Quantitative comparison of a human cancer cell surface proteome between interphase and mitosis

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Abstract

The cell surface is the cellular compartment responsible for communication with the environment. The interior of mammalian cells undergoes dramatic reorganization when cells enter mitosis. These changes are triggered by activation of the CDK1 kinase and have been studied extensively. In contrast, very little is known about molecular events at the cell surface during cell division. We undertook a quantitative proteomic comparison of cell surface-exposed proteins in human cancer cells that were tightly synchronized in mitosis or interphase. Six hundred and twenty-eight surface and surface-associated proteins in HeLa cells were identified; of these, 27 were significantly enriched at the cell surface in mitosis and 37 in interphase. Using imaging techniques, we confirmed the mitosis-selective cell surface localization of protocadherin PCDH7, a member of a family with anti-adhesive roles in embryos. We show that PCDH7 is required for development of full mitotic rounding pressure at the onset of mitosis. Our analysis provided basic information on how cell cycle progression affects the cell surface. It also provides potential pharmacodynamic biomarkers for anti-mitotic cancer chemotherapy.

Keywords cell cycle; cell rounding; cell surface; PCDH7; SILAC

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Cell Cycle; Post-translational Modifications, Proteolysis & Proteomics

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Introduction

The cell surface is responsible for communication with the environment and is critical for tissue development, homeostasis, and disease. The cytoplasm of mammalian cells undergoes dramatic reorganization during cell division. Almost all aspects of the cell’s interior are changed by the entry into mitosis under the influence of the master kinase Cdk1, and these changes have been extensively studied in many laboratories (Nurse, 1990; Nigg, 2001; Moseley & Nurse, 2009), including large-scale phosphoproteomic analysis (Dephoure et al, 2008; Olsen et al, 2010; Kettenbach et al, 2011). In contrast, very little is known about molecular events at the cell surface during cell division.

Cell surface morphology undergoes dramatic reshaping at the onset of mitosis. This is best characterized for adherent cells in culture. As cells enter mitosis, they transiently round up, which facilitates mitotic spindle assembly (Rosenblatt, 2008; Stewart et al, 2011b; Lancaster et al, 2013). During cytokinesis, the cell surface ingresses at the cleavage furrow, and in some cell lines, blebs at the poles. After cytokinesis, daughter cells spread back out. Mitotic rounding is not an artifact of tissue culture; similar changes occur during cell division in mesenchymal cells (Nakajima et al, 2013). Epithelial cells, which must maintain barrier integrity, undergo less dramatic morphological changes during division, but in tissues, they do tend to de-adhere from the basal lamina during mitosis and round up, while their tight junctions remain intact (Jingui & Ishikawa, 1992). Mitotic cell rounding is not well understood; it is driven in part by changes in the actin cytoskeleton and in part by the down-regulation of adhesive systems (Rosenblatt, 2008; Matthews et al, 2012). The small GTPase Rap1 may serve as a master regulator of these changes in adhesion (Dao et al, 2009). Regulation of intracellular pressure also contributes (Stewart et al, 2011a). One cellular system that changes internally, and probably causes cell surface changes as a result, is vesicular trafficking. Boucrot and Kirchhausen (2007) reported an alteration in the balance between exo- and endocytosis during mitosis. They showed that transferrin receptor is enriched on the mitotic cell surface as a result, but did not examine other cell surface proteins. Older studies reported biochemical changes at the cell surface during mitosis (Stein & Berestecky, 1974; Johnsen et al, 1975), but the molecular

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details were inaccessible with 1970s technology, and no systematic analysis has been reported using modern methods.

To compare surface-exposed proteins between cell cycle stages, we employed SILAC (Stable Isotope Labeling by Amino acids in Cell Culture) (Ong et al., 2002). We faced two technical challenges, accessing an appropriate cell cycle state, and the selective identification of plasma membrane proteins. To access mitotic cells in bulk, we synchronized using a thymidine washout followed by mitotic arrest with the kinesin-5 inhibitor S-trityl-L-cysteine (Brier et al., 2004). A kinesin-5 inhibitor was chosen for mitotic arrest because, in contrast to anti-microtubule drugs, it leaves microtubule dynamics intact (Skoufias et al., 2006). After evaluation of alternative approaches for surface enrichment, including membrane fractionation, labeling glycосylation sites on the cell surfaces (Schiess et al., 2009; Wollscheid et al., 2009; Zeng et al., 2009; Ramya et al., 2013; Deeb et al., 2014), we chose selective chemical labeling of protein amines with cell-impermeant reagents. The selectivity of this approach has been previously documented (Collins & Paulson, 2004; Elia, 2008; Schiess et al., 2009). We identified many cell surface and surface-associated proteins (> 600) and found that approximately 10% (> 60) proteins exhibited differential exposure at the cell surface between mitosis and interphase. These data will inform future studies of membrane dynamics during mitosis. A high priority was identification of surface-exposed markers for mitotic cells, which might serve as biomarkers for mitotic arrest by anti-microtubule drugs during cancer treatment. We identified one candidate, PCDH7, confirmed its surface enrichment during normal mitotic progression, and found a possible functional role in regulating mitotic rounding.

Results

Identification of surface-exposed proteins

To enrich for surface-exposed proteins, we treated cells still attached to plates with a non-permeable amine reactive biotinylation reagent, Sulfo-NHS-SS-Biotin, which contains a disulfide to facilitate release from immobilized streptavidin (Elia, 2008). We assessed surface labeling using fluorescent streptavidin after fixation and permeabilization. Amine biotinylation decorated the cell surface efficiently and selectively (Fig 1A). To evaluate labeling efficiency, we pulled down biotinylated proteins, released from streptavidin with DTT, alkylated with iodoacetamide to prevent disulfide scrambling, and analyzed initially by Western blot using EGFR (surface) and actin (interior) as markers. EGFR was efficiently enriched in the streptavidin-bound cell surface fraction as expected. The very abundant protein actin was significantly reduced but still detectable (Fig 1B). Potential causes of actin labeling include permeation of the biotinylation reagent and cell lysis during growth or labeling. We optimized incubation and washing steps to reduce actin recovery in the cell surface fraction, but were unable to completely eliminate it.

Quantitative comparison of cell surface proteins at different cell cycle stages

Our workflow is outlined in Fig 1C. Cells labeled with light lysine and arginine were arrested in interphase using thymidine, and cells labeled with heavy lysine and arginine (15N and 13C labeled) were arrested in monopolar mitosis by washout from thymidine into the kinesin-5 inhibitor S-trityl-L-cysteine. After separate surface labeling and washing, we lysed and pooled the two cell populations, enriched for cell surface proteins using streptavidin beads. Following a protease digestion, resulting peptides were separated using an IEF (Isoelectric focusing) fractionator and analyzed by LC-MS.

