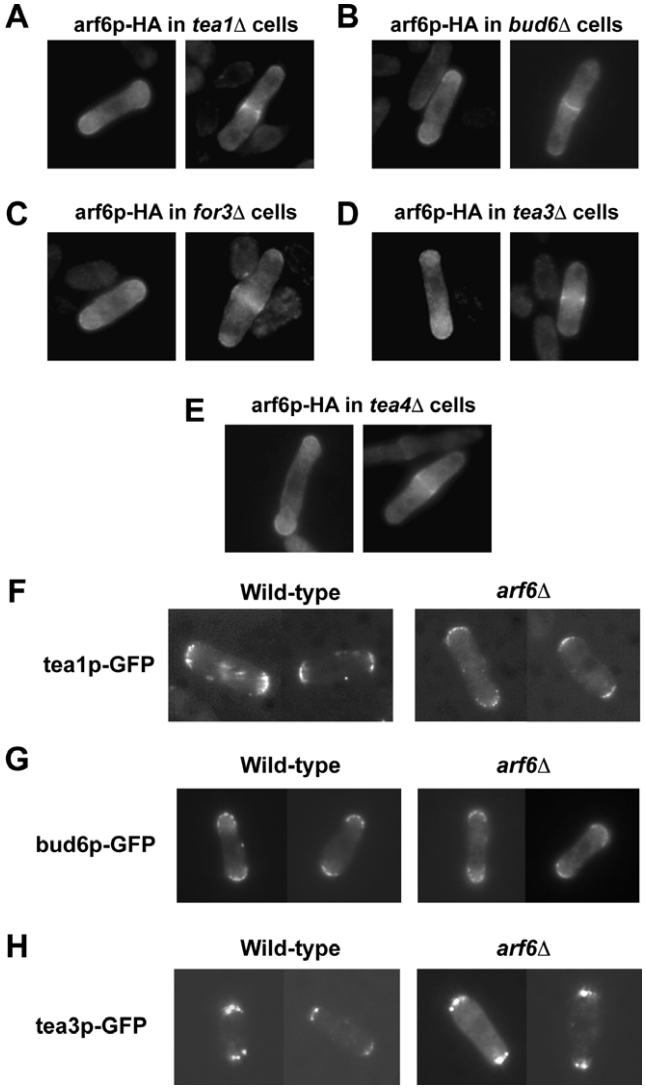

Fig. 3. Localization of arf6p in some mutants that have defects in NETO (A–E), and localization of some NETO factors in *arf6Δ* cells (F–H). Wild-type cells containing pARF6-HA were stained with anti-AlexaFluor-labeled HA antibody (A–E). Localization of arf6p-HA in *tea1Δ* cells (A), in *bud6Δ* cells (B), in *for3Δ* cells (C), in *tea3Δ* cells (D) and in *tea4Δ* cells (E). (F) Localization of tea1p-GFP in wild-type (left) and *arf6Δ* (right) cells containing pREP3X-*tea1*-GFP. (G) Localization of bud6p-GFP in wild-type (left) and *arf6Δ* (right) containing pREP3X-*bud6*-GFP. (H) Localization of tea3p-GFP in wild-type (left) and *arf6Δ* (right) cells containing pREP3X-*tea3*-GFP. These three plasmids used in (F–H), GFP-fused proteins were expressed from their native promoters.

HA were detected on cell ends and a presumptive septum in *for3Δ* cells (Fig. 3C). tea3p belongs to the Kelch-repeat protein family, and is a cell-end marker protein required for the proper localization of tea1p [26]. tea4p is a SH3-domain containing protein that binds tea1p directly at the cell tips and at the microtubule plus ends [27]. Localization of arf6p-HA was not affected by the absence of tea3p or tea4p (Fig. 3D and E). These results indicate that the polarized localization of arf6p is independent on tea1p, bud6p, for3p, tea3p, and tea4p.

Localization of some NETO factors in arf6Δ cells

Moreover, I examined whether the localization of tea1p, bud6p, or tea3p is affected in *arf6Δ* cells. I found that tea1p-GFP, bud6p-GFP and tea3p-GFP in *arf6Δ* cells retained at both cell tips same as in wild-type cells (Fig. 3F–H). Thus, arf6p is not required for the polarized localization of tea1p, bud6p, and tea3p.

Effect of the overexpression of arf6p and its mutants

I examined whether overexpression of arf6p has an effect on NETO. I introduced pMH1 which can overexpress *arf6*$^{+}$ from the *nmt1*$^{+}$-promoter on pREP3X. In wild-type cells, cells having *P* values under 0.18 were 82% (Fig. 4, gray bars). On the other hand, *arf6Δ* cells overexpressing arf6p having *P* values under 0.18 were 57% (Fig. 4, white bars). This result suggests that overexpression of arf6p can shift NETO to an early stage in cell cycle.

