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Targeting of the RhoGEF Ect2 to the Equatorial Membrane Controls Cleavage Furrow Formation during Cytokinesis

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SUMMARY

In animal cells, formation of the cytokinetic furrow requires activation of the GTPase RhoA by the guanine nucleotide exchange factor Ect2. How Ect2, which is associated with the spindle midzone, controls RhoA activity at the equatorial cortex during anaphase is not understood. Here, we show that Ect2 concentrates at the equatorial membrane during cytokinesis in live cells. Ect2 membrane association requires a pleckstrin homology domain and a polybasic cluster that bind to phosphoinositide lipids. Both guanine nucleotide exchange function and membrane targeting of Ect2 are essential for RhoA activation and cleavage furrow formation in human cells. Membrane localization of Ect2 is spatially confined to the equator by centralspindlin, Ect2's spindle midzone anchor complex, and is temporally coordinated with chromosome segregation through the activation state of CDK1. We propose that targeting of Ect2 to the equatorial membrane represents a key step in the delivery of the cytokinetic signal to the cortex.

INTRODUCTION

Animal cells divide by segregating sister chromatids to opposite poles before redrawing their boundaries in a process known as cytokinesis (Barr and Gruneberg, 2007; Eggert et al., 2006). During anaphase, the constriction of the plasma membrane leads to the formation of a cleavage furrow that separates the cytoplasm of the two nascent daughter cells. Cleavage furrow ingression is driven by a membrane-associated and actomyosin-based structure, called the contractile ring. Local activation of the small GTPase RhoA at the equatorial cell cortex in anaphase plays a key role in the assembly and constriction of the contractile ring (reviewed in Piekny et al., 2005).

By partitioning segregated sister genomes and centrosomes to daughter cells, cytokinesis prevents chromosomal instability and tumorigenesis (Ganem et al., 2007). Formation of the cleavage furrow has to be tightly coordinated with chromosome segregation so that cytokinesis occurs only after anaphase onset and only at the equator. Temporal control is exerted by cyclin-dependent kinase 1 (CDK1), which inhibits cytokinesis in early mitosis and is inactivated at the metaphase-to-anaphase transition (Barr and Gruneberg, 2007; Wurzenberger and Gerlich, 2011).

Cleavage furrow formation occurs at the plasma membrane. Yet, micromanipulation experiments and functional studies have established that the mitotic spindle positions the cleavage furrow and the zone of active RhoA in animal cells (D'Avino et al., 2005; Rappaport, 1985; von Dassow, 2009). Two components of the anaphase spindle that play a pivotal role in controlling the formation and positioning of the cleavage furrow at the equator are astral microtubules and the spindle midzone, also called the central spindle (Bringmann and Hyman, 2005; Dechant and Glotzer, 2003). The spindle midzone is a stable array of interdigitated microtubules that assembles at anaphase onset midway between segregating chromatids in the equatorial plane (Glotzer, 2009). The ability of microtubule-associated protein complexes to control cytokinetic events at the cell cortex and plasma membrane is a poorly understood but central aspect of cell division.

At the heart of cleavage furrow formation in animal cells lies the conserved Rho guanine nucleotide exchange factor (GEF) Ect2 (epithelial cell transforming sequence 2) (Prokopenko et al., 1999; Tatsumoto et al., 1999). Ect2 and its orthologs, such as Pebble in *Drosophila*, are essential for RhoA activation, contractile ring formation and cytokinesis (reviewed in Barr and Gruneberg, 2007; Piekny et al., 2005). The protein contains two N-terminal tandem BRCT (BRCA1 C-terminal) domains followed by a DH-type RhoGEF domain (Rossman et al., 2005) and a pleckstrin homology (PH) domain at the C terminus (Figure 1A). DH domains in RhoGEF proteins usually occur in conjunction with PH domains, potential phospholipid interaction elements (Lemmon, 2008). In vitro, full-length Ect2 protein and a C-terminal fragment containing the DH-PH region display guanine nucleotide exchange activity on RhoA (Kim et al., 2005; Tatsumoto et al., 1999).

During anaphase Ect2 is recruited to the spindle midzone and equatorial astral microtubules by binding to a conserved protein complex known as centralspindlin, a key structural component of the spindle midzone (Somers and Saint, 2003; Yüce et al., 2005). Centralspindlin is a heterotetramer composed of the mitotic kinesin Mklp1 and the Rho GTPase-activating protein MgcRacGAP (also called HsCyk-4 and RacGAP1) (Glotzer,