The experiment was repeated five times. To eliminate any possible heavy isotope incorporation bias, in three biological replicates, heavy mitosis/light interphase were analyzed, and in two experiments, the labels were reversed. By GO annotation, ~45% of proteins identified in the streptavidin binding fraction were membrane proteins and 24% were plasma membrane proteins. Supplementary Table S1 lists the information of all the biological replicates including enrichment percentages. Cell surface proteins were identified based their GO annotations cell surface and/or extracellular (Fig 1D).

Figure 2A (left) shows the distribution of all identified cell surface proteins with mitosis/interphase ratios displayed on a log2 scale. These data include the biological replicates where the labels were switched, relabeling heavy as light for the pooled analysis. The curve is notably skewed to the interphase side, implying that more surface proteins were biotinylated in interphase than mitotic cells. This global labeling bias presumably arose because the surface area exposed for biotinylation is higher in interphase. To facilitate identification of proteins whose surface enrichment changes, we normalized SILAC ratios within a given experiment using SILAC ratios of several abundant cell surface proteins (Table 1). These were chosen as standards since their SILAC ratios were similar to each other, and to the average values for that cell cycle state. The normalization process is explained in details in the Materials and Methods section. Normalization produced a distribution of SILAC values between cell cycle states that was more symmetrical around zero (Fig 2A, right).

Protein group identifications from all experiments were merged such that all those protein groups coinciding in at least one IPI identified were summarized into a single new protein group to avoid possible bias from analyzing the same protein in differing protein groups. Combining all the biological replicates, we identified 628 GO-annotated cell surface protein groups in total. Twenty-seven were significantly enriched in mitosis over five biological replicate experiments, and 37 were significantly enriched in interphase. Representatives of each category are shown in Table 1. All cell surface proteins that were significantly enriched on the cell surface in mitosis or interphase are listed in the Supplementary Table S2.

All the cell surface proteins that were not significantly changed between the two cell cycle states (after normalization) are reported in Supplementary Table S3. All proteins identified in our proteomic analysis are reported in Supplementary Table S4. When degrees of enrichment on the cell surface were calculated based on the median ratio in comparison with the reference proteins, about 15 of statistically significant mitosis-selective proteins had the median ratio above 2.5-fold (in the range of 2.5–8), including PCDH7 and PCDH1, those had the highest median SILAC ratios (5.15 and 7.78). Around 20 statistically significant interphase selective proteins had a median SILAC ratio of < 0.45. To our knowledge, only one literature report exists for comparison to Table 1 (Boucrot & Kirchhausen, 2007). They reported that the transferrin receptor (TFRC) was enriched on the surface of interphase cells. Our data confirm that prior report
Figure 1. Experimental outline of the cell surface labeling and preparation for mass spectrometry.

A Immunostaining of HeLa cells probed for FITC-labeled streptavidin and DNA and the merge streptavidin (red) and DNA (blue). Cell surface was labeled using amine biotinylation. Scale bar, 10 μm.

B Western blot analysis of cell surface preparation. The surface-biotinylated cells were lysed using a detergent containing lysis buffer, incubated with and then eluted from streptavidin beads (i.e., cell sur. fraction). Cell surface fraction and the whole-cell lysate were blotted against a cell surface protein, EGFR, and a cytoplasmic protein, actin.

C Workflow of the cell surface proteome. Cell surface labeling and isolation of cell surface proteins in SILAC-labeled interphase versus mitotic HeLa cells are shown.

D Schematic of all identified proteins (blue circle) and cell surface proteins (pink circle). Cell surface/plasma membrane proteins were identified based their GO annotations; their distribution is shown in a pie chart (top).

Source data are available online for this figure.
Table 1. Examples of mitosis- and interphase-enriched and unchanged cell surface proteins.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein</th>
<th>P-value</th>
<th>Median ratio</th>
<th>No. of Pep.</th>
<th>% of Cov.</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA</td>
<td>MHC class I antigen</td>
<td>Std</td>
<td>1.03</td>
<td>22</td>
<td>73%</td>
<td>No Change</td>
</tr>
<tr>
<td>CD97</td>
<td>CD97 antigen</td>
<td>Std</td>
<td>1.10</td>
<td>15</td>
<td>27%</td>
<td>No Change</td>
</tr>
<tr>
<td>FAS</td>
<td>Tumor necrosis factor receptor superfamily</td>
<td>Std</td>
<td>1.03</td>
<td>8</td>
<td>30%</td>
<td>No Change</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
<td>Std</td>
<td>0.80</td>
<td>42</td>
<td>28%</td>
<td>No Change</td>
</tr>
<tr>
<td>ITGB1</td>
<td>Integrin-beta 1</td>
<td>0.73</td>
<td>1.19</td>
<td>39</td>
<td>43%</td>
<td>No Change</td>
</tr>
<tr>
<td>CD109</td>
<td>CD109 antigen</td>
<td>0.79</td>
<td>0.96</td>
<td>24</td>
<td>10%</td>
<td>No Change</td>
</tr>
<tr>
<td>TGM2</td>
<td>Tissue transglaminase</td>
<td>0.01</td>
<td>4.04</td>
<td>9</td>
<td>16%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>CLIC4</td>
<td>Chloride intracellular channel protein 4</td>
<td>0.01</td>
<td>3.08</td>
<td>6</td>
<td>37%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>CLIC1</td>
<td>Chloride intracellular channel protein 1</td>
<td>0.01</td>
<td>2.35</td>
<td>8</td>
<td>39%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>PCDH7</td>
<td>Protocadherin-7</td>
<td>0.01</td>
<td>5.15</td>
<td>5</td>
<td>6%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>PCDH1</td>
<td>Protocadherin-1</td>
<td>0.04</td>
<td>7.78</td>
<td>3</td>
<td>4%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>Neurogenic locus notch homolog protein 1</td>
<td>0.04</td>
<td>4.61</td>
<td>2</td>
<td>1%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>NOTCH2</td>
<td>Neurogenic locus notch homolog protein 2</td>
<td>0.04</td>
<td>1.67</td>
<td>13</td>
<td>8%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>ROBO1</td>
<td>Roundabout homolog 1</td>
<td>0.01</td>
<td>1.77</td>
<td>26</td>
<td>19%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>SORD</td>
<td>Sorbitol dehydrogenase</td>
<td>0.01</td>
<td>2.65</td>
<td>9</td>
<td>32%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>LGALS3</td>
<td>Galactose-specific Lectin 3</td>
<td>0.02</td>
<td>2.09</td>
<td>6</td>
<td>28%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>MUC16</td>
<td>Mucin-16</td>
<td>0.04</td>
<td>2.51</td>
<td>12</td>
<td>3%</td>
<td>Mitosis</td>
</tr>
<tr>
<td>SLC6A12</td>
<td>Solute carrier family 12 member 6</td>
<td>0.04</td>
<td>0.11</td>
<td>2</td>
<td>5%</td>
<td>Interphase</td>
</tr>
<tr>
<td>M6PR</td>
<td>Cation-dependent mannose-6-phosphate receptor</td>
<td>0.02</td>
<td>0.23</td>
<td>4</td>
<td>18%</td>
<td>Interphase</td>
</tr>
<tr>
<td>ABC5</td>
<td>Multidrug resistance-associated protein 5</td>
<td>0.04</td>
<td>0.27</td>
<td>7</td>
<td>5%</td>
<td>Interphase</td>
</tr>
<tr>
<td>TFRC</td>
<td>Transferrin receptor protein 1</td>
<td>0.01</td>
<td>0.27</td>
<td>52</td>
<td>61%</td>
<td>Interphase</td>
</tr>
<tr>
<td>GPR56</td>
<td>G protein-coupled receptor 56</td>
<td>0.01</td>
<td>0.29</td>
<td>2</td>
<td>4%</td>
<td>Interphase</td>
</tr>
<tr>
<td>IGF2R</td>
<td>Cation-independent mannose-6-phosphate receptor</td>
<td>0.01</td>
<td>0.35</td>
<td>36</td>
<td>17%</td>
<td>Interphase</td>
</tr>
<tr>
<td>ECE1</td>
<td>Endothelin-converting enzyme 1</td>
<td>0.01</td>
<td>0.35</td>
<td>7</td>
<td>15%</td>
<td>Interphase</td>
</tr>
<tr>
<td>LRP1</td>
<td>Prolow-density lipoprotein receptor-related protein</td>
<td>0.01</td>
<td>0.42</td>
<td>65</td>
<td>18%</td>
<td>Interphase</td>
</tr>
<tr>
<td>AXLUFO</td>
<td>Tyrosine protein kinase receptor</td>
<td>0.02</td>
<td>0.48</td>
<td>4</td>
<td>8%</td>
<td>Interphase</td>
</tr>
<tr>
<td>F11RJAM1</td>
<td>Junctional adhesion molecule 1</td>
<td>0.01</td>
<td>0.49</td>
<td>13</td>
<td>40%</td>
<td>Interphase</td>
</tr>
<tr>
<td>PTPRK</td>
<td>Protein tyrosine phosphatase receptor type K</td>
<td>0.04</td>
<td>0.51</td>
<td>4</td>
<td>5%</td>
<td>Interphase</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>Catenin delta-1</td>
<td>0.04</td>
<td>0.53</td>
<td>9</td>
<td>12%</td>
<td>Interphase</td>
</tr>
</tbody>
</table>

