Basic-hydrophobic sites are localized in conserved positions inside and outside of PH domains and affect localization of Dictyostelium myosin 1s

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**ABSTRACT** Myosin 1s have critical roles in linking membranes to the actin cytoskeleton via direct binding to acidic lipids. Lipid binding may occur through PIP3/PIP2-specific PH domains or nonspecific ionic interactions involving basic-hydrophobic (BH) sites but the mechanism of myosin 1s distinctive lipid targeting is poorly understood. Now we show that PH domains occur in all Dictyostelium myosin 1s and that the BH sites of Myo1A, B, C, D, and F are in conserved positions near the β3/β4 loops of their PH domains. In spite of these shared lipid-binding sites, we observe significant differences in myosin 1s highly dynamic localizations. All myosin 1s except Myo1A are present in macropinocytic structures but only Myo1B and Myo1C are enriched at the edges of macropinocytic cups and associate with the actin in actin waves. In contrast, Myo1D, E, and F are enclosed by the actin wave. Mutations of BH sites affect localization of all Dictyostelium myosin 1s. Notably, mutation of the BH site located within the PH domains of PIP3-specific Myo1D and Myo1F completely eradicates membrane binding. Thus, BH sites are important determinants of motor targeting and may have a similar role in the localization of other myosin 1s.

**INTRODUCTION** Class-I myosins, the first-discovered unconventional myosins (Pollard and Korn, 1973), are one of the most widely expressed and ancient members of the myosin superfamily (Pollard and Korn, 1973; Richards and Cavalier-Smith, 2005; Odonitz and Kollmar, 2007; Kollmar and Muhlhausen, 2017). These ubiquitous motors have a wide range of cellular functions (McConnell and Tyska, 2010; Maxeiner et al., 2015; Kittelberger et al., 2015; Kittelberger et al., 2016; McIntosh and Ostap, 2016; Ouderkirk-Pecone et al., 2016; Visuttijai et al., 2016; Caridi et al., 2018; Juan et al., 2018; McIntosh et al., 2018; Nevzorov et al., 2018). All myosin 1s are monomeric and have a single heavy chain with an N-terminal head (motor domain), a neck with one or more IQ sites that bind light chains, and a C-terminal SH3 domain (see Figure 1; Pollard et al., 1991; Sellers, 2000; Berg et al., 2001). Most organisms harbor multiple isoforms of myosin 1s. For example, Dictyostelium discoideum has three short-tail (DdMyo1A, DdMyo1E, and DdMyo1F), and three long-tail (DdMyo1B, DdMyo1C, and DdMyo1D) myosin 1s, while Homo sapiens have six short-tail (HsMyo1A, HsMyo1B, HsMyo1C, HsMyo1D, HsMyo1E, and HsMyo1F) myosin 1s, and two long-tail (HsMyo1E and HsMyo1F) myosin 1s.

Dictyostelium myosin 1 isoforms may have unique as well as redundant functions because removal of multiple isoforms usually causes stronger phenotypic defects than removal of any single one (Novak et al., 1995; Jung et al., 1996; Ostap and Pollard, 1996; Falk et al., 2003; Rivero, 2008; Chen et al., 2012). Most of...
the known functions of myosin 1s require binding to membranes via acidic phospholipids. Mapping of the critical regions required for membrane targeting of each myosin 1 is needed for a full understanding of their individual and shared functions and how their lipid-binding sequences dictate their specific intracellular targeting and function. The binding of myosin 1s to lipids occurs through the basic TH1 domain either by a PIP3/PIP2-specific PH domain (Hokanson and Ostap, 2006; Komaba and Coluccio, 2010) or by non–lipid-specific ionic interactions (Brzeska et al., 2008, 2010, 2012, 2016; Feerer et al., 2010; Mazerik and Tyska, 2012). Three Dictyostelium myosin 1s (Myo1D, Myo1E, and Myo1F) bind PIP3 with high specificity, whereas the other three (Myo1A, Myo1B, and Myo1C) do not (Chen et al., 2012). Dictyostelium Myo1B and Myo1A bind to acidic phospholipids principally through a short basic-hydrophobic (BH) region located within the TH1 domain of their tails (Brzeska et al., 2010, 2012, 2014, 2016) as shown originally for Acanthamoeba myosin 1C (Brzeska et al., 2008). These BH sites all have a high content of basic and hydrophobic residues but no sequence homology (Brzeska et al., 2010).

PH domains have low sequence homology, similar to BH sites, but a highly conserved structure (seven β-sheets and a C-terminal α-helix). One of the major functions of PH domains is lipid binding (Lemmon et al., 2002; Schefzik and Welti, 2012; Feng et al., 2018); however, most PH domains do not display any lipid specificity (Lemmon et al., 2002). A high-resolution structure of HsMyo1C identified all the conserved structural elements of its PH domain (Lu et al., 2015), and the presence of PH domains was predicted for other human myosins 1s (Hokanson et al., 2006; Lu et al., 2015).

Dictyostelium cells are extremely motile; they undergo fast and dramatic morphological changes, form impressive actin waves, and macropinocytose robustly, making them an excellent model for studying mechanisms of myosin 1s targeting. The cellular localization of long-tail DdMyo1B (Brzeska et al., 2012, 2014) and short-tail DdMyo1A, DdMyo1E, and DdMyo1F (Brzeska et al., 2016) have been previously shown for cells in different motile states. To directly compare the localization of all myosin 1 isoforms in a single cell type, the dynamic localizations of long-tail DdMyo1C and DdMyo1D have now been characterized. We also investigated the importance of the BH sites for localization of Dictyostelium myosins 1C, 1D, 1E, and 1F.