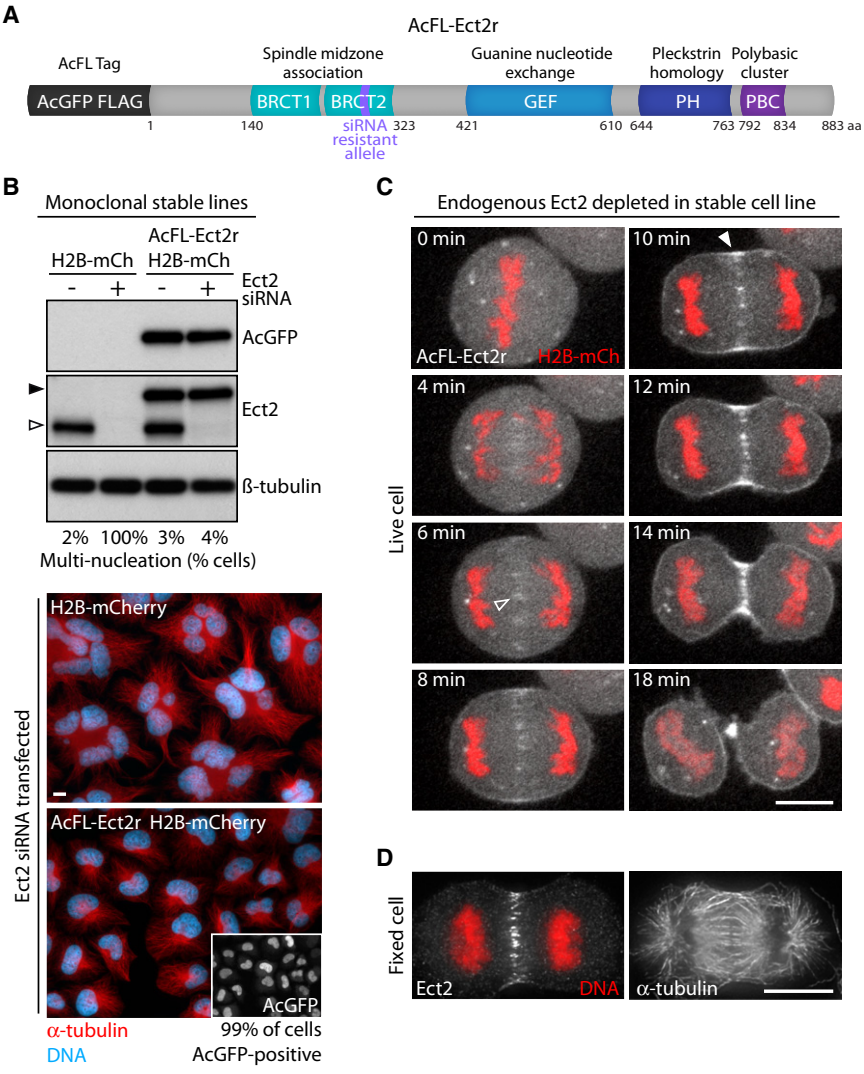

Figure 1. Ect2 Localizes to the Equatorial Membrane during Cytokinesis

(A) Domain organization of AcFL-tagged and RNAi-resistant human Ect2 protein.

(B) Immunoblot analysis of protein extracts prepared from stable HeLa Kyoto cell lines expressing H2B-mCherry or coexpressing AcFL-Ect2r and H2B-mCherry (top panel). Extracts were prepared 48 hr after transfection with control siRNA (–) or Ect2 siRNA (+) duplexes. Extracts were probed with antibodies directed against AcGFP, Ect2 and β-tubulin. Endogenous Ect2 protein and transgenic AcFL-Ect2r are indicated by open and filled arrowheads, respectively. The percentage of binucleated or multinucleated interphase cells (multinucleation) 48 hr after siRNA transfection is indicated below the blot lanes ($n > 600$ cells). Immunofluorescence (IF) microscopy analysis of cell lines stably expressing H2B-mCherry or stably coexpressing AcFL-Ect2r and H2B-mCherry 48 hr after transfection with Ect2 siRNA (bottom panel). Scale bar in this panel and the following panels represents 10 μm.

(C) Confocal live-cell imaging of the monoclonal cell line stably coexpressing AcFL-Ect2r (white) and H2B-mCherry (red). The cell line was characterized in (B). Recording started 30 hr after transfection with Ect2 siRNA. Time point t = 0 min was set to the metaphase-to-anaphase transition. The open and filled arrowheads indicate localization to the spindle midzone and cell cortex, respectively. For full sequence, see Movie S1.

(D) IF analysis of Ect2 localization during anaphase. Endogenous Ect2 protein and α-tubulin were detected by antibodies in methanol-fixed HeLa Kyoto cells.

expression studies in human cells have revealed that a C-terminal region of Ect2 containing the PH domain localizes to the cell periphery (Chalamalasetty et al., 2006). While Ect2 is readily visualized at the spindle midzone, a fraction of the protein has also been detected at the cell cortex in anaphase cells (Chalamalasetty et al., 2006; Nishimura and Yonemura, 2006). These findings raise the possibility that the controlled delivery of Ect2 to the equatorial plasma membrane at anaphase could represent a key but hitherto uncharacterized step in RhoA activation and cleavage furrow formation.

2009). Complex formation between Ect2 and centralspindlin is directed by the binding of Ect2's N-terminal region and BRCT domains to MgcRacGAP (Somers and Saint, 2003; Yüce et al., 2005). In mammalian cells, this interaction requires the phosphorylation of MgcRacGAP by the mitotic kinase Plk1, which creates a binding site for Ect2's tandem BRCT domains (Burkard et al., 2009; Wolfe et al., 2009). Ect2, MgcRacGAP, and, in mammalian cells, Plk1 are required for the activation of RhoA and cleavage furrow formation (Barr and Gruneberg, 2007). This has lead to the formulation of models proposing that recruitment of the RhoGEF Ect2 to peripheral midzone microtubules or equatorial astral microtubules activates RhoA at the adjacent equatorial membrane, leading to the formation of the contractile ring and the cleavage furrow (D'Avino et al., 2005; Nishimura and Yonemura, 2006; Piekny et al., 2005; Somers and Saint, 2003; Yüce et al., 2005).

