Figure S1
Distribution of Rnd3 and active RhoA in membrane blebs.
(A) Tricolor map of membrane blebs in freely-moving DLD1 cells (Cell Migration) and apoptotic DLD1 cells (early and late stage). Angular coordinates are shown on the horizontal axis, and time is shown on the vertical axis. Red zones represent expansion, blue zones represent retraction, and white zones represent no movement.
(B) Membrane blebbing of freely-moving DLD1 cells transfected with Lifeact–RFP and GFP-tagged Rnd3. The localization of Rnd3 at the plasma membrane gradually disappeared upon the initiation of the retraction of membrane blebs (t =25 s). Scale bar, 2 μm.
(C) Membrane blebbing of freely-moving DLD1 cells transfected with Lifeact–RFP and GFP-tagged Anillin homology domain (AHD), which specifically binds to GTP form of RhoA. Scale bar, 2 μm.
(D) Membrane blebbing of freely-moving DLD1 cells transfected with Lifeact–RFP and GFP-tagged p190B RhoGAP. Scale bar, 2 μm.
(E) Membrane blebbing of freely-moving DLD1 cells transfected with Lifeact–RFP and GFP-tagged ROCK1. Scale bar, 2 μm.
Construction of mathematical simulation on bleb dynamics

(A) The mathematical model of bleb dynamics was constructed based on the molecular networks of blebbing in freely-moving DLD1 cells as follows;

Equation (1)

\[
\begin{align*}
\frac{dx}{dt} &= (a - hzx) \left(1 - \frac{x}{k_x}\right) \\
\frac{dy}{dt} &= (b - (ex + gw)y) \\
\frac{dz}{dt} &= c(y - z) - (ex + gw)z \\
\frac{dw}{dt} &= fz \left(1 - \frac{w}{w_{max}}\right) \\
\frac{dS}{dt} &= -kwS + mp
\end{align*}
\]

Equation (1a) represents the change in the amount of Rnd3 at the plasma membrane, x. Rnd3 is equally distributed at the plasma membrane at a constant concentration during the expansion phase (Fig.S1B). Therefore, we assume that Rnd3 is supplied to the plasma membrane from the cytoplasm at a constant rate a. Rnd3 was assumed to be removed at a rate, hzx, proportional to the concentration of active ROCK1 (z) and Rnd3 itself (x). This reflects the fact that Rnd3 is phosphorylated by active ROCK1 and sequestered in the cytoplasm via binding to 14-3-3. We assume that there is an upper limit to the amount of Rnd3 at the plasma membrane \(K_x\) because the area of plasma membrane of the expanding blebs is restricted.

Equation (1b) represents the change in the amount of active RhoA at the plasma membrane, y. We assume that RhoA changes from an inactive state to an active state at a constant rate b. Here we assume most RhoA are inactivated and reside at the plasma membrane because RhoA is tethered to the plasma membrane via lipid modification (Mitin et al., 2012) (Fig.S1C). RhoGEFs activate RhoA at rate c. RhoA changes from an active state to an inactive state at a rate of \((ex + gw) y\). It undergoes inactivation in proportion to the amount of Rnd3, x. This corresponds to the fact that Rnd3 activates p190-Rho-GAP and p190-Rho-GAP inactivates RhoA (Figs.S1B and S1D). We assumed that p190-Rho-GAP inactivates RhoA regardless of its binding to ROCK1. We also assume the inactivation occurring at rate proportional to the amount of actin at the plasma membrane, w, because components of mature actin cortex recruit Rho-GAPs, such as FilGAP (Saito et al., 2012).

Equation (1c) represents the change in the amount of active ROCK1 at the plasma membrane, z. Active ROCK1 increases at a rate of \(c(y-z)\), which means that active ROCK1 increases in proportion to the amount of active RhoA that is not yet bound to ROCK1 (Figs.S1B and S1E). Considering that ROCK1 binds to RhoA on a one-to-one basis and maintains its activity while binding, the amount of active ROCK1, z, is equal to the amount of RhoA already bound to ROCK1. Therefore, the amount of active RhoA that can activate ROCK1 is described as \((y-z)\). RhoA changes from an active state to an inactive state at a rate of \((ex + gw) z\), which reflects the fact that ROCK1 is inactivated when Rnd3 and actin inactivate RhoA bound to ROCK1.