RESULTS

Identification of BH sites within the PH domains of Dictyostelium and human myosin 1s

Myosin 1s bind to membranes via their TH1 domain. The recently published crystal structure of the tail region of human Myo1C (Lu et al., 2015) revealed the secondary structure of its TH1 region and of the PH domain within it. We aligned the sequences of Dictyostelium and human myosin 1s (Edgar, 2004) and superimposed the structural elements from the crystal structure of human HsMyo1C tail (Lu et al., 2015) and identified the positions of the BH sites (Supplemental Figures S1 and S2, and Table 1). BH sites are defined as amino acid residues with a BH score above 0.6 and the BH search identifies BH sites by including nine residues before and after the BH residues. The alignments of the human and Dictyostelium TH1 domain sequences unexpectedly reveal that the BH sites of all Dictyostelium myosin 1s except Myo1E are in or near the β3/β4 loop of their PH domains (Figure 2A). The only BH site of Myo1E and the second BH sites of Myo1C and Myo1F are N-terminal to their PH domains but still within the TH1 tail segment (Figure 2B and Table 1). Based on the homology to human myosin 1C, these BH-N sites are located in the vicinity of the α4/α5 loop of their TH1 domains. We found only four phylogenetically diverse myosin 1s with BH sites in homologous positions (Figure 2B). Interestingly, the BH sites of all human myosin 1s except HsMyo1B are instead found in the vicinity

FIGURE 1: Dictyostelium myosin 1s. Schematic representation of Dictyostelium Myo1A-Myo1F. The heads (motors), necks, and regions within the tails are color coded, and their boundaries are marked on the top according to Cymobase (Odronitz and Kollmar, 2006; Kollmar and Muhlhausen, 2017). The tails (but not the motors) are drawn in proportion. The BH sites are marked according to BH search (Brzeska et al., 2010). Positions of PH domains are additionally marked by red arrows on the top. Light-chain binding sites (IQ motifs) are marked according to Crawley et al. (2011). The exact locations that are not marked in the figure by numbers are as follows: MIA: IQ1: 725–746; IQ2: 747–768; PH: 765–865; BH: 802–923; BH: 820–923; MIA: IQ1: 703–720; IQ2: 721–738; IQ3: 739–760; BH-N: 775–780; PH: 809–907; BH: 837–854, MID: IQ: 692–713; PH: 760–864; BH: 804–815; MIA: IQ1: 698–719; IQ2: 720–741; BH: 804–815; PH: 850–952, MIF: IQ1: 741–762; IQ2: 781–802; BH-N: 871–878; PH: 912–1010; BH: 947–955.

Motor Neck TH1 PH GPQ SH3 IQ BH

Molecular Biology of the Cell

102 | H. Brzeska et al.
TABLE 1: Localization of BH peaks in the tails of Dictyostelium and human myosin 1s.

| Myosin | Tail length | Name of BH peak | Location | Sequence |
|--------|-------------|----------------|----------|----------|
| DdMyo1A | Short | BH | β3/β4 | (858) KGDSWFAI (865) |
| DdMyo1B | Long | BH | β3/β4 | (802) KKVVLHTLR (811) |
| DdMyo1C | Long | BH-N | α4/α5 | (837) TKQAYLIKLQKKNLAT (854) |
| DdMyo1D | Long | BH | β3/β4 | (804) KRPWITYVQKRR (814) |
| DdMyo1E | Short | BH-N | α4/α5 | (804) YDFHGGKKKWDF (815) |
| DdMyo1F | Short | BH-N | α4/α5 | (871) RKKEWDCR (878) |
| HsMyo1A | Short | BH-N | α3 | (830) KRF (832) |
| HsMyo1B | Short | BH | β1/β2 | (909) T |
| HsMyo1C | Short | BH | β1/β2 | (915) KRFHLWRCKKYRDQ (929) |
| HsMyo1D | Short | BH | β1 | (894) DRKGYKPR (901) |
| HsMyo1E | Long | BH | β1/β2 | (856) NVLFSCH (862) |
| HsMyo1F | Long | BH-C | ? | (777) FKGVKKRDLT (787) |
| HsMyo1G | Short | BH | β1/β2 | (773) RRRKPKRDLI (783) |
| HsMyo1H | Short | BH | β1/β2 | (927) RKGMAKKGKP (936) |

The positions of BH peaks were defined by comparing BH plots of myosins (Supplemental Figures S1 and S2) to their sequence alignments (Figures 1 and 2 and Supplemental Figure S3). BH sites are defined as amino acid residues with BH score higher than 0.6 but nine additional residues on both sides contribute to their appearance (Brzeska et al., 2010).

of the β1/β2 loop of their PH domains (Figure 2A), that is, in a region of the PH domain N-terminal to that harboring the BH sites of Dictyostelium myosin 1s. The single BH site of HsMyo1B and a second BH site of HsMyo1A are in the C-terminus of the neck region (BH-N in Table 1). BH sites in this location are also present in myosins 1s from diverse species (Supplemental Figure S3). The long-tailed HsMyo1F has a second BH site in the proline-rich region of the tail (BH-C in Table 1).

Identification of the BH sites of Dictyostelium myosin 1s in or near the unstructured β3/β4 loop of PH domains was supported by an analysis of predicted protein folding using the PHYRE2 Protein Fold Recognition Server (Kelley et al., 2015). All PH domains of Dictyostelium myosin 1s matched the human Myo1C tail crystal structure (PDB 4R8G) with 96–100% confidence, higher than the confidence of the match with all other PH domains in the Protein Data Bank. The predicted secondary and tertiary structures of the putative Dictyostelium myosin 1 PH domains agree with the secondary structure of the PH domain of human Myo1C and consist of seven β-sheets and a C-terminal α-helix (Figure 3). The exact boundaries and lengths of these structures for the individual myosins 1s are slightly different in the Phyre2 model (see Supplemental Figure S4) than in the sequence alignment shown in Figure 2 but in both alignments the BH sites are in the vicinity of the β3/β4 loop.

In vivo imaging of myosin 1s and their mutants

Dictyostelium cells were transfected with the plasmids encoding GFP-Myo1s listed in Table 2 and RFP-LifeAct, and the localization of the expressed proteins monitored by fluorescence microscopy. The expression and stability of each GFP-myosin fusion was examined by immunoblotting of SDS–PAGE of cell extracts. GFP-myosin heavy-chain bands were observed at the expected molecular weights and no lower molecular weight bands were present in the blots (Supplemental Figure S5). The blotting results along with the absence of fluorescent autophagosomes in microscopy images argues against any significant proteolysis and therefore also against unfolding of expressed proteins. Although the expression of the GFP-myosin 1s, as seen in total cell extracts, varied between cell lines expressing different myosins and mutants (Supplemental Figure S5), the level of expression in individual cells within the same cell line also varied. Therefore, there was always a considerable pool of cells with similar expression level for comparison (Supplemental Figure S6).

The localization of each mutant myosin was quantified in at least 100 cells in two independent transfections in parallel with wild-type protein, and the images shown are representative of a large population of cells for a given cell line.