Small GTPases cycle between active GTP-bound and inactive GDP-bound states, and act as molecular switches in numerous signaling pathways [4]. Cellular functions of mammalian ARF6 are approached by using dominant-negative (ARF6^{T27N}) and constitutively active mutants (ARF6^{Q67L}) [11–13]. To analyze the function of arf6p in fission yeast, I constructed two mutants of arf6p: arf6p^{T35N} and arf6p^{Q75L}, in which asparagine is replaced by threonine at position 35 (as a dominant-negative mutant, Fig. 1A), and glutamine is replaced by leucine at position 75 (as a constitutively active mutant, Fig. 1A), respectively. Overexpression of arf6p^{T35N} had little effect. It could not complement the NETO defect in *arf6Δ* cells (data not shown). On the other hand, the overexpression of arf6p^{Q75L} had toxic effects; cells did not grow (data not shown). Previous studies showed that ARF6 cycles between active and inactive forms (GDP/GTP cycle) were necessary for its proper function [28,29]. A human ARF6 mutant, ARF6^{T157N} (threonine is replaced by asparagine at position 157), had been identified as a fast GDP/GTP-cycling mutant [28]. It decreases the affinity for both nucleotides, which results in fast cycling between its GDP- and GTP-bound states. The 157th residue (threonine) of the human ARF6 corresponds to the 165th residue (leucine) of arf6p (Fig. 1A). Thus, I constructed a mutant, arf6p^{L165N}, as a fast GDP/GTP-cycling mutant of arf6p and examined its effect. I made a construct to produce arf6p^{L165N} from the *nmt1*$^{+}$-promoter (pREP3X-*arf6*-L165N). I scored the *P* values of cells overexpressing arf6p^{L165N} in *arf6Δ* strain. Cells having *P* values over 0.24 were 16% in arf6p^{L165N}-overexpressing cells (Fig. 4, black bars) versus 7.5% in arf6p^{WT}-overexpressing cells (Fig. 4, white bars), suggesting that cells overexpressing arf6p^{165N} undergo NETO at an early stage than the cells expressing wild-type arf6p. This finding strongly suggests that Arf6p has a pivotal role as a molecular switch in NETO. However, regarding arf6p^{L165N}, the corresponding residues in human Arfs are different (threonine) (Fig. 1A). It is therefore difficult to predict the validity of this mutation. Further characterization using biochemical and cell biological assays remains to be required.

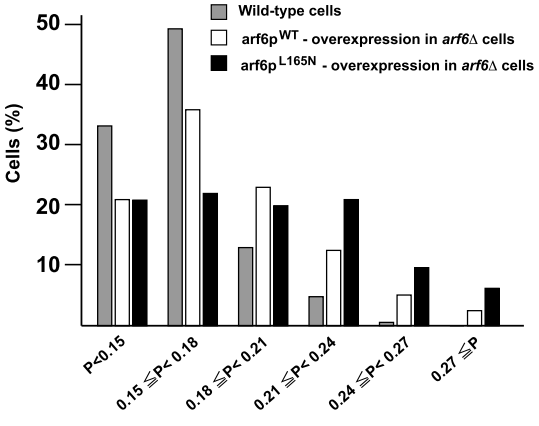

Fig. 4. Overexpression of wild-type arf6p (arf6p^{WT}) and a fast GDP/GTP-cycling mutant (arf6p^{L165N}) induced the shift of timing of NETO. *arf6Δ* cells overexpressing arf6p^{WT} or arf6p^{L165N} were stained with Calcofluor. *P* values were scored in each strains ($n = 200$ cells) and presented in the histogram (white bars, arf6p^{WT}—overexpression in *arf6Δ* cells; black bars, arf6p^{L165N}—overexpression in *arf6Δ* cells; gray bars, wild-type cells as control).

Discussion

Fission and budding yeasts serve as genetic model organisms for the study of the mechanisms involved in cell polarity [1]. Budding yeast, *Saccharomyces cerevisiae*, has Arf3p that is a sole member of class III Arf in this organism. Previous study showed that Arf3p is also involved in the regulation of cell polarity; disruption of *S. cerevisiae ARF3* caused a defect in bud-site selection [30]. Both *S. cerevisiae* Arf3p and *S. pombe* arf6p do not require the actin cytoskeletons to achieve their polarized localization. Thus, these two proteins may have a common role to generate (or to orient) polarized growth during the bud-site selection in budding yeast and NETO in fission yeast. In mammalian cells, ARF6 has a dual function; actin cytoskeleton remodeling and plasma membrane trafficking [5,6,9]. Thus, in fission yeast, arf6p may be involved in supplying membrane to the growing new end.

Studies on NETO in fission yeast seem to be relevant to understanding regulation of cell polarity in other cell types. Although the disruptions of previously identified genes

involved in NETO (*tea1*$^+$, *tea3*$^+$, *tea4*$^+$, *bud6*$^+$, and *for3*$^+$) do not exhibit a complete defect in NETO, *arf6Δ* cells completely fail to undergo NETO. Thus, *arf6*$^+$ is the first identification of a gene that is essential for NETO. The polarized localization of arf6p does not depend on microtubules and some NETO factors (tea1p, tea3p, tea4p, bud6p, and for3p), suggesting that microtubules and these factors are important for NETO, but not sufficient. How does arf6p regulate NETO? arf6p is dispensable for the growth at old ends, but is essential for the activation of growth at new ends. Although the polarized localization of actin coincides with active growth zones (Fig. 1F), actin patches disappear at the new ends after septation, and reappear at new ends when NETO occurs. On the other hand, arf6p does not disappear after septation at new ends (Fig. 2A). The concentrated arf6p at new ends may activate an unknown landmark as the future growing sites possibly with some factors (its GDP/GTP exchange factor and its GTPase-activating protein). The downstream target of arf6p remains unclear. Further studies should elucidate the precise mechanism of the arf6p-mediated NETO-signalling pathway. It will also be of interest to know whether arf6p-mediated signal transduction is conserved in higher eukaryotic cells for the activation of sites for polarized cell growth.

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