Equation (1d) represents the change in the amount of actin filaments at the plasma membrane, w. Based on our previous observations, phosphorylation of Ezrin by ROCK1 is essential for reassembly of the actin cytoskeleton during the retraction phase of membrane blebs (Aoki et al. 2016). The amount of actin at the plasma membrane increases in proportion to the amount of active ROCK1 localized to the membrane at a rate of fz. Here we assume that only a subset of Ezrin is activated and that most of Ezrin is inactivated at the cytoplasm. In other words, the rate of activating Ezrin by ROCK1 is much smaller than the rate of inactivating Ezrin. From this assumption, active Ezrin does not decrease by binding to actin due to its rapid replenishment from the inactive Ezrin pool. Then we can consider the increase rate of actin as proportional to the amount of active ROCK1. As with Rnd3, we assume the amount of actin on membrane does not increase infinitely and the maximum value is set to \(w_{max}\).

Equation (1e) represents the change in the surface area of bleb, S. The surface area of
bleb increases at rate $mp$ proportional to the intracellular pressure $p$. Conversely, the surface area of bleb decreases in proportion to the amount of actin on membrane, $w$, and surface area itself, $S$. This represents the surface tension of the bleb surface, the strength of which is proportional to the amount of actin $w$.

(B) The three metrics that characterize bleb behavior were defined as follows; *Peak value* indicates the size of bleb when bleb expanded most; *Peak time* indicates the time when bleb expanded most; and *Retraction time* indicates the time taken for bleb to retract from the peak size to a size 1.05 times that at time $= 0$.

(C) The changes in three metrics when the value of each parameter was either doubled or halved. This table shows the ratio of the value of each cases to that of standard case. Parameter $a$ indicates the rate at which Rnd3 is supplied to the plasma membrane. It is predicted from simulation that the size of bleb and the time taken for retraction increase as the value of $a$ increases. This is because the increase of $a$ upregulates the amount of Rnd3 at the plasma membrane ($x$), which in turn inhibits activation of RhoA-ROCK1 pathway according to equations (1b) and (1c), resulting in the delay of actin accumulation and thus bleb retraction.

Parameters $b$ and $c$ are functionally related and they account for the rate of RhoA activation and ROCK1 activation by RhoA, respectively. Increasing Parameter $b$ upregulates RhoA activity at the plasma membrane ($y$) and subsequently ROCK1 activity ($z$), the rate of which is Parameter $c$. Since both parameters promote actin reassembly and accelerate the onset of retraction phase, the simulation predicts that the bleb size and the time taken for retraction decrease as $b$ and $c$ increase.

Parameter $h$ stands for the rate at which Rnd3 activity is inhibited by ROCK1. When phosphorylated by ROCK1, phosphorylated Rnd3 is removed from the plasma membrane. Since increasing $h$ downregulates the amount of Rnd3 at the plasma membrane ($x$), according to the equation (1a), the simulation predicts that blebs become smaller as the value of $h$ increases, which results in the activation of RhoA-ROCK1 pathway.

Parameter $e$ represents the rate of RhoA inhibition by RhoGAP. If the value of $e$ is increased, RhoA activity at the plasma membrane ($y$) and ROCK1 activity ($z$) decreases. Therefore, it is predicted by the simulation that an increase of value $e$ leads to the formation of larger blebs.

Parameter $f$ indicates the rate at which actin polymerizes as a consequence of activating the RhoA-ROCK1 pathway. Although this parameter is also difficult to modulate experimentally, the simulation predicts that the increasing $f$ causes the upregulation of the amount of actin at the plasma membrane ($w$) and downregulation of bleb size ($S$).

Parameter $g$ shows the rate at which RhoA is inhibited by actin filaments. When the value of $g$ is increased, RhoA activity at the plasma membrane ($y$) and ROCK1 activity ($z$) decreases. Therefore, it is predicted that increase of $g$, the value of which cannot be experimentally controlled, leads to the formation of larger blebs.

Parameter $k$ indicates the rate at which bleb expansion decelerates due to the elasticity of the cell membrane as the surface area of the bleb increases. Parameter $mp$ represents the internal pressure of the cell. If the value of $k$ is increased or if the value of $mp$ is decreased, the bleb size ($S$) decrease.

We chose values of parameters used for figures in the main text as follows. We estimated the model parameters based on experimental data and regenerated the time series of abundances of four different molecules in the model (Rnd3, RhoA, ROCK1, and actin). We noted that the measurements of the absolute abundance of each molecule includes errors more than the temporal pattern. In parameter fitting, we focused on the temporal pattern of the abundance of molecules rather than their absolute abundances. For this purpose, we normalized the abundance of each molecule by setting the largest and the smallest values to be 1 and 0, respectively. We then recorded the representative time when the normalized values were 0.1, 0.2, ... 0.9.

We also noted considerable variation of timing of the process between Bleb events. To cope with this, we divided the times obtained by the time when normalized abundance of actin was 0.5. We used these empirical values for parameter fitting of the model.