Localization of PIP3-specific long-tail Myo1D

Three Dictyostelium myosin 1s display PIP3-binding; two short-tailed myosins, Myo1E and Myo1F, and the long-tailed Myo1D that has a GPQ domain that targets Myo1B to actin waves. It is not known whether these three myosins have identical cellular localizations and whether the BH site plays a role in their targeting. The dynamic localization of Myo1D largely resembles that of both...
Myo1E and Myo1F. Expressed Myo1D localized sharply and uniformly to the plasma membrane of nonmotile cells (Figure 4A), diffusely at the front of chemotaxing cells (Figure 4F), as seen for all other Dictyostelium myosin 1s (Brzeska et al., 2012, 2014, 2016, and see below), and localized to cell–cell contacts (Figure 4B).

A striking difference between Myo1D and the other long-tailed (but not PIP3 specific) Myo1B localization in actin waves was observed (Figures 4E and 5E). Myo1D localized, like short-tail Myo1E and Myo1F, in the PIP3-rich region encircled by the actin wave (Figure 4E), with only occasional enrichment in the actin wave itself (see 0 and 14 min in Figure 5E), whereas Myo1B colocalizes only with the actin (Brzeska et al., 2012, 2014). Thus, in spite of the presence of an actin-binding domain (the GPQ region), the Myo1D PIP3-binding site in the TH1 region plays a dominant role in Myo1D localization.

All myosin 1s except Myo1A are associated with macropinosomes during their formation and internalization (Brzeska et al., 2016, and see below). Myo1D is also found in pinocytic protrusions and cups, and freshly internalized vesicles (Figure 4C, D, and G). It is localized along the entire cup surface (Figure 5, A and B), and, after cup closure, along the entire vesicle surface (Figure 5, C and D). In contrast to Myo1B, Myo1D is not enriched at the sites of cup closure (Figures 5C, 6 s, and 6D, 0 s). Myo1D is seen at cup edges before closure, although it is not highly enriched, in contrast to Myo1E and Myo1F that are largely absent from this location (compare Myo1D in Figure 5, A and B, with Myo1F in Supplemental Figure S7B, 0 s, and with Myo1F and Myo1E in Brzeska et al., 2016). Myo1D dissociated from macropinosomes before actin (Figure 5, C, 18 s, and D, 24 s).
Differential association of Myo1E and Myo1F with macropinocytic vesicles

Myo1E and Myo1F both have PIP3-specific PH domains (Chen et al., 2012) and localize to the plasma membrane of nonmotile cells, to cell–cell contacts and macropinocytic structures, the PIP3-rich region encircled by actin waves, and the engulfing mouth and front of chemotaxing cells (Chen et al., 2012; Brzeska et al., 2016, and see below). The localizations of both Myo1E and Myo1F resemble that of Myo1D shown in Figures 4 and 5 except that Myo1E and Myo1F are excluded from the edges of pinocytic cups (Brzeska et al., 2016). The localizations of Myo1E and Myo1F during the process of macropinocytosis were examined in more detail to determine whether they were identical.

Myo1E has previously been shown to remain associated with pinocytic vesicles for a longer time than Myo1B (Brzeska et al., 2016). We now show that Myo1E dissociates from macropinocytic vesicles before actin (Figure 6, A, 24 s, and B, 22 s), that is, earlier than Myo1F that remains associated with pinocytic vesicles after actin dissociation (Brzeska et al., 2016, and see later in this article). Thus, while the overall localization of Myo1F and Myo1E during macropinocytosis is similar, Myo1F stays associated with internalized vesicle longer than Myo1E. Thus far, this is the only observed difference in the localizations of Myo1E and Myo1F.

PIP3-specific myosin 1s associate strongly with freshly ingested macropinosomes

All myosin 1s except Myo1A associated with macropinosomes during their formation and internalization (Brzeska et al., 2012, 2016, and this article). While the levels of actin remained constant during this process, the fluorescence of PIP3-specific Myo1D, Myo1E, and Myo1F increased transiently before dissociating from the early macropinosome (Figure 7; see also Figures 5D, 6 s, and 6B, 16 s). The long-tailed Myo1B is similarly localized to the early macropinosomes and dissociates before F-actin, but its levels do not change during macropinosome internalization (Brzeska et al., 2016).

Mutation of BH site located in PIP3-specific PH domain of Myo1D abolishes membrane association

BH sites are essential for in vivo membrane association of Myo1B (Brzeska et al., 2012) and Myo1A (Brzeska et al., 2016) and for in vitro binding of Acanthamoeba Myo1C (Brzeska et al., 2008). Peptides corresponding to BH sites at this position in Myo1D, Myo1B, and Myo1C bind to acidic lipid vesicles in vitro and scrambling the peptide sequence does not affect its affinity for lipids (Brzeska et al., 2010). Dictyostelium Myo1D has a single BH site located in the β3/β4 loop of its PIP3-specific PH domain (Figures 1 and 8 and Table 1). Therefore, it seemed reasonable to consider that the BH site of Myo1D contributes to its in vivo membrane association by direct binding to acidic membrane lipids. Mutation of Arg and Lys residues in the BH site to Ala, which eliminates its single BH peak (Figure 8 and Table 2), completely abolished the association of Myo1D with the plasma membrane of nonmotile cells, cell–cell contacts, macropinocytic structures (Figure 4, A–D), the PIP3-rich membrane region encircled by actin waves (Figure 4E), and the engulfing mouth of chemotaxing cells (Figure 4G). However, Myo1D-BH-Ala still localized diffusely at the front of chemotaxing cells (Figure 4F), consistent with this localization being due to myosin association with actin by the myosin head, as shown earlier for Myo1B and Myo1A (Brzeska et al., 2012, 2016). These results reveal for the first time the importance of a BH site located within a PIP3-specific domain for membrane-lipid binding.

Role of the N-terminal BH site in myosin 1E membrane association

Myo1E is the only Dictyostelium myosin 1 with a single BH site at the N-terminus of its TH1 region and none in its PH domain (Figure 1 and Table 1). Mutation of this BH site (Table 2 and Figure 8) strongly inhibited Myo1E localization to the plasma membrane of nonmotile cells (Figure 9A) but the effect of mutation was not substantial when cells became motile. Myo1E-BH-N-Ala associated with macropinocytic protrusions more weakly than Myo1E (especially at the early stages of starvation, Figure 9B) but it associated strongly with macropinocytic vesicles (Figure 9, B and C) and localized to the region encircled by actin waves, cell–cell contacts of randomly moving cells, and the front and engulfing mouth of chemotaxing cells in a manner indistinguishable from Myo1E (Figure 9, D and E).