The listed proteins are analyzed in multiple biological repeats with inverted isotopes (heavy mitosis: light interphase or heavy interphase: light mitosis). For each protein, the following information is shown: adjusted P-values, median ratio (mitosis/interphase), number of quantified razor peptides in a replicate, percentage of maximum coverage in a replicate, direction. The P-values of reference proteins were not calculated and indicated as std (standard). The peptides were identified with an FDR of 5%, and proteins were identified with an FDR of 1% using MAXQUANT.

Finding with high confidence (median SILAC ratio 0.27), indeed, TFRC is one of the most interphase-enriched surface proteins. Abundance ratios measured by SILAC tend to underestimate true changes due to limited dynamic ranges (Asara et al. 2008). To retest SILAC results, we used Western blot analysis on two proteins from Table 1 where antibodies were available—EGFR, which does not change by mitosis-arrested cells in tumors treated with anti-mitotic drugs. We focused on protocadherins, a family that was strikingly enriched on the surface (see PCDH7 and PCDH1 in Table 1). Protocadherins are involved in cell–cell interactions and have been intensively studied in the Drosophila nervous system (Zipursky & Sanes, 2010; Lefebvre et al., 2012). Vertebrate protocadherins have been relatively little studied. They score as homophilic cell adhesion proteins in some assays (Schreiner & Weiner, 2010) and as anti-adhesion proteins that antagonize canonical cadherin-mediated adhesion in frog embryos (Chen & Gumbiner, 2006a,b). Thus, their role in cell adhesion may be context dependent.

We first retested cell surface exposure of PCDH7 by biotinylation, isolation, and Western blotting (Fig 2B). Consistent with the mass spectrometry results, PCDH7 protein was enriched in the mitotic cell surface fraction and barely detectable in the interphase cell surface.

Protocadherin-7 localizes to the cell surface during mitosis

The highest priority for our study was to identify proteins that might serve as surface markers for mitotic cells. These could be used to isolate mitotic cells from tissue for analysis and potentially to image mitosis-arrested cells in tumors treated with anti-mitotic drugs.
Figure 2. SILAC-based quantitative analysis of cell surface proteins in interphase and mitosis.

A  Histogram of the isotope ratio distribution before (left) and after (right) normalization and centering. The x-axis shows the mitosis/interphase ratio on a log2 scale. Note that more cell surface proteins were recovered from the interphase cells prior to normalization.

B Western blot confirmation of the mitosis-selective cell surface localization of PCDH7. Western blotting analysis of cell surface protein preparation from mitosis and interphase cells. The whole-cell lysate and elution from streptavidin beads were blotted against TFRC, EGFR, actin, and phospho-Histone H3. TFRC protein was enriched in interphase cell surface preparation in comparison with mitosis preparation, while PCDH7 protein was enriched in mitotic cell surface preparation.

C Western blot confirmation of the mitosis-selective cell surface localization of PCDH1. Whole-cell lysate samples were subjected to SDS–PAGE and blotted with the anti-PCDH1, anti-β-actin, and anti-phospho-Histone H3 antibodies, as indicated on the left. Cell surface fraction samples were blotted with the anti-PCDH1 and anti-EGFR antibodies.

Source data are available online for this figure.
fraction. According to the quantitative comparison of PCDH7 protein bands using densitometry, mitotic cell surface enrichment of PCDH7 is calculated at around threefold (Supplementary Table S5). Total PCDH7 protein expression was not different in mitosis versus interphase lysates (Fig 2B), suggesting most PCDH7 is sequestered in an intracellular compartment during interphase. Next, we tested the cell surface exposure of PCDH1. Similar to PCDH7, total PCDH1 protein expression was not different in mitosis versus interphase lysates (Fig 2C, top); it was enriched in the mitotic cell surface fraction and was barely detectable in the interphase cell surface fraction (Fig 2C, bottom). Quantification of PCDH1 bands in the mitotic versus interphase cell surface fraction suggested that mitotic selective enrichment of PCDH1 is around threefold (Supplementary Table S5). Anti-PCDH1 antibody did not work in the alternative applications tested below, so we focused on PCDH7.