Despite our detailed understanding of the mechanism underlying the spindle midzone association of Ect2, the molecular basis for how Ect2 delivers the cytokinetic signal from microtubules to the plasma membrane remains unknown. Transient

RESULTS

Live-Cell Imaging Reveals Ect2 Localization to the Equatorial Membrane during Cytokinesis

To investigate the function and localization of Ect2 during cytokinesis, we generated a genetic complementation system in human HeLa "Kyoto" cells. We created a stable cell line coexpressing histone H2B-mCherry and an siRNA-resistant allele of Ect2 (Ect2r) that was N-terminally tagged with AcGFP (Aequora coerulescens GFP) and a single FLAG epitope (AcFL) (Figure 1A). The engineered line expressed transgenic AcFL-Ect2r in all cells of the population and at a level indistinguishable from the one

observed for the endogenous counterpart (Figure 1B). siRNA-mediated depletion of endogenous Ect2 protein in a line that only expressed H2B-mCherry converted all cells of the population into bi-nucleated or multinucleated cells, indicative of complete cytokinesis failure (Figure 1B). This phenotype was completely suppressed in the cell line coexpressing H2B-mCherry and siRNA-resistant AcFL-Ect2r despite efficient removal of the endogenous Ect2 protein (Figure 1B). Thus, transgenic and tagged Ect2 is able to entirely replace the cytokinetic function of the endogenous protein.

The established model allowed us to track Ect2 during cytokinesis in live cells at native protein level but in the absence of a competing endogenous counterpart (Movie S1 available online). Time-lapse imaging after depletion of the endogenous protein revealed that AcFL-Ect2r remained in the cytoplasm until the metaphase-to-anaphase transition (Figure 1C). Within 4–6 min after anaphase onset AcFL-Ect2r accumulated at the spindle midzone (Figure 1C, open arrowhead). This spindle midzone localization was maintained throughout cleavage furrow ingression. Eight minutes after anaphase onset the protein also became concentrated at the cell periphery. Shortly before and during cleavage furrow ingression peripheral AcFL-Ect2r became progressively enriched at the equator (Figure 1C, closed arrowhead). After completion of furrow ingression, the protein accumulated around the midbody before being imported into the reforming daughter nuclei during mitotic exit. Our live-cell analysis establishes that Ect2 first localizes to spindle midzone after anaphase onset and then becomes strongly concentrated at the equatorial cortex or membrane during cytokinesis (30/30 cells) (Movies S1 and S2). The localization of Ect2 to the periphery is largely lost during cell fixation (Figure 1D) but can to some extent be preserved using special fixation conditions (Chalamalasetty et al., 2006). Importantly, the peripheral accumulation of Ect2 is tightly correlated in both space and time with the formation of the cleavage furrow at the equator during anaphase (Figure 1C). This raises the possibility that this membrane-proximal pool of the RhoGEF protein Ect2 plays a key role in the activation of RhoA and the initiation of cytokinesis.

Mechanistic Basis for Ect2 Association with the Plasma Membrane

We next sought to determine the mechanism for the association of Ect2 with the cell cortex or plasma membrane. A previous study has demonstrated that the carboxy-terminal half of Ect2 can localize to the cell periphery (Chalamalasetty et al., 2006). We therefore focused our attention on a region of Ect2 (Ect2CT, aa 414–883) (Figure 2A; Figure S1A) that encompasses the GEF domain, PH domain and Tail region but lacks the two BRCT repeats responsible for spindle midzone association of Ect2 (Chalamalasetty et al., 2006; Tatsumoto et al., 1999; Yüce et al., 2005). Similar to a lipid-modified marker protein (Myr-Palm-FLAc), transiently expressed AcFL-Ect2CT was efficiently recruited to the cell periphery in anaphase cells (Figure 2B). The AcFL tag alone remained cytoplasmic (Figure 2B). In contrast to the full-length protein (Figure 1C), transiently expressed Ect2CT was not enriched at the cell equator during anaphase (Figure 2B). Expression of Ect2CT caused severe changes in interphase cell morphology, such as cell rounding, and prevented cleavage furrow ingression during cytokinesis (Figure 6B and data not shown) presumably due to ectopic Rho activation. Although Ect2CT colocalized with cortical actin in solvent treated cells, the protein accumulated in peripheral regions that were largely devoid of cortical actin in dihydrocytochalasin B-treated anaphase cells (Figure 2C) (20/20 cells). This finding together with our observation that Ect2 binds to phospholipids in vitro (Figure 2D, see below) suggests that Ect2 associates directly with the plasma membrane and not with the cortical actin cytoskeleton.