The Myo1E-BH-N-Ala mutant was transiently enriched on pinocytic vesicles after their ingestion (Figures 9C, 30 s, and 7), and dissociated from pinocytic vesicles before actin (Figure 9C, 40 s) as seen for the wild-type Myo1E (Figure 6A). However, the

| Protein name               | Description                          | Mutation          |
|----------------------------|--------------------------------------|-------------------|
| Myo1C                      | Full length, 1–1182                  | None              |
| Myo1C-BH-Ala               | BH site mutated                      | K838A, K845A, K847A |
| Myo1C-BH-N-Ala             | BH-N site mutated                    | R776A, R783A, K784A |
| Myo1C-2BH-Ala              | Both BH sites mutated                | R776A, R783A, K784A, K838A, K845A, K847A |
| Myo1D                      | Full length, 1–1109                  | None              |
| Myo1D-BH-Ala               | BH site mutated                      | K804A, R805A, K812A, R831A |
| Myo1E                      | Full length, 1–1005                  | None              |
| Myo1E-BH-N-Ala             | BH-N site mutated                    | K810A, K811A, K812A |
| Myo1F                      | Full length, 1–1071                  | None              |
| Myo1F-BH-Ala               | BH site mutated                      | K946A, K947A, K951A, K952A |
| Myo1F-BH-N-Ala             | BH-N site mutated                    | R871A, K872A, K873A, R878A, R879A, K880A |
| Myo1B-BH-Myo1D             | BH sites swapped                     | KKKVLVHTLIRR/RKKRPWIYVQKRR |

In Myo1B-BH-Myo1D the BH site in Myo1B (801)KKKVLVHTLIRR(812) was replaced with the BH site of Myo1D (802)RKKRPWIYVQKRR(814).

**TABLE 2: Myosins and mutants used in this study.**
BH-N-Ala mutation decreased the time that Myo1E remained associated with pinocytic vesicles (Figure 10). On average, Myo1E dissociated from vesicles $\sim 2$ s before actin, whereas Myo1E-BH-N-Ala dissociated $\sim 6$ s before actin, indicating that the Ala mutant associated with pinocytic vesicles more weakly than wild-type Myo1E. Thus, the single BH site of Myo1E, located outside the PH domain, contributes to Myo1E localization, especially in nonmotile cells. However, in contrast to Dictyostelium Myo1D, it is not the main determinant of Myo1E intracellular localization.

**FIGURE 3:** 3D models of the PH domains of Dictyostelium myosin 1s. 3D models of the PH domains of Dictyostelium Myo1A, Myo1B, Myo1C, Myo1D, and Myo1F were created in Phyre2 (Kelley et al., 2015) using the crystal structure of the PH domain of human Myo1C (Lu et al., 2015) as a template. The arrows point to the BH sites (marked in purple) that are located in the vicinity of the $\beta_3/\beta_4$ loop of Dictyostelium myosin 1s. DdMyo1E does not have a BH site at this region. The BH sites of human myosin 1s (unpublished data) are located in the vicinity of the $\beta_1/\beta_2$ loop.

**FIGURE 4:** Localization of Myo1D and the effect of BH-Ala mutation. Myo1D (left panels) localizes uniformly on the plasma membrane in nonmotile cells (A), at cell–cell contacts (B), in macropinocytotic protrusions (C), at micropinocytic vesicles (D), in the region encircled by actin wave (E), at the front of chemotaxing cells (F), and in engulfing mouth (G). Myo1D-BH-Ala mutant (right panels) is absent from all these locations (A–E, G) except the front of chemotaxing cells (F). Cells in row A were fixed and live cells are shown in all other rows. Arrows point to regions of interest. Experiments were done in AX2 cells. The localization of MID was the same in Myo1B null cells (unpublished data). Bars are 10 $\mu$m. The number of independent transfections was four for Myo1D and two for Myo1D-BH-Ala. At least two independent experiments were performed for each transfection under each condition. The images are representative of more than 100 cells observed under each condition.

The two BH sites of Myo1F are required for membrane association

Myo1F is the only myosin 1 that stays associated with pinocytic vesicles longer than F-actin (Figure 11A). Myo1F has two BH sites: one located in the $\beta_3/\beta_4$ loop of its PH domain and a second one outside the PH domain at the N-terminus of TH1 region, in the same region as the sole Myo1E BH site (Figures 1 and 8 and Table 1). The contribution of the two BH sites of Myo1F to its localization was tested by mutating each separately (see Tables 1 and 2 and Figure 8 for mutations). When the BH site in the PH domain was mutated, Myo1F-BH-Ala did not localize to the plasma membranes of nonmotile cells, or to macropinocytic structures, or to regions encircled by actin waves (see Myo1F-BH-Ala in Figure 11, B–D). This effect was very similar to the effect of mutation of the homologous site in
Cellular localization of long-tail Myo1C is very similar to the localization of long-tail Myo1B

The finding that localization of long-tail Myo1D and Myo1B in actin waves and macropinosomes differs raised the question of whether the third long-tail Dictyostelium myosin 1, Myo1C, which does not display PIP3 specificity (Chen et al., 2012), behaves similarly to Myo1D or to Myo1B. The localization of Myo1C was found to be quite similar to that of Myo1B (Brzeska et al., 2012, 2014, 2016); it is distributed sharply and uniformly along the plasma membrane of nonmotile cells, and tightly localized to cell–cell contact sites, macropinocytic structures of randomly moving cells, and the “mouth” of a cell engulfing another cell during chemotaxis (Figure 12, A–C and F). Like all other Dictyostelium myosin 1s, Myo1C is localized diffusely at the front of chemotaxing cells (Figure 12E). Myo1C is associated with F-actin in actin waves (Figure 12D), similar to Myo1B.