We next examined the localization of PCDH7 in unsynchronized cells using immunofluorescence where cells were not permeabilized prior to staining, so only surface-exposed proteins were accessible to antibodies. PCDH7 was detected using a commercial antibody directed to the extracellular part of PCDH7. In HeLa cells which had progressed naturally into mitosis (evident from condensed chromosomes and the formation of mitotic spindle, arrows in Fig 3A), PCDH7 localized to the entire cell surface (arrows), which was obviously brighter than the surface of interphase cells.

![Image](image_url)
Nurhan Ozlü et al  Quantitative analysis of the cell surface

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To further investigate the sub-cellular localization of PCDH7, we generated a GFP fusion protein and expressed it in U2OS cells by transient expression. We observed that in mitotic cells, PCDH7 decorated the whole cell surface (Fig 3C, top row), whereas in interphase cells, PCDH7 only strongly localized to cell–cell contact regions (Fig 3C, bottom row). We were concerned that the strong cell–cell contact staining in interphase might be an artifact of over-expression in transient transfection experiments. To localize PCDH7 at more physiological expression levels, we utilized a BAC transgenomics pipeline in which HeLa kyoto cell lines stably express GFP-tagged proteins are under control of their own promoter (Poser et al., 2008). We expressed GFP-tagged PCDH7 controlled by its endogenous promoter in a bacterial artificial chromosome. We observed a similar cell cycle–dependent localization of PCDH7 as in the indirect immunofluorescence and transient transfection experiments, where the protein was localized all over the surface of mitotic cells, but restricted to cell–cell interaction in interphase (Fig 3D).

To gain more insight into cell cycle dynamics of PCDH7, we performed live imaging studies in transiently transfected U2OS cells. In interphase cells, the PCDH7 signal showed diffuse localization in the cytoplasm. As the cell progressed into prometaphase and become rounded and less adhesive, PCDH7 localized to the retraction fibers around the cortex and during metaphase PCDH7 appeared to localize to the cell cortex. In the post-mitotic daughter cells, PCDH7 mostly localized to the cell–cell contact margin and not to the rest of the cortex (see Supplementary Videos S1 and S2).

To gain mechanistic insight into cell cycle regulation of surface exposure of PCDH7, we tested the role of the cytoplasmic domain. PCDH7 has a large extracellular domain (~900 aa) toward the N-terminus with seven extracellular cadherin (EC) domains and a relatively short cytoplasmic domain (~170 aa). We generated a construct where the majority of the cytoplasmic domain (100 amino acid from the C-terminus) was truncated (ACyt-PCDH7). As expected the wild-type (full length) PCDH7::GFP fusion protein localized to the cell surface only at mitosis, but not interphase. In contrast, the cytoplasmic deleted PCDH7::GFP was easily detectable on the cell surface during interphase (Fig 4A). To quantify the distribution of the wild-type and truncated constructs, we measured the GFP fluorescence intensity on the cell surface and in the cytoplasm during mitosis and interphase from single focal planes and calculated the ratio of integrated densities. The surface/interior ratios showed that wild-type PCDH7 was highly surface-enriched during mitosis (~fourfold) and significantly interior enriched during interphase. The cytoplasmic domain truncated mutant, in contrast, showed a more even distribution (Fig 4B).

To evaluate where PCDH7 localizes in interphase when it is not on the cell surface, we tested for co-localization with other subcellular markers. First, we tested the wild-type GFP::PCDH7 transfected interphase cells and co-stained with the endoplasmic reticulum (ER) markers KDEL and calnexin. In interphase cells, GFP::PCDH7 localized close to ER marked by KDEL and partially co-localized (Fig 4C, top). ER staining in interphase might be an artifact of GFP::PCDH7 over-expression in transient transfection experiments, resulting in mis-folding. To examine the endogenous PCDH7 localization, we localized endogenous protein with PCDH7 antibody, in this case permeabilizing cells to allow staining of protein in interior compartments. During interphase, PCDH7 again partially co-localized with the endoplasmic reticulum (ER) marker KDEL in HeLa cells (Fig 4C, middle), whereas during mitosis, KDEL and PCDH7 had completely different staining patterns, PCDH7 decorated the cell cortex, and KDEL was in the cytoplasm (Fig 4C, bottom). Similar staining patterns were observed in PCDH7 and calnexin co-stained interphase and mitotic cells (Supplementary Fig S1). We next probed PCDH7 localization during interphase by sub-cellular fractionation. Endoplasmic reticulum, mitochondria, and cytoplasm from HeLa cells were partially separated by density gradient fractionation (Bozidis et al., 2007). Total cell lysate, cytoplasm, mitochondria, and ER fractions were analyzed by Western blotting using KDEL (ER), actin (cytoplasm), cox-2 (mitochondria), and PCDH7. PCDH7 was enriched in the ER fraction compared to the cytoplasmic, mitochondria, and the total cell lysate fractions (Fig 4D). These data support a model where PCDH7 is mostly retained in the ER during interphase and allowed to reach the cell surface during mitosis.

PCDH7 is required for development of full mitotic rounding pressure

PCDH7 is the first known protein that exhibits increased surface exposure during mitosis. To test for a functional role in the morphology changes that accompany cell division, we pursued knockdown experiments. PCDH7 and PCDH1 share 46% homology, and according to our SILAC and Western blot data, both are enriched at the cell surface during mitosis, suggesting possible redundant function. For this reason, both PCDH1 and PCDH7 were knocked down using esiRNA in HeLa cells. On Western blots, anti-PCDH7 antibody recognized a single band of 120 kDa that disappeared in PCDH7 esiRNA and PCDH1 and PCDH7 double-esiRNA-treated cells (Fig 5A, right). Similarly, a single band that was recognized by anti-PCDH1 antibody was significantly reduced following PCDH1 esiRNA (Fig 5A, left). Initial imaging by fixed immunofluorescence and time-lapse phase contrast of knocked down cells showed no obvious effects on timing or progression of mitosis (not shown). To perform a more quantitative analysis, we used a recently developed AFM method to measure the force of mitotic rounding (Stewart et al., 2011a, 2012). In this assay, rounding pressure (force per unit area) of mitotic cells singly and doubly depleted for PCDH7 and PCDH1 was measured using a tipless cantilever. RNAi of luciferase and MYH9 were used as negative and positive controls, respectively. Myosin II knockdown was previously shown to strongly reduce mitotic rounding force (Toyoda et al., 2011). Luciferase depletion did not effect, while MYH9 depletion more strongly reduced rounding pressure, as expected. Depletion of PCDH1 and PCDH7 significantly reduced cell rounding pressure (Fig 5B, right). Next, we tested whether mouse PCDH-LAP-tagged BAC transgenes could rescue the reduced rounding pressure (Fig 5B, left). This experiment was only performed for PCDH1 due to lack of mouse PCDH7 BAC clone. Using Western blotting, we confirmed that mouse PCDH1::GFP is not affected by the esiRNA treatment, whereas internal PCDH1 protein level significantly reduced (Fig 5A, right). Compared to the parental HeLa cells, this...
Figure 4. Cytoplasmic domain of PCDH7 is required for its cell cycle-dependent cell surface localization.