We next investigated which elements within Ect2CT direct membrane targeting of the protein. The localization of Ect2CT to the plasma membrane was not affected by point mutations in the DH domain that abolish the GEF activity of Ect2 on RhoA in vitro (GEF^{4A}) (Figures 2B and 3D). This indicates that the guanine nucleotide exchange activity of Ect2 is dispensable for the recruitment of Ect2 to the membrane. Deletion of the conserved PH domain, a potential lipid interaction module (Lemmon, 2008), severely compromised but, unexpectedly, did not completely abrogate membrane association of Ect2CT (ΔPH) (Figure 2B) suggesting the presence of an additional membrane interaction region. Deletion of the carboxy-terminal Tail region (ΔTail) as well as simultaneous deletion of the PH domain and Tail region abrogated cortical localization of AcFL-Ect2CT (ΔPHΔTail) (Figure 2B). Analysis of the Tail sequence revealed the presence of a polybasic cluster (PBC) between amino acid 792 and 834 of human Ect2 (Figure 2A; Figure S1B). The polybasic nature of this region is strongly conserved in Ect2 orthologs of other animal species (Figure S1B), with *Caenorhabditis elegans* being a notable exception. Interestingly, the C-terminal region containing the PBC has previously been shown to be required for the transforming activity of human Ect2 (Solski et al., 2004) and may contribute to the control of Ect2 protein turnover after mitotic exit (Liot et al., 2011). Polybasic clusters are frequently found at the carboxy-termini of small GTPases and target the proteins to the plasma membrane by binding to negatively charged phosphatidylinositol 4,5-bisphosphate ($PI[4,5]P_2$) and phosphatidylinositol 3,4,5,-triphosphate ($PI[3,4,5]P_3$) lipids (Heo et al., 2006). Expression of the polybasic cluster of Ect2 fused to AcFL revealed that the region alone can be sufficient for plasma membrane targeting (PBC) (Figure 2B). Our transient expression analysis suggests that two conserved regions within the carboxy terminus of Ect2, a PH domain and a polybasic cluster, cooperate to target the protein to the plasma membrane.

To test the ability of Ect2 to directly interact with lipids in vitro, we expressed maltose-binding protein (MBP)-tagged Ect2 protein and variants thereof using the insect cell-baculovirus system (Figure 2D). Purified recombinant proteins were incubated with an array of lipids that were immobilized on a support membrane. MBP fusions of both full-length Ect2 protein and Ect2CT bound to phosphatidylinositol 4-phosphate (PI[4]P), $PI[4,5]P_2$, and $PI[3,4,5]P_3$ (Figure 2D). Consistent with the in vivo membrane targeting experiments described above, simultaneous deletion of the PH domain and the Tail region, which contains the polybasic cluster, abolished the ability of MBP-Ect2CT to interact with phosphoinositides in vitro (Figure 2D). The specificity of the lipid interaction assay was validated by the selective binding the PH domain of GRP1 to $PI(3,4,5)P_3$

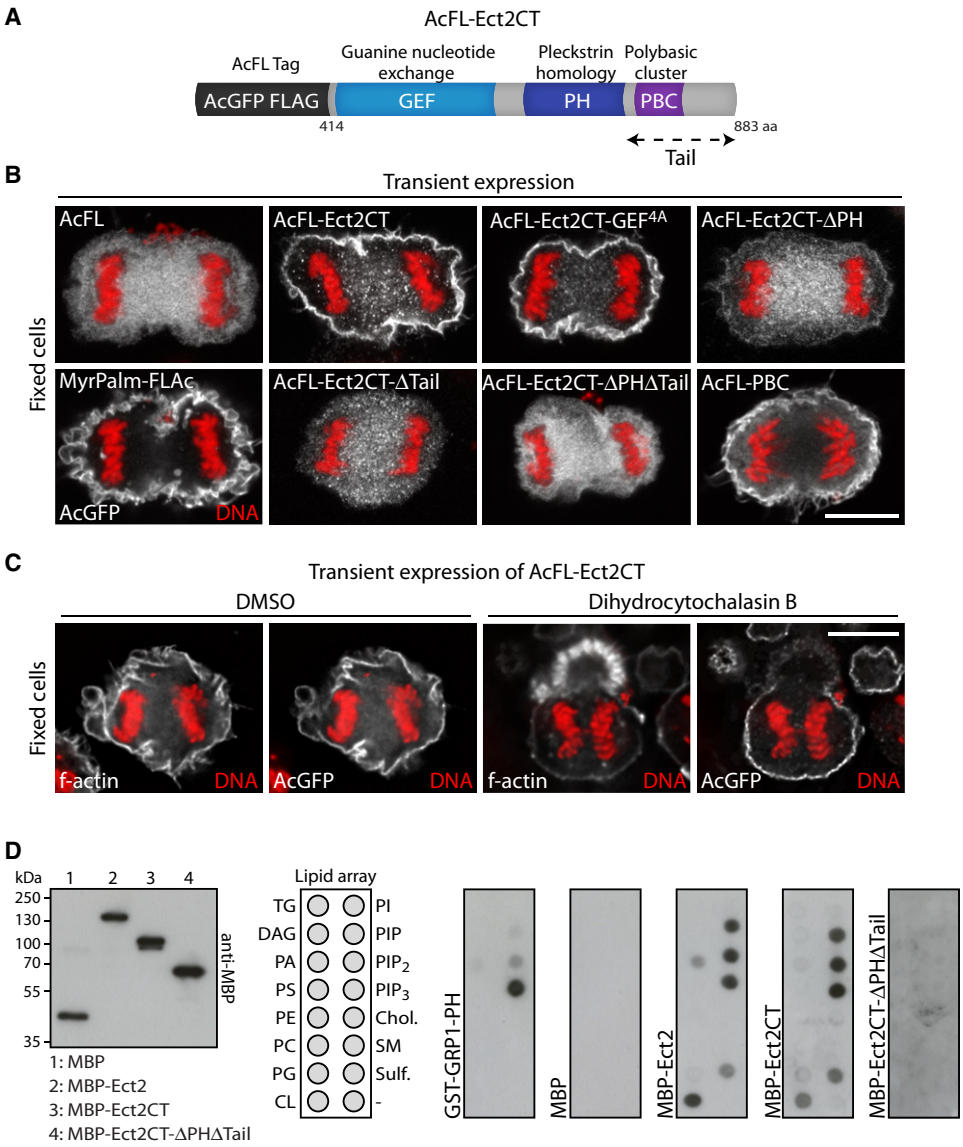

Figure 2. Mechanism of Ect2 Association with the Plasma Membrane

(A) Domain organization of AcFL-Ect2CT spanning aa 414 to 883 of human Ect2.