Myo1C is significantly enriched in the edges of macropinocytic cups (Figure 13, A and B), similar to Myo1B (Brzeska et al., 2016) and in contrast to Myo1D (Figure 13D). Following cup closure, Myo1C remained associated with newly internalized macropinocytic vesicles for a short period of time but dissociated from these vesicles before actin (Figure 13C, 12 s). Myo1C was also diffusely enriched in a “closing patch” after vesicle ingestion (Figure 13C, 3 and 6 s), and often found sharply localized to the plasma membrane at the site of previous vesicle ingestion, which sometimes converted to a new macropinocytic cup (Figure 11D, 20 s, and Supplemental Figure S7A). Interestingly, vesicle enclosure at the plasma membrane site was often first detected by the accumulated fluorescence of GFP-labeled Myo1C followed by enclosure by F-actin (Figure 13C, 3 and 6 s). Similar phenomena were also observed for expressed Myo1F (Supplemental Figure S7B). The localization of expressed Myo1C was the same in wild-type AX2 cells and Myo1B null cells (Figure 13, A and B), in agreement with our earlier data showing that the presence or absence of endogenous myosin 1s did not affect the localization of expressed myosin 1s (Brzeska et al., 2016).

Myo1D, emphasizing the importance of the BH site in the PH domain for membrane interactions. Mutation of the BH site at the N-terminus of the TH1 region, Myo1F-BH-N-Ala, also completely abolished membrane association of Myo1F, and its binding to the interior of actin waves (see Myo1F-BH-N-Ala in Figure 11, B–D), a much stronger effect than mutation of the homologous site in Myo1E. These results show that each of the two BH sites of Myo1F make significant contributions to the association of Myo1F with membranes.

Mutation of each of the two BH sites weakens, and mutation of both BH sites abolishes membrane association of Myo1C

Long-tail Myo1C has two BH sites: one located within the PH domain, like the single BH site of Myo1B and Myo1D, and one at the N-terminus of the TH1 region (Figures 1 and 8 and Table 1). Mutation of the BH site within the PH domain (Myo1C-BH-Ala in Figure 8 and Table 2) significantly weakened, but did not completely abolish, Myo1C association with membranes.
The presence of the second BH site N-terminal to the PH domain (BH-N) on Myo1C membrane association might be explained by the role and this is largely determined by membrane-binding sequences unique functions (McConnell and Tyska, 2010; McIntosh and Ostap, 2016). The localization of a particular myosin 1 dictates its cellular role and this is largely determined by membrane-binding sequences in the common TH1 domain. Comparison of the localizations of all of Dictyostelium myosin 1s and identification of sequences critical for the membrane targeting (Tables 3 and 4) now provides a more complete understanding of the distribution of myosin 1 isoforms in a

(Figure 12, A–F), the effect being most pronounced in nonmotile cells (Figure 12A). We quantified the difference by measuring the ratio of the maximum fluorescence intensity on the plasma membrane to the average fluorescence intensity in the cytoplasm of Myo1C and Myo1C-BH-Ala images in two independent experiments (Figure 14). The ratios were 2.45 ± 0.146 SEM for Myo1C and 1.41 ± 0.067 SEM for Myo1C-BH-Ala, clearly demonstrating that the BH-Ala mutation strongly affected plasma membrane association in these cells.

The differences between Myo1C and Myo1C-BH-Ala were less pronounced in motile cells. Myo1C-BH-Ala was detected in cell–cell contacts, although the signal was very weak (Figure 10B). The BH-Ala mutant clearly associated with macroin pylotic protrusions and vesicles (Figure 12C), although less robustly than the wild-type Myo1C and was detectable in the engulfing mouth of chemotaxing cells (Figure 12F). Myo1C-BH-Ala and Myo1C associated diffusely at the front of chemotaxing cells (Figure 12E), not surprisingly as diffuse associations with the front had been shown to be independent of the BH site of other myosin 1s (Brzeska et al., 2010, 2016).

The weak effect of the Ala mutation of the BH site in the PH domain on Myo1C membrane association might be explained by the presence of the second BH site N-terminal to the PH domain (BH-N-Ala site in Table 1). We tested the importance of this BH site by mutating it separately or together with the first site (Myo1C-2BH-Ala; Figures 1 and 8 and Tables 1 and 2). The effect of mutating the BH-N site alone (Myo1C-BH-N-Ala in Figures 1 and 8, and Tables 1 and 2) was similar to the effect of mutating only the BH site within the PH domain abolishing association of Myo1C with the plasma membrane of nonmotile cells but not its association with macroin pylotic protrusions and actin waves (Figure 12, G–I). However, when both BH sites were mutated (Myo1C-2BH-Ala) association with membranes was completely abolished in nonmotile cells, macroin pylotic protrusions, and in actin waves (Figure 10, G–I); that is, both BH sites contribute to Myo1C membrane targeting, whereas either BH site may be sufficient to localize the Myo1C to actin waves, presumably due to the presence of the actin-binding GPQ domain.

A stronger BH site increases Myo1B membrane association but not its localization

Our earlier in vitro data showed that BH sites bind membrane lipids by nonspecific interactions whose overall strength is proportional to the positive charge density of the BH site (Brzeska et al., 2008) and independent of its sequence (Brzeska et al., 2010). We replaced the BH site of non–PIP3-specific Myo1B with the stronger BH site of PIP3-specific Myo1D (Figure 15A). The BH peak of the resulting Myo1B-BH-Myo1D mutant is significantly stronger than that of Myo1B

(Figure 15A). It is also stronger than the BH peak of Myo1D because amino acids on both sides of a BH site affect the BH peak size. We compared the plasma membrane association of Myo1B and of the Myo1B-BH-Myo1D mutant in cells after starvation for 10 min. Myo1B-BH-Myo1D was localized uniformly to the plasma membrane in 66% of cells but Myo1B in only 18% (Figure 15B), demonstrating that strengthening the BH site of Myo1B causes stronger membrane association in vivo consistent with our in vitro data that lipid binding by BH sites depends on their amino acid composition rather than their sequence (Brzeska et al., 2010). However, Myo1B-BH-Myo1D mutant colocalized with actin in actin waves and was enriched at the macrop inocytic cup edges (Figure 15C) similarly to Myo1B and not like Myo1D. This demonstrates that the BH site of Myo1D does not carry any lipid specificity by itself.