A Confocal images representing the localization of PCDH7. U2OS cells expressing full-length (WT) PCDH7 at mitosis (top) and interphase (middle). Cytoplasmic deleted (CytΔ) PCDH7-expressing cells at mitosis (middle) and interphase (bottom). PCDH7, green; DNA, blue. Scale bar, 20 μm.

B Quantification of PCDH7::GFP (WT and CytΔ) protein localization on the cell surface in mitosis [WT (n = 11), CytΔ (n = 6), red] and interphase [WT (n = 25), CytΔ (n = 20), blue]. Interphase CytΔ PCDH7 localizes to the cell surface more than WT (full length) PCDH7 (insets). Bars show mean ± SEM.

C In interphase co-localization between PCDH7 and ER marker KDEL. HeLa cells were transiently transfected with PCDH7::GFP (green) (ectopic), fixed and stained for ER marker KDEL (red) (interphase, top panel). Interphase (middle panel) and mitotic (bottom panel) HeLa cells were fixed and stained against (endogenous) PCDH7 and KDEL. Scale bar, 5 μm.

D PCDH7 and PCDH1 enriched on endoplasmic reticulum fraction. Whole-cell lysate, cytoplasmic, endoplasmic, and mitochondrial fractions were blotted against an endoplasmic reticulum protein, KDEL, a cytoplasmic protein, actin, a mitochondrial protein Cox-2 and PCDH7, PCDH1.

Source data are available online for this figure.
cell line had a general lower pressure; nevertheless, reduced rounding pressure in the depletion of PCDH1 was recovered in a mouse PCDH7-LAP-tagged BAC transgenes stably expressing HeLa cells (Fig 5B, left). We conclude that PCDH expression is required for development of full mitotic rounding pressure, consistent with PCDH1 and PCDH7 exposure at the cell surfaces having an anti-adhesive effect.

To test whether PCDH1 and PCDH7 depletion has any affect on the rate of cell rounding during mitotic entry, we measured the rate of rounding in control versus knockdown cells. After esiRNA-mediated depletion of PCDH1 and PCDH7, the length of the longer axis of dividing cells was measured as a function of time in movies, and its slope was calculated as a readout speed of mitotic rounding. We observed that there is a slight decrease in the speed of mitotic rounding in PCDH1-depleted cells (Supplementary Fig S2A).

To test whether the extracellular domain of a PCDH protein could affect cell rounding, we expressed a truncated construct in

![Graphs](image)

Figure 5. PCDH7 is required on the cell cortex for building rounding pressure.

A PCDH7 antibody detects a single band of ~110 kDa in luciferase RNAi (Luc), PCDH1 RNAi HeLa cells lysates, but this band is not detectable in PCDH7 and PCDH1 and PCDH7 RNAi cells. α-tubulin was used as a loading control (left). HeLa Kyoto cells stably expressing PCDH7-LAP-tagged BAC transgenes treated with luciferase RNAi (control) and 150 nM (1×) and 300 nM (2×) PCDH1 esiRNA and blotted against anti-GFP, anti-PCDH1, and anti-actin antibodies. PCDH1 bands were reduced in PCDH1 RNAi cells, whereas mouse GFP::PCDH1 was not affected by the esiRNA treatment (right).

B Rounding pressure plots of MYH9 (positive control, n = 7), Luc (negative control, n = 12), PCDH7 (n = 11), PCDH1 (n = 12), and PCDH1 and PCDH7 (n = 11) RNAi cells. Bars show mean ± SEM (left). Rounding pressure plots HeLa Kyoto cells stably expressing PCDH7-LAP-tagged BAC transgenes treated with MYH9 (positive control, n = 7), Luc (negative control, n = 14), PCDH1 (n = 13) esiRNA. *P < 0.05; **P < 0.01; ***P < 0.001; ns, P > 0.05 (non-significant).

Source data are available online for this figure.
which the extracellular domain is secreted (Supplementary Fig S2C), which we refer to as an ectodomain. Expression of this domain induced cell rounding in interphase and loss of focal adhesions (Supplementary Fig S2B). These data are consistent with an anti-adhesive function of the ectodomain. However, on longer-term culture, the ectodomain-expressing cells died, so we cannot rule out that the cell rounding was due to a toxic effect, for example, induced by ER stress.

Protocadherin-7 as a mitotic marker to sort mitotic cells

One of the goals of our study was to identify a surface marker that could be used to isolate mitotic cells. We tested whether PCDH7 could serve this role using flow cytometry. HeLa cells were enriched for mitotic cells by a short treatment with S-trityl-L-cysteine, incubated with the PCDH7 antibody that recognized the extracellular part of PCDH7, then fixed, permeabilized, and stained with the standard mitotic marker phospho-Histone H3, followed by secondary antibodies and DNA staining as illustrated in Fig 6A. Flow cytometry analysis revealed that phospho-Histone H3-positive cells were 87.3% in G2/M by DNA analysis, PCDH7-positive cells were 82.1% in G2/M, and all cells were 42% in G2/M (Fig 6B). Forty-two percent of the phospho-Histone H3-positive cells were PCDH7-positive (Fig 6B, middle), and 79% of the PCDH7+ cells were phospho-Histone H3-positive. Thus, PCDH7 staining before permeabilization marked mitotic cells almost as accurately as the classic phospho-Histone H3 stain.

Discussion

This study used quantitative proteomics to identify cell surface-exposed proteins that change between interphase and mitosis. Six hundred and twenty-eight cell surface and cell surface-associated proteins were identified, and their surface exposure was quantified at two cell cycle stages corresponding to interphase and mitotic rounding—one of the largest, quantitative cell surface datasets to our knowledge. Sixty-four proteins were reproducibly enriched on the mitotic or interphase cell surface. In general, cell cycle-regulated proteins were involved in cell adhesion, receptor, and endosome/lysosome biology, but no strong biological mechanisms emerged from the list of regulated proteins. Deeper bioinformatics analysis, for example, of cytoplasmic motifs, might reveal clues to regulatory mechanisms.