(B) IF analysis of indicated control proteins and AcFL-tagged Ect2 fragments in formaldehyde-fixed anaphase cells following transient transfection. Transiently expressed proteins were detected using an antibody directed against the AcGFP moiety of the AcFL and FLAc tags. Domain organization of all transfected constructs is shown in Figure S1A. For alignment of polybasic cluster see Figure S1B. Scale bar in this panel and the following panels represents 10 μm.

(C) IF analysis of transiently expressed AcFL-Ect2CT in formaldehyde-fixed anaphase cells. Cells were treated with dimethyl sulfoxide (DMSO) or 20 μM dihydrocytochalasin B for 20 min prior to fixation. AcFL-Ect2CT and filamentous actin (f-actin) were detected using anti-AcGFP antibodies and fluorophore-conjugated phalloidin, respectively.

(D) Immunoblot analysis of recombinant maltose-binding protein (MBP) and MBP-Ect2 fusion proteins (left panel). MBP fusion proteins were expressed in insect cells using the baculovirus system and purified using amylose resin. Lipid arrays were incubated with the indicated recombinant proteins before being probed by anti-GST (for GST-GRP1-PH) or anti-MBP antibodies (right panel). Key for lipid array: triglyceride (TG), diacylglycerol (DAG), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylethanolamine (PE); phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylinositol (PI), PtdIns(4)P (PIP), PtdIns(4,5)P_2 (PIP_2), PtdIns(3,4,5)P_3 (PIP_3), cholesterol (Chol.), sphingomyelin (SM), sulfatide (Sulf.).

See also Figure S1.

(Figure 2D) (Klarlund et al., 1997). These results suggest that the targeting of Ect2 to the membrane could be directed by the interaction of the PH domain and polybasic cluster with negatively charged phosphoinositides in the inner leaflet of the plasma membrane.

Cytokinesis Requires Ect2's Guanine Nucleotide Exchange Activity and Its Membrane Association Domains

To scrutinize the function of Ect2's exchange activity and its membrane association domains during cell division, we

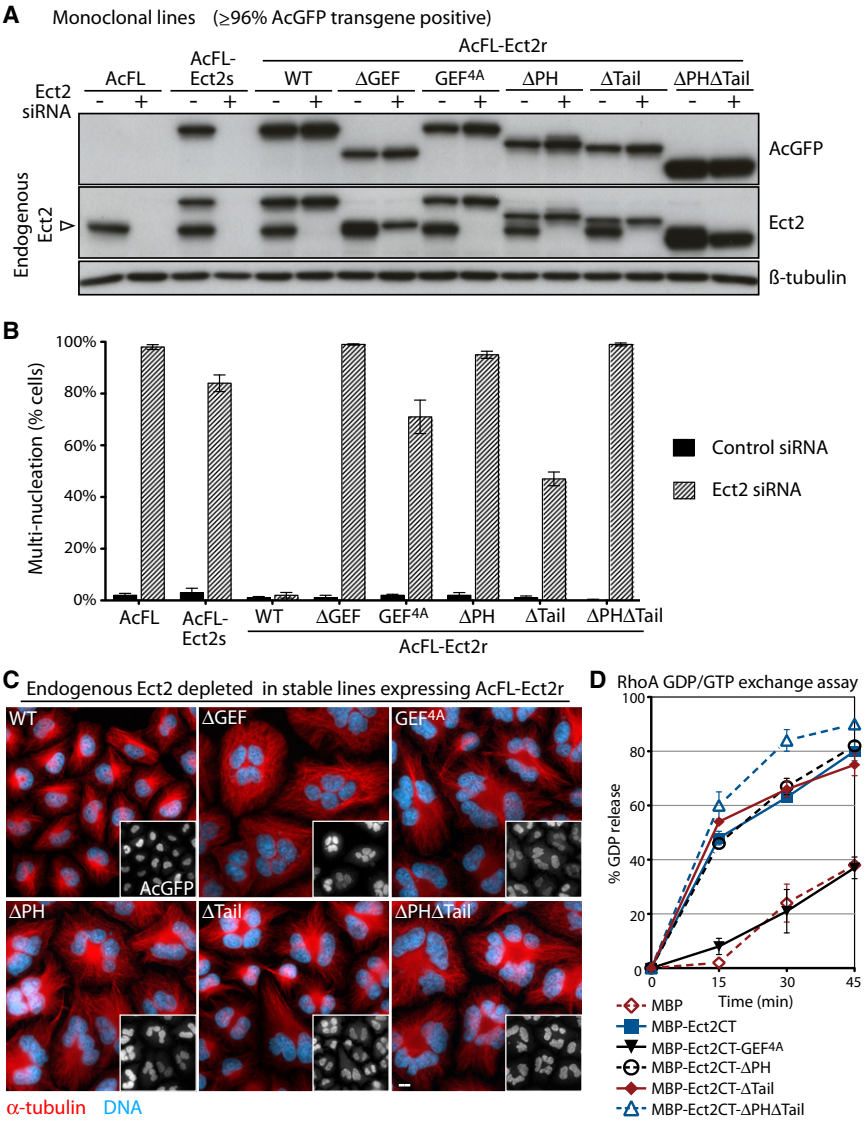

Figure 3. GEF Activity and Membrane Interaction Domains of Ect2 Are Required for Cytokinesis