DISCUSSION

Cells typically express more than one myosin 1 with both shared and unique functions (McConnell and Tyska, 2010; McIntosh and Oستap, 2016). The localization of a particular myosin 1 dictates its cellular role and this is largely determined by membrane-binding sequences in the common TH1 domain. Comparison of the localizations of all of Dictyostelium myosin 1s and identification of sequences critical for the membrane targeting (Tables 3 and 4) now provides a more complete understanding of the distribution of myosin 1 isoforms in a
FIGURE 7: Fluorescence of myosin 1s intensifies after vesicle incorporation, whereas fluorescence of F-actin remains stable. The images of cells taken at indicated time points are shown in the left panels (Myo1D, Myo1E, Myo1E-BH-Ala, and Myo1F, top to bottom). Arrows show the direction of line scans for myosins and F-actin shown in the right panels, and 0 distance is the beginning of the arrow. The images of each Myo1 are representative of more than 100 cells observed in two independent experiments.

single cell and insight into the molecular determinants that tune their intracellular localizations.

Differential localizations of Dictyostelium myosin 1s

All six Dictyostelium myosin 1s localize sharply to the plasma membrane of nonmotile cells and diffusely at the front of chemotaxing cells (Table 3). The tails of the myosins are responsible for the former and the heads for the latter. However, the dynamic localizations of the six myosin 1s in actin waves and macropinocytic structures are different, most strikingly for actin waves (Table 3).

Myo1B, Myo1C, Myo1D, Myo1E, and Myo1F, but not Myo1A, associate with macropinocytic structures (protrusions, cups, and vesicles), consistent with their involvement in endocytosis (Novak et al., 1995; Neuhaus and Soldati, 2000; Durrwang et al., 2006; Chen et al., 2012). The overall pattern of myosin 1s localizations suggests that the isoforms are likely to have different roles in macropinocytosis. Myo1B and Myo1C are localized to the leading edges of pinocytic cups (Table 3) and concentrated at the site of membrane sealing on the newly internalized vesicle indicating that these myosins may be involved in the closure of macropinocytic cups. Myo1B, Myo1C, and F-actin levels remain constant on the forming macropinosome; however, shortly after vesicle ingestion, there is a transient enhancement of Myo1D, Myo1E, and Myo1F on the vesicle membrane. This likely reflects an enhancement of PIP3, to which Myo1D, Myo1E, and Myo1F bind specifically. The myosin 1s associate with pinocytic vesicles for different lengths of time. Myo1B, Myo1C, and Myo1D first dissociate from the newly formed macropinosome, followed by Myo1E, then F-actin. Myo1F remains on these endocytic vesicles after actin dissociation (Brzeska et al., 2016, and this article) and is more likely to be involved in later stages of macropinocytosis.

The dynamic localization of myosin 1s differs prominently in actin waves (Table 3).
Actin waves have been compared with large macropinocytic cups (Bretschneider et al., 2009; Gerisch, 2010), the common features being PIP2 and actin in the periphery, and PIP3 enrichment in the center (Gerisch et al., 2012; Veltman et al., 2016). The PIP3-specific short-tail Myo1E and Myo1F, and long-tail Myo1D, localize to the membrane region encircled by the actin wave (see Table 3), consistent with the presence of PIP3 in these structures. These three myosins may contribute to stabilizing PIP3-rich patches. In contrast, Myo1B and Myo1C localize to regions of the waves containing PIP2 and enriched in actin. Myo1B and Myo1C fully colocalize with dynamic actin in the wave, and both the lipid-binding BH site and the actin-binding GPQ region are required for this localization (Brzeska et al., 2014). Myo1B and Myo1C may be involved in the recruitment of actin to these waves. It is of interest to note that Myo1D, that has both PIP3-binding specificity and actin-binding GPQ domain in the tail, is predominantly associated with PIP3-containing membranes, indicative of a higher affinity for PIP3 than for actin. Thus, the ability to bind PIP3 directs a subset of myosin 1s to the membrane encircled by the actin wave while myosin 1s that have the GPQ region and do not show PIP3 specificity target the actin wave itself. Interestingly, myosin 1s that colocalize with actin in actin waves also contain an SH3 domain that enables CARML-mediated interaction between myosin 1 and Arp 2/3 (Jung et al., 2001; Bretschneider et al., 2009).

BH sites and membrane binding of Dictyostelium myosin 1s
All of the Dictyostelium myosin 1s have a BH site and a putative PH domain in the membrane-binding TH1 domain of their tails. Interestingly, the BH site of five myosin 1s, Myo1A, Myo1B, Myo1C, Myo1D, and Myo1F, is contained within their PH domains. These BH sites are located near the β3/β4 loop of the PH domain that in our models of the PH domains of Dictyostelium myosin 1s, as well as in a crystal structure of human Myo1C (Lu et al., 2015), protrude from the PH domain, making them perfect candidates for intramolecular interactions. The number and the location of BH sites can vary. The sole BH site of Myo1E and the second BH sites of Myo1C and Myo1F lie outside the canonical PH domain but near the α4/α5 loop of the extended PH domain (Lu et al., 2015) at the N-terminus of the TH1 region.

Elimination of each of the tail-located BH sites abolished or weakened membrane association of all Dictyostelium myosin 1s (Table 4). The basic amino acid/Ala mutations of the BH sites in the PH domains abolished all membrane binding of PIP3-specific Myo1D and
Myo1F (this article) and of non–PIP3-specific Myo1A and Myo1B (Brzeska et al., 2012, 2016) while weakening membrane binding of non–PIP3-specific Myo1C (this article). Mutation of the N-terminal BH sites of Myo1E, Myo1C, and Myo1F that are within the basic TH1 tail segment abolished the association of Myo1F with membranes and weakened the plasma membrane association of Myo1E and Myo1C. The greater effect of mutation of the BH sites of Myo1C and Myo1E in nonmotile cells suggests that the plasma membrane of these cells may have a lower negative charge density than specific regions of the plasma membrane in motile cells.

BH sites are defined by their amino acid composition and lack a consensus sequence homology (Brzeska et al., 2010). Therefore the BH sites themselves are unlikely to provide a conserved steric specificity required for selective binding to certain phospholipids. Indeed, replacing the BH site of non–PIP3-specific Myo1B with the BH site of PIP3-specific Myo1D resulted in a mutant that was not PIP3 specific and localized like the parent Myo1B.

Although increasing the size of the single BH peak in Myo1B strengthened its membrane association, there are factors other than the BH site that may affect myosin 1s localizations and membrane associations. These may include a functional PH domain, more than one lipid-binding site, different degrees of BH site exposure, and the interaction of the myosin with other proteins. For example, we know that the heads of Myo1B (Brzeska et al., 2012) and Myo1A (Brzeska et al., 2016) are required for the relocation of myosin from the plasma membrane of nonmotile cells to motile protrusions and the front of polarized cells. However, the fact that the Myo1B-BH-Myo1D mutant associates with the same membrane sites as Myo1B argues against the BH site being involved in any sequence-specific inter- or intramolecular protein interactions.

