<table>
<thead>
<tr>
<th>System</th>
<th>% Enucleated cells</th>
</tr>
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<tbody>
<tr>
<td>Mouse spleen enucleation assays (after incubation – before incubation)</td>
<td>12 – 18</td>
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<tr>
<td>Mouse fetal liver enucleation assays</td>
<td>15 - 30</td>
</tr>
<tr>
<td>Human primary erythroid culture from CD34(^+) cells on day 14</td>
<td>30 - 45</td>
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Table S1. The range of percentage of enucleation observed in control conditions in various enucleation assay systems used in this study.
Figure S1

Flow cytometry analysis of the effect of small molecule inhibitors of cytokinesis on enucleation

Flow cytometry profiles of representative enucleation assays performed on primary mouse spleen erythroblasts are shown. Syto 16 and SytoX Red are permeable and non-permeable nucleic acid dyes, respectively. The Syto 16+/SytoX<sub>low</sub> population includes live nucleated cells, whereas SytoX<sup>+</sup> cells represent dead cells. The boxed gate (Syto 16<sub>low</sub>/SytoX<sub>low</sub>) shows the percentage of enucleated cells. The topmost left panel shows the baseline profile of percoll-gradient purified mononuclear cells from spleen prior to incubation. All other panels depict profiles after 5 hours of incubation with small molecule inhibitors or DMSO.
Small molecule inhibitors of cytokinesis affect cell cycle progression of MEL cells. To verify that the doses of small molecule inhibitors used in enucleation assays of figure 2 and S2 block cytokinesis, we treated cultures of proliferating MEL cells with titrations of blebbistatin, hesperadin, nocodazole, cytochalasin D, SU6656, or DMSO alone. After 48 hours, cells were washed, fixed in 75% ethanol at -20°C overnight, stained with propidium iodide (PI) for half an hour on ice and analyzed by flow cytometry. Linear scale histograms of DNA content for various conditions are shown. Major peaks include 2N (diploid cells), 4N and 8N (polyploid cells). Note that cells treated with blebbistatin, hesperadin, or SU-6656 all show have increased percentages of cells with 4N and 8N DNA content when compared to vehicle control treated cells. Treatment with nocodazole, a microtubule stabilizing agent that blocks progression through mitosis, resulted in a strong G2/M phase arrest with DNA content of 4N. Cells treated with cytochalasin D, which also blocks cytokinesis, resulted in accumulation of a population of cells with 4N and 8N DNA content in addition to cells with sub G0 DNA content, indicative of dying cells.
**Figure S3**

**Hemoglobin synthesis does not correlate with enucleation efficiency.** To test the effect of vesicle trafficking inhibitors on hemoglobin synthesis during late erythropoiesis, primary mouse fetal liver erythroblasts at 38 hours of culture were treated with MiTMAB, monensin, dynasore or their respective solvent and incubated for 10 hours. Then, a portion of the cells were used to estimate % of enucleated cells (bottom) and the rest were utilized to prepare cell lysates and quantitate for their hemoglobin content (top) using a protocol described in methods section. Bar graphs represent mean ± standard deviation for 3 independent experiments. * P value <0.05, ** P value <0.01, and *** P value <0.001. Note that, even though there is no significant difference in enucleation between control and dynasore control, the absence of serum proteins had significant effect on hemoglobin synthesis. Conversely, MiTMAB blocked enucleation without affecting hemoglobin synthesis. Thus we conclude that hemoglobin synthesis does not influence enucleation.
**Figure S4**

(A) Enucleation assays of primary mouse splenic erythroblasts were performed in the presence of BTS (N-Benzyl-\(p\)-toluenesulphonamide) or BDM (2, 3-Butanedione 2-Monoxime). CytoD, cytochalasin D. Bars depict mean ± standard deviation for 3 independent experiments. * P value <0.05, ** P value <0.01.

(B) Representative flow cytometry plots of enucleation assays are shown. Details are similar to those in figure S2.

BDM, a non-specific inhibitor of myosin ATP-ase activity, blocks erythroblast enucleation.
MiTMAB and monensin inhibit enucleation in primary human erythroblasts. A) Human primary erythroid cells differentiated from CD34+ cells were treated with MiTMAB, monensin or solvent on day 13, incubated for 30 hours, washed and cyto spun onto poly (L) lysine coated slides and stained with benzidine and hematoxylin (B) and percentage of enucleated cells were determined. Bar graphs show percentages of enucleated cells relative to the total number of benzidine positive cells as mean ± standard deviation for three independent experiments. * P value <0.05, ** P value <0.01. B) Representative microscopic fields of stained cytopspins are shown.
Figure S6

A) The relative transcript levels of clathrin heavy chain (top) or caveolin-1 (bottom) expressed during various stages of differentiating primary human erythroblasts obtained as in figure 6 are shown. Day3 – BFU-E; Day7 – CFU-E through basophilic erythroblasts; Day10 – Polychromatic erythroblasts; Day14 – Orthochromatic erythroblasts. Bars depict mean ± standard deviation for 3 independent experiments.

B) An immunoblot of lysates from primary human erythroblasts show absence of caveolin-1 expression as opposed to continued expression of clathrin heavy chain during differentiation. Murine heart tissue lysate is a positive control for caveolin-1 expression and HSC-70 is used as loading control.
Colchicine does not block enucleation. Colchicine, a microtubule toxin, did not interfere with enucleation of primary mouse spleen erythroblasts. Mean ± standard deviation of 3 independent experiments is shown.