(A) Immunoblot analysis of protein extracts prepared from monoclonal HeLa Kyoto cell lines expressing the indicated transgenes. Extracts were prepared 48 hr after transfection with control siRNA (–) or Ect2 siRNA (+). Extracts were probed with antibodies directed against AcGFP, Ect2, and β-tubulin. Domain organization of all constructs used is shown in Figure S2A.

(B) Quantification of the fraction of bi-nucleated and multinucleated interphase cells (multinucleation) in monoclonal lines expressing the indicated transgenes. Error bars indicate standard deviation of three experiments (n > 200 cells each). Cells were analyzed by IF 48 hr after transfection with control or Ect2 siRNA. See Figure S2B for the characterization of a second set of independent monoclonal cell lines.

(C) IF analysis of monoclonal cell lines expressing the indicated transgenes. Cells were analyzed 48 hr after transfection with Ect2 siRNA. Scale bar represents 10 μm.

(D) In vitro RhoA GDP/GTP exchange assay. [^{3}H]GDP-loaded GST-RhoA was incubated with recombinant MBP or MBP fusions of the indicated Ect2 fragments in the presence of unlabelled GTP. [^{3}H]GDP release was determined by measuring the remaining protein-associated radioactivity at the indicated time points. Error bars indicate standard deviation of two experiments. A characterization of the recombinant proteins used is shown in Figure S2C.

See also Figure S2.

employed the potent genetic complementation system introduced earlier to generate a series of Ect2 mutant alleles (Figure 3A; Figure S2A). To assess the requirement of the GEF function, the DH domain was deleted (ΔGEF). Alternatively, a stretch of 4 highly conserved residues (565PVQR568) within the CR3 helix of the DH domain was replaced by 4 alanine residues (GEF^{4A}). The CR3 helix is critical for the guanine nucleotide exchange activity of DH domains (Rossman et al., 2005) and mutations therein have previously been shown to block the transforming activity of Ect2 (Saito et al., 2004). Introduction of the GEF^{4A} mutations abolished the GDP/GTP exchange activity of recombinant MBP-Ect2CT on RhoA in vitro (Figure 3D; Figure S2C). To investigate the requirement of Ect2 membrane targeting for cytokinesis, the PH domain (ΔPH) and the PBC containing Tail region (ΔTail) were deleted separately or in conjunction (ΔPHΔTail). Crucially, neither individual nor combined removal of the PH and Tail regions reduced the GDP/GTP exchange activity of recombinant Ect2CT on RhoA in vitro (Figure 3D; Figure S2C). Although we cannot rule out that the PH domain and PBC have functions in addition to membrane targeting, our in vitro experiments suggest that the guanine nucleotide exchange activity of Ect2 can be separated from the ability of the protein to associate with the plasma membrane.

We generated monoclonal HeLa Kyoto lines that express AcFL-tagged RNAi-resistant alleles of Ect2 (AcFL-Ect2r) in at least 96% of cells and at levels close to the one observed for the endogenous protein (Figure 3A; Figure S2A). Test experiments revealed that AcFL-Ect2r expressed at less than 20% of the level of the endogenous counterpart was sufficient to successfully execute cytokinesis in the absence of the endogenous protein (data not shown). To investigate whether specific alleles of Ect2 can support cytokinesis, we depleted the endogenous protein in transgenic monoclonal lines and determined the emergence of bi-nucleated and multinucleated cells, a signature phenotype of defective cytokinesis (Figures 3). Loss of endogenous Ect2 protein abrogated cytokinesis in cells expressing the AcFL tag or an RNAi-sensitive transgenic version of Ect2 (AcFL-Ect2s) (Figure 3B). In contrast, expression of an RNAi-resistant wild-type allele of Ect2 (AcFL-Ect2r-WT) fully rescued the cell division defect caused by depletion of the endogenous protein (Figures 3B and 3C). Deletion of Ect2's GEF domain

(ΔGEF) completely abolished the rescue activity of AcFL-Ect2r (Figures 3B and 3C). Point mutations within the CR3 helix of the GEF domain (GEF^{4A}) severely compromised the ability of AcFL-Ect2r to support cytokinesis in the absence of endogenous protein (Figures 3B and 3C). The difference in phenotypic penetrance between the ΔGEF and GEF^{4A} variants of Ect2 could be caused by either residual exchange activity exhibited by the GEF^{4A} allele in vivo or by an additional function of the GEF domain that is distinct from its exchange activity. Importantly, deletion of the PH domain (ΔPH) and combined deletion of both PH domain and Tail region (ΔPHΔTail) completely abrogated the ability of AcFL-Ect2r to support cytokinesis in the absence of endogenous Ect2 (Figures 3B and 3C). Removal of the Tail region alone (ΔTail) had a weaker effect and blocked cell division in about half of the cell population (Figures 3B and 3C). The results obtained with mutant transgenic alleles of Ect2 were confirmed in a second set of independently isolated monoclonal cell lines (Figure S2B). Our genetic complementation analysis and in vitro assays demonstrate that Ect2's guanine nucleotide exchange activity and its membrane association domains are crucial for the execution of cytokinesis in human cells.