**BH sites and PH domains in other proteins**

BH sites are present within and outside the PH domains of several myosin 1s, other than those in Dictyostelium, as well as in other lipid-binding proteins (Brzeska et al., 2010), but their roles in membrane binding have not been systematically studied. For example, 67 of 77 Dictyostelium proteins containing PH domains listed in Park et al. (2008) have BH peaks located within their PH domains.

BH sites might provide initial nonspecific binding to membranes required for PIP3-specific binding by other residues within the PH domain. Such a nonspecific interaction is required for Myo1D binding to PIP3-enriched regions in vivo. Conversely, because the interaction of BH sites with membranes is proportional to the negative charge density of the membrane, PIP2/PIP3-enriched regions would enhance BH binding because of their higher charge.

To our knowledge there are only two reports describing mutations of BH sites in mammalian myosin 1s. Mutations of each of the two BH sites of mammalian Myo1A abolished its targeting to...
microvilli (Mazerik and Tyska, 2012), suggesting that the role of the BH sites in mammalian Myo1A is similar to their role in Dictyostelium myosin 1s. Interestingly, the BH sites of mammalian Myo1A are located outside the PH domain and mutation of conserved residues of the PH domain did not eliminate targeting of mammalian Myo1A. The mutation of the BH site located within the PH domain of mammalian Myo1A has not been tested. Mutation of residues within the BH site of mammalian Myo1E did not abolish lipid binding (Feese et al., 2010) but this mutation also did not abolish the BH peak so this result is not conclusive and further studies on the role of the Myo1E BH site are needed.

In general, mutations of conserved residues within the PH domains of myosin 1s have given mixed results, abolishing membrane association in some cases but not in others. Mutation of a conserved residue within the PH domain of Acanthamoeba Myo1C had very little effect on its non–PIP3-specific lipid binding driven by the BH site (Brzeska et al., 2008). In contrast, mutation of conserved residues in the PH domain of Dictyostelium Myo1E, that does not have a BH site in its PH domain, caused the loss of lipid binding (Chen et al., 2012), while mutation of the BH site located N-terminal to the PH domain weakened membrane association (this article). Mutation of conserved residues abolished lipid binding of mammalian Myo1B (Komaba and Coluccio, 2010) and Myo1F (Chen et al., 2012) but had no effect on Myo1E (Feese et al., 2010). The mutation of conserved residues within the PH domain also abolished lipid binding for mammalian Myo1C (Hokanson et al., 2006), but this myosin may interact with lipids by nonspecific ionic interactions (McKenna and Ostap, 2009). Similarly, mutation of a conserved residue within the PH domain abolished membrane association of mammalian Myo1G,
FIGURE 12: Localization of Myo1C and its BH mutants. (A–F) Localization of Myo1C and Myo1C-BH-Ala. Myo1C localizes sharply on the plasma membrane in nonmotile cells (A), in cell–cell contacts (B), in macropinocytic protrusions and vesicles (C), together with actin in actin waves (D), diffusely at the front of elongated cells (E), and sharply at the mouth of chemotaxing cells (F). Myo1C-BH-Ala is barely detectable in plasma membrane of nonmotile cells (A) and at cell–cell contacts (B) but it is clearly present in macropinocytic protrusions and vesicles (C), in actin waves (D) at the front of chemotaxing cells (F). Myo1C localization was observed in AX2 and Myo1B null cells. Images shown are of AX2 cells. Bars are 10 μm. Eight Myo1C independent transfections were done and two independent experiments were performed for each transfection. The images are representative of more than 100 cells observed.

FIGURE 13: Localization of Myo1C in macropinocytic structures. (A, B) Myo1C is enriched at cup edges in Myo1B null cells (A) and in AX2 cells (B). (C) Myo1C dissociates from the vesicle before actin and Myo1C is diffused in closing patches (12 s). Note that Myo1C fully surrounds the vesicle before it is fully enclosed by F-actin (3 s). (D) Comparison of Myo1C (left panels) and Myo1D (right panels). Myo1C is enriched at the edge of the macropinocytic cup where Myo1D is also present but not enriched (0 s). After vesicle ingestion Myo1C localizes sharply to the site of vesicle closure (20 s). Both myosins seal the vesicle entrance site before actin does (20 s for Myo1C and 3 s for Myo1D). All images are of live cells; Myo1B null cells are shown in row A and AX2 cells in other rows. Bars are 10 μm. Eight Myo1C independent transfections were done and two independent experiments were performed for each transfection. The images are representative of more than 100 cells observed.
but this myosin also engages regions other than the PH domain in lipid binding (Patino-Lopez et al., 2010). Taken together, these results suggest that nonspecific ionic interactions may play a role in the association with membranes of other myosin 1s with functional PH domains, similarly as shown for Dictyostelium myosin 1s.

It is important to keep in mind, however, that the BH search identifies only potential lipid-binding sites. The basic-hydrophobic composition of BH sites also makes them good candidates for inter- or intramolecular interactions with acidic-hydrophobic regions of other proteins, and only some of the BH sites that have been tested are involved in lipid binding (Lanier et al., 2016).


### TABLE 3: Summary of in vivo localization of Dictyostelium myosin 1s.

| Myosin | Tail length | Mutated BH site | BH site location within TH1 domain | Mutation of BH site affects membrane association |
|--------|-------------|-----------------|-----------------------------------|-----------------------------------------------|
| Myo1A  | Short       | BH              | β3/β4                             | +                                             |
| Myo1B  | Long        | BH              | β3/β4                             | +                                             |
| Myo1C  | Long        | BH              | β3/β4                             | +/-                                           |
|        |             | BH-N            | β3/β4                             | +/-                                           |
|        |             | β3/β4 and α4/α5 |                                  | +                                             |
| Myo1D  | Long        | BH              | β3/β4                             | +                                             |
| Myo1E  | Short       | BH-N            | α4/α5                             | +/-                                           |
| Myo1F  | Short       | BH              | β3/β4                             | +                                             |
|        |             | BH-N            | α4/α5                             | +                                             |

The sources of the data in the table are as follows: MIA, Brzeska et al., 2016; MIB, Brzeska et al., 2012, 2014, 2016; MIC, this article; MID, this article; MIE, Brzeska et al., 2016, and this article; MIF, Brzeska et al., 2016, and this article.