To reduce the number of parameters in the model, we introduced the following non-dimensional variables and parameters:

$$
\tilde{x} = \frac{x}{K_x}, \quad \tilde{y} = \frac{y}{b}, \quad \tilde{z} = \frac{z}{b}, \quad \tilde{w} = \frac{w}{w_{max}} \quad (S1).
$$
\[
\dot{a} = \frac{a}{K_a}, \quad \dot{h} = bh, \quad \dot{e} = eK_e, \quad \dot{f} = \frac{bf}{w_{max}}, \quad \dot{g} = w_{max}g.
\]

Then equations can be rewritten as the following four differential variables and parameters only:

\[
\begin{align*}
\frac{dx}{dt} &= (\dot{a} - \dot{h} \dot{x})(1 - \dot{x}) \\
\frac{dy}{dt} &= 1 - (\dot{e} \dot{x} + \dot{g} \dot{w})y \\
\frac{dz}{dt} &= c(\dot{y} - \dot{z} - (\dot{e} \dot{x} + \dot{g} \dot{w}) \dot{z}) \\
\frac{d\dot{w}}{dt} &= \dot{f} \dot{z}(1 - \dot{w})
\end{align*}
\]

We generated time series of variables for a range of parameter values, and calculated the temporal patterns of four molecules in the same manner applied to the empirical data explained above. We searched for the parameter values that generate time series close to the empirical data. We adopted the Nelder-Mead Method for exploring best-fitting parameter set. We repeated the procedure 100 times with initial parameter set chosen randomly within the range of 0.01 to 1 and we identified the parameter set which has the smallest squared error. The values of the best fitted parameters are as follows; \(\dot{a} = 2.496, c = 8.438, \dot{h} = 15.928, \dot{e} = 3.619, \dot{f} = 1.190,\) and \(\dot{g} = 0.394.\)

Based on these calculations, parameters in the mathematical simulation were optimized based on measurement values obtained from experimental data. In the case of freely-moving DLD1 cells (Standard case), each parameter was determined as follows; \(a=5, b=1, c=8.4, e=1.8, f=2.4, g=0.2, h=15.8, m=1, p=1, k=1, K_e=2,\) and \(W_{max}=2.\)

In order to simulate membrane blebs in early phase of apoptosis, we modified the model as follows:

\[
\begin{align*}
\frac{dx}{dt} &= (a - h(z + z_c)x)(1 - \frac{x}{K_x}), \quad (2a) \\
\frac{dw}{dt} &= (f z + f_c z_c)(1 - \frac{w}{w_{max}}). \quad (2b)
\end{align*}
\]

Here we assumed that there is another cascade for activating ROCK1 by caspase other than RhoA. \(z_c\) represents the amount of ROCK1 activated by caspase, and \(f_c\) represents the rate at which activated ROCK1 promotes actin polymerization. The total amount of activated ROCK1 is \(z + z_c\). We regarded \(z_c\) as a constant value because in the experiments the amount of ROCK1 activated by caspase does not change during apoptosis. The modified model showed that removal of Rnd3 from the plasma membrane and reassembly of actin cortex occurred earlier in the expansion phase as compared to the cell migration bleb model. Each parameter was determined as follows; \(a=5, b=1, c=8.4, e=1.8, f=2.4, g=0.2, h=15.8, m=1, p=1, k=1, K_e=2, W_{max}=2, z_c = 1,\) and \(f_c = 2.4.\)

In order to simulate bleb dynamics when ROCK1 is constitutively active at a constant level regardless of the Rnd3-RhoGAP pathway, we used following parameters. Parameters are: \(z=2, a=5, b=1, c=8.4, e=1.8, f=2.4, g=0.2, h=15.8, m=1, p=1, k=1, K_e=2,\) and \(W_{max}=2.\)

**Supplemental Movie 1**
Membrane blebbing in freely-moving DLD1 cells. DLD1 cells expressing Lifeact–RFP were imaged. Frames were taken every 5 s. (Scale bar, 10 μm.)

**Supplemental Movie 2**
Membrane blebbing in early-phase apoptotic DLD1 cells. DLD1 cells expressing Lifeact–RFP were treated with anti-Fas antibody (250 ng/mL) and cycloheximide (10 mg/mL) for 2 hours and imaged. The cells formed smaller blebs as compared to the freely-moving DLD1 cells. Frames were taken every 5 s. (Scale bar, 10 μm.)

**Supplemental Movie 3**
Membrane blebbing in late-phase apoptotic DLD1 cells. DLD1 cells expressing Lifeact–RFP were treated with anti-Fas antibody (250 ng/mL) and cycloheximide (10 mg/mL) for 4 hours and imaged. The retraction speed of membrane blebs was retarded in late-stage apoptotic cells. Frames were
taken every 5 s. (Scale bar, 10 μm.)