How might a protein be enriched on the surface of interphase or mitotic cells? The only proposed mechanism, to our knowledge, is that exocytosis is down-regulated in mitosis, while clathrin-mediated endocytosis is not (Boucrot & Kirchhausen, 2007). This was proposed to account for enrichment of the transferrin receptor on the surface of interphase cells. Inspection of our list of interphase-enriched proteins shows that several proteins are indeed endocytosed by clathrin-dependent mechanisms such as TFRC, M6PR, IGF2R, LRPI, and ECE1 (Anderson et al, 1977; Nair et al, 2003; Padilla et al, 2007; Grant & Donaldson, 2009; Martins et al, 2011; Mulkearns & Cooper, 2012). However, other such proteins were not detectable interphase-enriched in our dataset, such as EGFR (Carpenter, 2000), CD63 (Duffield et al, 2003), and ATP7A (Lane et al, 2004). We suspect that the mechanism proposed by Boucrot and Kirchhausen (2007) is relevant, but that is does not affect all endocytosed proteins equally. Many, perhaps all, cell surface proteins can be endocytosed, but the precise mechanism of endocytosis and the regulation of endocytosis vary enormously. Deeper bioinformatic analysis of our data might reveal clues as to sub-classes of endocytosed proteins that are selectively depleted in mitosis.

According to our analysis, one of the most prominent classes of proteins whose cell surface exposure changes during progression through mitosis is adhesion molecules.

Several adhesion-related proteins were down-regulated on the mitotic cell surface (GPR56, F11R/JAM1, CTNND1, PTPRK, AXL, TPBG, MSLN, and ITGA6), while two were up-regulated (PCDH7 and PCDH1). Mitotic regulation of adhesion receptors is expected because cells alter their adherence properties dramatically as they progress into mitosis. At the onset of mitosis, cells transiently lose their adherence and round up by poorly understood mechanisms. Not all adhesion proteins are affected; the number of fibronectin receptors on CHO cells was not altered by mitotic arrest in one study (Pomies & Block, 1992). Our analysis confirmed lack of major changes in cell surface exposure of integrins. Instead, the activity of integrins may be regulated by the small GTPase Rap1 (Dao et al, 2009). Junction adhesion molecule 1 (JAM1) was down-regulated on the mitotic cell surface, which might be relevant to Rap1 regulation since knockdown of JAM1 in a previous study resulted in a decrease in β1 integrin protein levels and decreased Rap1 activity (Mandell et al, 2005). GPR56 is another interphase selective adhesion molecule, which is also implicated in integrin signaling, blocking its function induced cell rounding and reduced cell adhesion (Shashidhar et al, 2005; Ke et al, 2007; Jeong et al, 2013). Regulation of adhesion during mitosis is still poorly understood. Our findings of differential surface exposure of candidate adhesion regulators will help solve this mechanistic problem, which is probably most important in the tissue context.

Three proteins involved in cell-to-cell signaling in tissues, NOTCH1, NOTCH2, and ROBO1, were all up-regulated on the surface of mitotic cells (Table 1). This suggests the interesting possibility that neighboring cells might detect the presence of a dividing cell using the NOTCH or ROBO pathways. Neighboring cells in epithelia are known to detect and respond to cells undergoing apoptosis (Rosenblatt et al, 2001), whether they detect and respond to dividing neighbors is not known.

Chloride intercellular channel (CLIC) proteins were also notably enriched on the mitotic cell surface. They are members of the glutathione S-transferase superfamily; they were shown to form anion channels in an artificial membrane system; however, their function in cells appears to be controversial (Cromer et al, 2002; Ashley, 2003). CLIC proteins present in the cell in two forms, either as a water-soluble cytoplasmic form or as membrane-bound form (Cromer et al, 2002). Interestingly, RhoA activation leads to translocation of CLIC4 protein from cytoplasm to the plasma membrane (Ponsioen et al, 2009). RhoA is activated during mitosis and may contribute to cell rounding (Maddox & Burrage, 2003), which might explain mitotic enrichment of CLIC1 and 4.

The most strikingly mitosis-enriched surface protein was PCDH7, a member of protocadherin family. We confirmed this result using different methods including Western blotting, immunostaining of endogenous protein, and live imaging of transiently expressed
PCDH7::GFP fusion gene and by imaging PCDH7 under its own promoter using bacterial artificial chromosome (BAC) transgenomics. Another protocadherin protein PCDH1 also had a high median ratio toward, which was confirmed by Western blotting analysis (Fig 2C). Protocadherins are widely expressed cell surface proteins that were initially thought to be homophilic cell–cell adhesion molecules on the basis of domain similarity to canonical cadherins. However, the Xenopus protocadherin PAPC was shown to negatively regulate cell–cell adhesion mediated by cadherins (Chen & Gumbiner, 2006b; Chen et al, 2009). Thus, surface-exposed PCDH1 and PCDH7 could function to down-regulate adhesion during mitosis. Consistent with this possibility, knockdown of PCDH1 and PCDH7 caused a decrease in mitotic rounding pressure. Further work is required to determine whether this decrease is due to more surface adhesion, reduced actomyosin contractile activity, or reduced turgor pressure, all of which are implicated in mitotic rounding (Stewart et al., 2011a,b). In interphase cells, PCDH7 appeared to localize to endomembranes that partially co-localized with the ER marker calnexin and co-fractionated with ER by density gradient (Fig 4C and D). We hypothesize that PCDH1 and PCDH7

Figure 6. Protocadherin-7 as a mitotic marker.
A Workflow of flow cytometry analysis.
B Flow cytometry analysis of HeLa cells treated with anti-PCDH7 antibody, fixed and stained for phospho-Histone H3 and DNA. Forty-two percent of unsorted HeLa cells are in G2/M phase. When cells are sorted based on their phospho-Histone H3 (middle) expression, phospho-Histone H3+ (high expression) cells are preferentially mitotic (87.3% in G2/M phase) and 42% of those are PCDH7+. Similarly, PCDH7+ (high expression) cells are preferentially mitotic (82.1% in G2/M phase) and 79% of those are phospho-Histone H3+ (bottom).
are held in the ER during interphase and released to traffic to the surface as cells enter mitosis. A relatively short cytoplasmic domain (170 amino acids) of PCDH7 was required for retention in this compartment during interphase—constructs lacking this domain went to the cell surface in interphase (Fig 4A and B). Manipulation of this domain should allow us to test this ER retention model. It is also possible that PCDH7 is retained in some specialized compartment whose localization and density approximates that of the ER.