The Pleckstrin Homology Domain and C-Terminal Polybasic Cluster Target Ect2 to the Plasma Membrane during Cytokinesis

To investigate the role of the DH, PH and Tail domains (Figure 4A) in controlling the distribution of Ect2 during cell division, the localization of transgenic Ect2 variants was first determined in fixed cells. Following depletion of the endogenous protein in transgenic monoclonal cell lines, all mutant Ect2 variants associated with the spindle midzone during anaphase (Figure 4B) demonstrating that DH, PH and Tail regions within the carboxy-terminal half of Ect2 are dispensable for spindle midzone association of the protein. This is consistent with the fact that Ect2′s N-terminal BRCT repeats are responsible for the recruitment of the protein to the midzone (Chalamalasetty et al., 2006; Tatsumoto et al., 1999; Yüce et al., 2005).

To scrutinize the localization of Ect2 and its association with the membrane in live cells, we depleted the endogenous protein and used confocal time-lapse microscopy to track the transgenic counterparts (n > 35 cells for each mutant allele). Wild-type Ect2 accumulated at the spindle midzone and the equatorial membrane during anaphase (Figure 4C). Deletion or mutation of the GEF domain (ΔGEF and GEF^{4A}) did not abrogate the localization of the protein to the equatorial membrane (Figure 4C). Ect2-GEF^{4A} showed stronger accumulation at the equatorial membrane than the wild-type counterpart and was partially depleted from the spindle midzone. This indicates that inactivating mutations in the DH domain could lead to the retention of the protein at the equatorial periphery. Despite accumulation at the equatorial membrane, cleavage furrow formation was severely compromised in cells expressing ΔGEF and GEF^{4A} alleles of Ect2 (Figures 4C and 5A). Loss of the PH domain (ΔPH) significantly reduced the association of Ect2 with the membrane (Figure 4C). Crucially, deletion of both PH domains and Tail region (ΔPHΔTail) completely abolished the localization of the protein to the equatorial membrane in anaphase cells, while leaving spindle midzone localization unaltered (Figure 4C). Thus, the localization of Ect2 to the equatorial membrane during cytokinesis requires the protein's PH domain and its PBC containing Tail region. Concomitant with the loss of membrane association, the ΔPHΔTail variant of Ect2 was unable to support cleavage furrow formation (Figures 4C and 5A). While the membrane interaction domains of Ect2 are essential for cell division in vivo (Figures 3B and 3C), they are dispensable for Ect2's exchange activity on RhoA in vitro (Figure 3D). These results suggest that the guanine nucleotide exchange activity and the ability to associate with the plasma membrane are two distinct but key aspects of Ect2 function during cytokinesis.

RhoA Activation and Cleavage Furrow Formation Require the Guanine Nucleotide Exchange Function and Membrane Association of Ect2

To investigate the nature of the cytokinetic defect observed in mutant alleles of Ect2 we used bright-field time-lapse microscopy. This analysis revealed that loss of Ect2 in cells expressing only the AcFL tag prevented cleavage furrow formation in the majority of mitotic cells (Figure 5A). A minor fraction of cells were capable of transiently forming a furrow before ultimately also failing to divide. This suggests that furrow ingression followed by regression represents a hypomorphic Ect2 loss-of-function phenotype. Expression of transgenic wild-type Ect2 (AcFL-Ect2r) completely restored cleavage furrow formation and cytokinesis in the absence of the endogenous counterpart (Figure 5A). In contrast, transgenic alleles of Ect2 lacking either the GEF domain (ΔGEF) or both membrane association domains (ΔPHΔTail) were unable to support cleavage furrow formation (Figure 5A). The phenotype of the two alleles was similar to that of a total loss of Ect2 protein indicating that the deleted regions are indispensible for Ect2's cytokinetic function. Consistent with our terminal phenotype analysis (Figure 3B), inactivating point mutations within the GEF domain (GEF^{4A}) had a slightly weaker impact on furrow formation (Figure 5A). In contrast to the loss of both membrane association domains (ΔPHΔTail), Ect2 variants lacking either the PH domain (ΔPH) or Tail region (ΔTail) were able to transiently support furrowing in the majority of the cells examined (Figure 5A). This hypomorphic phenotype is consistent with our finding that both domains cooperate to direct the association of Ect2 with the membrane (Figure 2B). Our live-cell analysis suggests that formation of a cleavage furrow in human cells requires the guanine nucleotide exchange activity of Ect2 and the interaction of the protein with the plasma membrane.