### TABLE 4: Localization of BH sites in Dictyostelium myosin 1s and effect of their mutation on membrane association.

Additionally, many lipid-binding proteins do not have clearly defined BH peaks.

The BH search identified known lipid-binding sites in proteins other than myosin 1s (Brzeska et al., 2010) and previously unknown lipid-binding sites in varied proteins including kinases (Barbosa et al., 2016; Lee et al., 2018; Gerganova et al., 2019), phosphatases (Itoh et al., 2014), ion channels (Bernier et al., 2013; Giblin et al., 2019), CARMIL (Lanier et al., 2016), and proteins of PAR polarity complex (Bailey and Prehoda, 2015; Ramanujam et al., 2018). In addition, the basic region located within the β3/β4 loop of the PH domain of the metastatic factor, P-Rex-1, was recently found to serve as a nonspecific membrane localization element (Cash et al., 2016, 2019) and this region corresponds to a strong BH peak in the BH profile of P-Rex-1 (unpublished data). BH sites may prove to be good mutagenic targets for some other lipid-binding proteins as well as for other than Dictyostelium myosin 1s because elimination of BH site(s) abolishes lipid binding independently of the myosin 1s lipid specificity. This may be especially useful for proteins for which mutation of conserved residues in putative PH domains has no effect.

### MATERIALS AND METHODS

**Cell lines, cell culturing, and cell imaging conditions**

Cells were grown, transfected, and prepared for microscopy as described earlier (Brzeska et al., 2014). Briefly, GFP-Myo1 expression plasmid and the RFP-LifeAct plasmid were cotransfected into AX2 or Myo1Bnull cells (Brzeska et al., 2014) by electroporation (Gaudet et al., 2007). We have shown earlier that the localizations of expressed myosin 1s are not affected by the presence or absence of other endogenous myosin 1 isoforms (Brzeska et al., 2016). Cells were grown in 10-cm Petri dishes in HL5 media (Formedium; HLG0101; Sussman, 1987) in the presence of appropriate selective antibiotics at the following concentrations: G418 sulfate (Mediatech) at 12 μg/ml for expressing myosin 1s and their mutants, and hygromycin (Invitrogen) at 50 μg/ml for expressing LifeAct (Riedl et al., 2008). Myo1B null cells were grown in the presence of blasticidin S HCl (Invitrogen) at 7 μg/ml. For microscopy, cells were transferred to chambered cover glass (Nalge Nunc International; 1555383) and allowed to attach for 30 min. HL5 media was then exchanged for starvation solution (20 mM phosphate buffer, pH 6.2, 0.2 mM CaCl2, 2 mM MgCl2). For monitoring of nonmotile cells,
attachment time was shortened to 20 min and cells were fixed immediately after solution exchange. Random movement and macropinocytosis were observed in live cells between 0.5 and 4 h of starvation and chemotaxis after overnight incubation at 4°C. Actin waves were induced by adding 1 μM latrunculin (latrunculin A; Sigma) into the cells that were starved for 30 min. Waves persisted for at least 3 h in the presence of latrunculin and were recorded in live cells.

Image recording and processing
Cells were viewed with a Zeiss 780 confocal microscope with a 63× plan Achromat 1.4 objective. Image recording and profile scanning were done using Zeiss ZEN software and final illustrations were prepared using Zeiss ZEN software, Photoshop, and Excel.

Protein sequence alignments and modeling
Sequences of myosin 1s were aligned using MUSCLE (multiple sequence comparison by log-expectation) (Edgar, 2004) available on the PIR web site (https://pir.georgetown.edu/pirww/ search/multialign.shtml) or the EMBL-EBI web site (www.ebi.ac.uk/Tools/msa/muscle/), the latter with applying Person/Fasta parameters. The positions of BH sites were determined using BH search (Brzeska et al., 2010; https://hpccwebapps.cit.nih.gov/bhsearch/). The 3D modeling was done using the PHYRE2 Protein Fold Recognition Server (Kelley et al., 2015; www.sbg.bio.ic.ac.uk/phyre2/html/). The locations of other myosin 1 domains were according to Cymobase (Odronzit and Kollmar, 2006, 2007; Kollmar and Mühlhausen, 2017; www.cymobase.org/cymobase).

Amino acid sequences of all myosin 1s were obtained from Cymobase and their accession names are listed in Figure 2 and Supplemental Figure S3.

DNA constructs
The myosins and their mutants used in this study are shown in Table 1. Base clones for each myosin 1 gene (myoC, myoE, myoD, and myoF) were generated by TA cloning (Stratagene system; Agilent) of a full-length genomic fragment generated by PCR using AX2 genomic DNA as a template. Q5 mutagenesis (New England Biolabs) was then used to introduce mutations into the region of each gene encoding one or both of the BH regions (see Supplemental Table S1). The sequence of all PCR-generated clones was verified (University of Minnesota BioMedical Genomics Center). The wild-type or mutant full-length genes were then restriction enzyme cloned into a low copy number origin of replication extrachromosomal expression plasmid carrying a G418 resistance cassette, pTX-GFP (Levi et al., 2000). The resulting plasmid encoded each of the myosin 1s with GFP fused at its N-terminus. The plasmid carrying RFP-LifeAct and hygromycin resistance was described earlier (Riedl et al., 2008; Veltman et al., 2009; Brzeska et al., 2014).

Immunoblotting
Cells from each strain were collected and dissolved in 100 μl of urea-containing Laemmli sample buffer (Novak et al., 1995) to a final concentration of (3–4) × 10^6 cells/ml and run on an 8–16% TGX separating gel (Bio-Rad Laboratories), transferred to nitrocellulose membrane (LI-COR Biosciences), and then probed simultaneously with anti-GFP (BioLegend; catalogue #902601, clone B4; used at 1:4000) and anti-Dictyostelium actin (Westphal et al., 1997; used at 1:1000) monoclonal antibodies. The blots were incubated in goat anti-mouse Alexa 800 (LI-COR Biosciences; catalogue #926-32210; used at 1:4000) and then scanned using an Odyssey infrared imaging system (LI-COR Biosciences).

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116 | H. Brzeska et al.

Molecular Biology of the Cell
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