One motivation for this study was to find cell surface biomarkers for mitotic arrest that might be to measure tissue responses to anti-mitotic drugs, and to isolate mitotic cells from tissue for analysis. PCDH proteins may have value in this respect. Using flow cytometry, we found that live cells incubated with anti-PCDH7 antibody and subsequently fixed can be enriched for the mitotic population based on their PCDH7 expression, and this enrichment is as effective as that obtained with the standard marker phospho-H3. Improved antibodies to the surface-exposed portion of PCDH7 might have considerable value as imaging agents for detecting mitotic cells in situ during optimization of anti-mitotic cancer chemotherapy.

Materials and Methods

Cell growth and cell arrest

For SILAC experiments, HeLa S3 cells were grown as described in (Ozlu et al, 2010). Cells were arrested at mitosis and interphase as described in Ozlu et al (2010). Briefly, HeLa S3 cells grown on plates were treated with a double thymidine block protocol for interphase synchronization. To arrest at mitosis, cells were treated with 10 μM S-trityl-l-cysteine (STC) for 12 h after a single thymidine block and release. More than 95% of cells were arrested in mitosis by this method. For the interphase population, we used double thymidine arrested cells with > 95% arrested cells. Mitotic and interphase populations were differentially labeled with 15N and 13C lysine and arginine (Ozlu et al, 2010), separately washed with ice-cold PBS and then pooled for subsequent labeling and analysis. The wash was gentle in order to leave proteins that do not have membrane-spanning regions attached to the cells, for example, secreted proteins and domains released by proteolysis that adhere to the cell surface. Human PCDH7-LAP-expressing HeLa cells were generated as described in Poser et al (2008). U2OS cells expressing mCherry-tubulin cell line were a gift from Dr. Alex Bird, MPI-CBG, Dresden.

Cloning and transfection

cDNAs for PCDH7 was a gift from Prof. Sumio Sugano, Laboratory of Functional Genomics, Department of Medical Genome Sciences, The University of Tokyo. PCDH7 was cloned into eGFP-N1 vector and sequenced. The PCDH7::GFP wt and truncated constructs were transfected using protocols described by the manufacturer into various cell lines using Lipofectamine 2000 (Invitrogen). For generating cytA PCDH7::GFP truncated construct for eGFP-N1 vector, following primers were used: 5’-GGCGCAAGCTTATGTGAGGATGCGGACC-3’ and 5’-GGCGCGATCCCTCCTCAGTGTTGACTGG-3’.

Antibodies and Western blotting

For immunostaining or Western blotting, the following primary antibodies and reagents were used: streptavidin, Alexa Fluor 488 (s-32354; Invitrogen), TFRC-FITC conjugated (ab34670; Abcam), EGFR (2232; Cell Signaling), PCDH7 (E-14) (sc-104576; Santa Cruz and ab170692; Abcam), PCDH1 (ab55504; Abcam), anti-phospho-Histone H3 (06-570; Upstate), actin (MA1-91399; Pierce). For immunostaining, the following secondary antibodies were used: donkey anti-mouse, anti-rabbit, and anti-goat conjugated to Alexa Fluor 488, 594, or 647 (Invitrogen).

For Western blot analyses, samples were separated by molecular weight using SDS–PAGE gels and transferred to a nitrocellulose membrane. The membrane was blocked with 0.1% Tween-20 with 5% w/v nonfat dry milk and probed with 1 μg/ml of the described primary antibody, and the signal was detected using ECL (Amersham) detection of the HRP-conjugated anti-rabbit secondary antibody (Bio-Rad Laboratories). Blots were visualized using X-ray films. Images of X-ray film were captured using Kodak Gel Logic 2200 Imaging System and quantified by densitometry using Carestream Molecular Imaging Software (Version 5.0).

Immunostaining, microscopy and quantification

U2OS or HeLa cells were fixed in 2% formaldehyde for 10 min. For permeabilization, cell was treated with PBS-0.5% Triton X-100 for 10 min. Cells were blocked with 1.5% BSA in PBS for 30 min. Cells were incubated with primary antibodies in 1.5% BSA in PBS overnight at 4°C or for 1 h at room temperature, washed, and then incubated with secondary antibodies and DAPI. Finally, coverslips were mounted in ProLong Gold (Invitrogen) and sealed. Imaging was performed using either by a spinning disk confocal microscope (TE-2000; Nikon) controlled by the Metamorph imaging software (MDS Analytical Technologies) or with a wide-field Delta Vision microscope (Applied Precision), and the images were deconvolved with SoftWorx (Applied Precision). PCDH7 signal was quantified using the Fiji software. From single focal planes, integrated densities of surface to interior ratio were divided to the measured area.

Biotinylation of cell surface proteins and isolation of biotinylated proteins

Amine reactive biotinylation

HeLa S3 cells grown on plates (~2 × 10⁶ cells) were rinsed twice with PBS supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ and once with borate-buffer saline (BBS) (12 mM sodium borate and 125 mM NaCl, pH 8.3) and then incubated with 5 mM EZ-Link Sulfo-NHS-SS-Biotin (Pierce) and incubated for 30 min at 4°C with gentle shaking. Residual biotin was quenched with 100 mM glycine in BBS buffer. Cells were washed with PBS and snap-frozen after scraping from the cell plates.

Affinity purification and fractionation of biotinylated proteins

Biotinylated and frozen cells were cells were lysed in a buffer (10 mM Tris–Cl pH 7.5, 150 mM NaCl, 0.5% SDS, 1 mM EGTA, 1 mM EDTA, 2% Nonidet P-40, 10 mM iodoacetamide) containing...
protease inhibitor (Roche Applied Science) and phosphatase inhibitors (1 μM okadaic acid, 1 μM microcystin, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM β-glycerol phosphate, 1 mM sodium pyrophosphate). Lysates were pre-cleared at 2,800 g for 5 min. For SILAC experiments, cells lysates were mixed in an equal concentration, determined using BCA protein assay (Pierce). Cell lysates were incubated with streptavidin magnetic beads (Promega) or with Streptavidin Plus Ultrasil Resin (Pierce) overnight at 4°C. Beads were washed extensively with lysis buffer, twice with high salt lysis buffer (NaCl concentration was adjusted to 500 mM), and once with a Tris buffer (10 mM Tris–Cl pH 7.5, 1 mM EGTA, 1 mM EDTA). For the Western blotting analysis, biotinylated proteins were eluted by boiling in 1× SDS sample buffer including 100 mM DTT. For the proteomic analysis, biotinylated proteins were eluted by boiling in a buffer (2% SDS, 100 mM Tris–HCl pH 7.6, 0.1 M DTT). Eluted proteins were digested using Filter Aided Sample Preparation (FAPS) protocol as described in Wisniewski et al. (2009). An extra step was added, treating the resulting peptides with PNGase F (New England Biolabs) for 6 h prior to eluting from the filter units. Subsequently, the peptides were separated into 24 fractions using an Agilent 3100 OFFGEL fractionator and its High Res Kit (pH 3–10) (Agilent).

Sub-cellular fractionation and isolation of endoplasmic reticulum
Fractionation method was adapted from Bozidis et al. (2007). Briefly, four 150-mm culture plates of HeLa cells with 60–70% growth were used (12 million cells per plate). Cells were collected after trypsin treatment and centrifugation at 1,400 g for 5 min. Cell pellets were resuspended in STE buffer (270 mM sorbitol, 10 mM Tris Base, 0.1 mm EDTA, pH 7.4) with protease inhibitor cocktail (Pierce, Product# 88666F). Cell suspension was homogenized using sonicator, and cell lysate was centrifuged at 1,400 g for 10 min. Small fraction of the supernatant was collected as ‘total protein fraction’. Remaining supernatant was centrifuged at 15,000 g at 4°C for 10 min. Supernatant crude ER fraction is separated from mitochondrial pellet. Crude ER was loaded for discontinuous sucrose density gradient centrifugation (2, 1.5, and 1.3 M) at 152,000 g at 4°C for 45 min. Top ‘cytosolic fraction’ was collected whereas ER fraction visible as a white band at the intersection of 1.5 and 1.3 M sucrose fractions was collected using a 23-G needle. Collected band was diluted with additional ice-cold STE buffer and centrifuged at 126,000 g at 4°C for 45 min. The translucent pellet was resuspended in PBS, pH 7.4 and labeled as ‘ER fraction’. Mitochondrial pellet was washed with STE buffer to get rid of ER contaminant on the surface of mitochondrial pellet. Remaining pellet was resuspended in 0.8 ml of ice-cold STE buffer and loaded onto sucrose gradient (1.7 and 1.0 M) and centrifuged at 40,000 g for 22 min at 4°C. Mitochondrial band was collected at the interface of the sucrose gradient. Collected fraction was diluted with STE buffer and centrifuged at 15,000 g, 4°C for 10 min. Mitochondrial pellet thus formed was resuspended in PBS. All samples were frozen at –20°C.

FACS analysis
HeLa and MCF-7 cells were treated with 5 μM S-trityl-(R)-cysteine to enrich mitotic cells and scraped from the plates in ice-cold PBS. Cells were blocked for 15 min with 2% BSA at 4°C followed by anti-PCDH7 incubation for 45 min (E-14) (sc-104576; Santa Cruz). Subsequently, cells were fixed with 1% PFA in PBS for 10 min and stained for anti-phospho-Histone H3 (06-570; Upstate), secondary antibodies, and DAPI. Data were acquired on a FACS Aria II (BD Biosciences) and analyzed with the FlowJo software.

AFM measurement of mitotic cell pressure
Pressure of mitotic HeLa cells were measured essentially as previously described (Stewart et al., 2011a, 2012). In summary, cells grown on a glass-base dish (FD35; WPI Instruments) were mounted on a setup that consists of an atomic force microscope (NanoWizard II; JPK Instruments) and a light microscope (Axio Observer Z1; Zeiss). A tipless cantilever (NSC37-B, spring constant of 0.3 N/m; Mikromasch) was used to contact the single metaphase cells. First, the cells were compressed by the cantilever fixed as 14 μm height from the substrate. Then, the cantilever was lowered to 8 μm at 0.1 μm/s to measure the equilibrium force and to image the maximal cross section area of the compressed cell to calculate the rounding pressure.

Mass spectrometry and data acquisition
Peptides were analyzed by online C18 nanoflow reversed-phase HPLC (2D nanoLC; Eksigent) linked to an LTQ Orbitrap mass spectrometer (Thermo Scientific). Samples were loaded onto an in-house packed 100 μm i.d. × 15 cm C18 column (Magic C18, 5 μm, 200 Å; Michrom Biosource) and separated at 200 nl/min with 60 min linear gradients from 5 to 35% acetonitrile in 0.4% formic acid. Survey spectra were acquired in the orbitrap with the resolution set to a value of 30,000. Up to six of the most intense ions per cycle were fragmented and analyzed in the linear trap. Raw files were processed using version 1.1.1.21 of MaxQuant (Cox et al., 2009). Cysteine carbamidomethylation was used as a fixed modification, and oxidation (M), deamidation (N), N-acetyl (protein N-term) were set as variable modifications. Two missed tryptic cleavages were allowed, and the minimal length required for a peptide was six amino acids. The initial precursor mass tolerance was set to 10–20 ppm, and the fragment mass tolerance was set to 0.5 Da. The peptide false discovery rates (FDR) were set to 0.05, protein FDR were 0.01, and all other parameters were default settings. The datasets were searched against the International Protein Index human database (v.3.69). Labeling was set to doublets of 0/0 and 8/10. For protein quantification, razor and doublets of 0/0 and 8/10. For protein quantification, razor and unique peptides were used with two or more ratio counts. A protein SILAC ratio was calculated as the median of all SILAC peptide ratios. Plasma membrane, extracellular, and cell surface proteins were selected based on the GOCC annotation obtained from the MaxQuant Protein Groups table.

Statistical analysis
Heavy-to-light ratios were computed, and GO-based filtered cell membrane protein groups were used for the subsequent analysis and transformed to a binary logarithmic scale. Since significant variation between experimental repeats was observed as shown in Fig 2A left, all repeats were normalized. Quantile normalization (Bolstad et al., 2003), an established standard technique from the analysis of microarrays, was applied to all repeats and reduced
experimental variation. The resulting histogram of the joint distribution is shown in Fig 2A right.

CD97, HLA, EGFR, and FAS were chosen as a representative group of proteins that are not differentially regulated in interphase and mitosis and used as a standard for unchanged proteins for further comparison. For all measurements of this group of proteins, a variation within the interval from −0.524 to 0.997 with a median of 0.120 was observed. A linear normalization was used for all measurements to set the standard for unchanged proteins median to zero to simplify comparisons. For each protein group, a nonparametric two-sided Mann–Whitney U-test versus the standard for unchanged proteins was run and an individual $P$-value was computed. A Benjamini–Hochberg correction was applied to adjust for multiple testing and only protein groups with an adjusted $P$-value below 0.05 were considered as differentially regulated. Only regarded proteins identified and quantified in at least three measurements in the seven replicate experiments were considered. For the proteins, which are observed in less than three repeats, statistical significance is practically impossible.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

NO designed the experiments; NÖ, MHQ, GM, NEÖ, SB carried out the proteomic experiments and the cell biological follow-up studies; BYR carried out the computational analysis of the quantitative proteomics data; YT, AAH provided reagents; WT assisted with the initial computational analysis; NÖ, TJM, JAS conceived the project and wrote the manuscript with the input from the other authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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