To address the nature of this requirement, we analyzed the localization of the centralspindlin subunit Mklp1, the GTPase RhoA and the contractile ring component anillin (Eggert et al., 2006) in monoclonal lines expressing different Ect2 alleles after depletion of the endogenous Ect2 protein. Loss of Ect2's GEF function or membrane association domains did not affect the formation of the spindle midzone, as judged by the localization of Mklp1 to the central spindle at anaphase (Figure 4B). In cells complemented by transgenic wild-type Ect2, RhoA and the contractile ring component anillin, localized to the equatorial cell cortex during anaphase (Figure 5B). Loss of either Ect2's GEF function or the protein's ability to associate with the membrane prevented or severely compromised the accumulation of RhoA and anillin at the equator (Figure 5B). Consistent with the observed cleavage furrow phenotypes (Figure 5A),

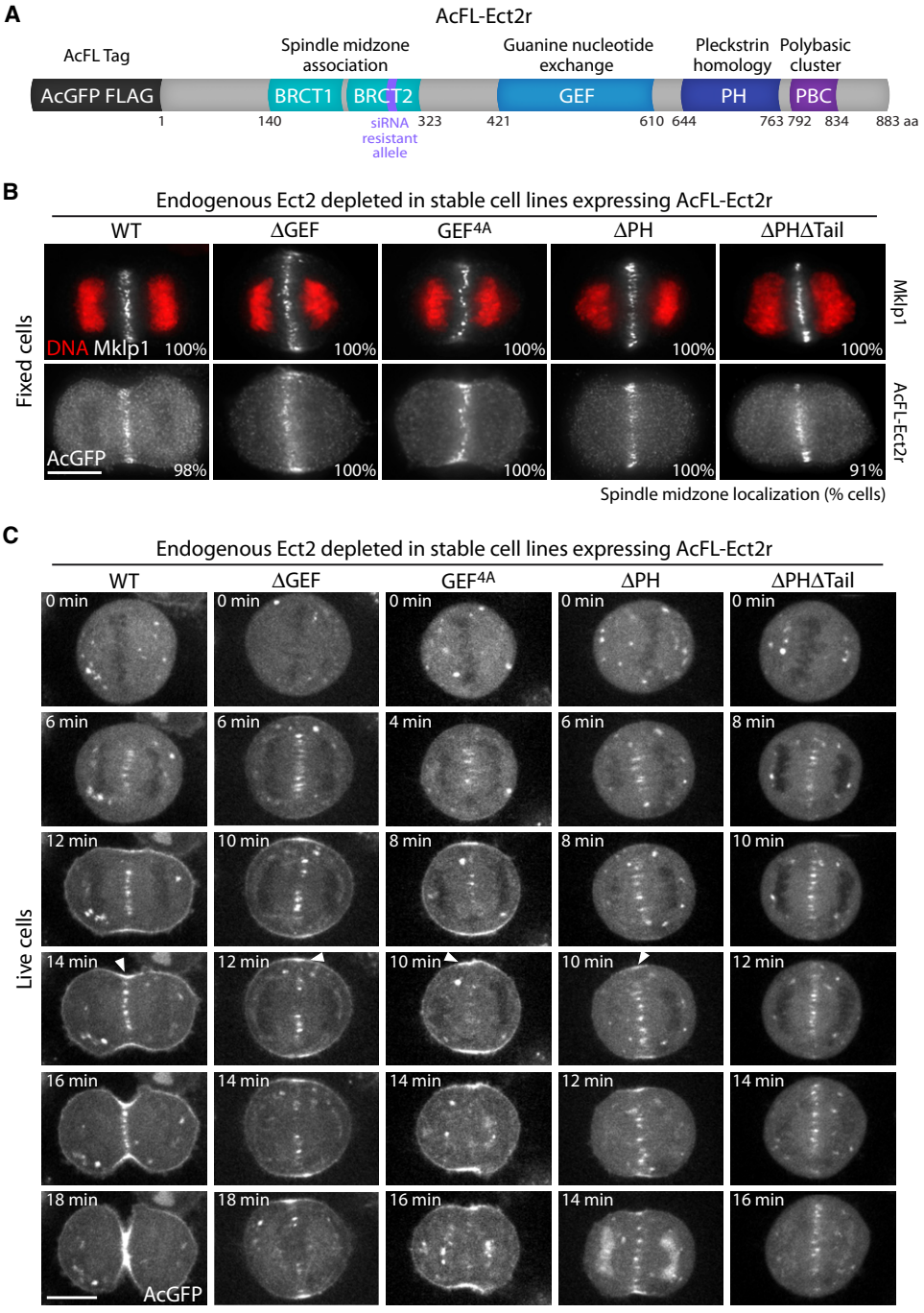

Figure 4. The PH domain and Polybasic Cluster Target Ect2 to the Equatorial Plasma Membrane during Cytokinesis

(A) Domain organization of AcFL-tagged and RNAi-resistant human Ect2 protein.

(B) IF analysis of cells expressing the indicated transgenes 36 hr after transfection with Ect2 siRNA (n > 50 anaphase cells each). Cells were costained with anti-Mklp1 and anti-AcGFP antibodies. Scale bars in this panel and the following one represent 10 μm.

(C) Confocal live-cell imaging of the indicated AcFL-Ect2r variants in the stable cell lines. Recording started 20 hr after transfection with Ect2 siRNA. Time point t = 0 min was set to the metaphase-to-anaphase transition. The filled arrowheads indicate localization to the cell cortex.

deletion of the GEF domain and simultaneous deletion of both membrane association regions had the most severe impact on RhoA and anillin localization (Figure 5B) and closely resembled a complete loss of Ect2 function condition. This analysis provides a mechanistic explanation for the cytokinetic defect caused by mutations in the DH, PH and Tail domains of Ect2. Ect2 variants lacking guanine nucleotide exchange function are unable to activate RhoA despite accumulating at the equatorial membrane. Conversely, Ect2 alleles lacking the ability to interact with the membrane are unable to locally activate RhoA at the

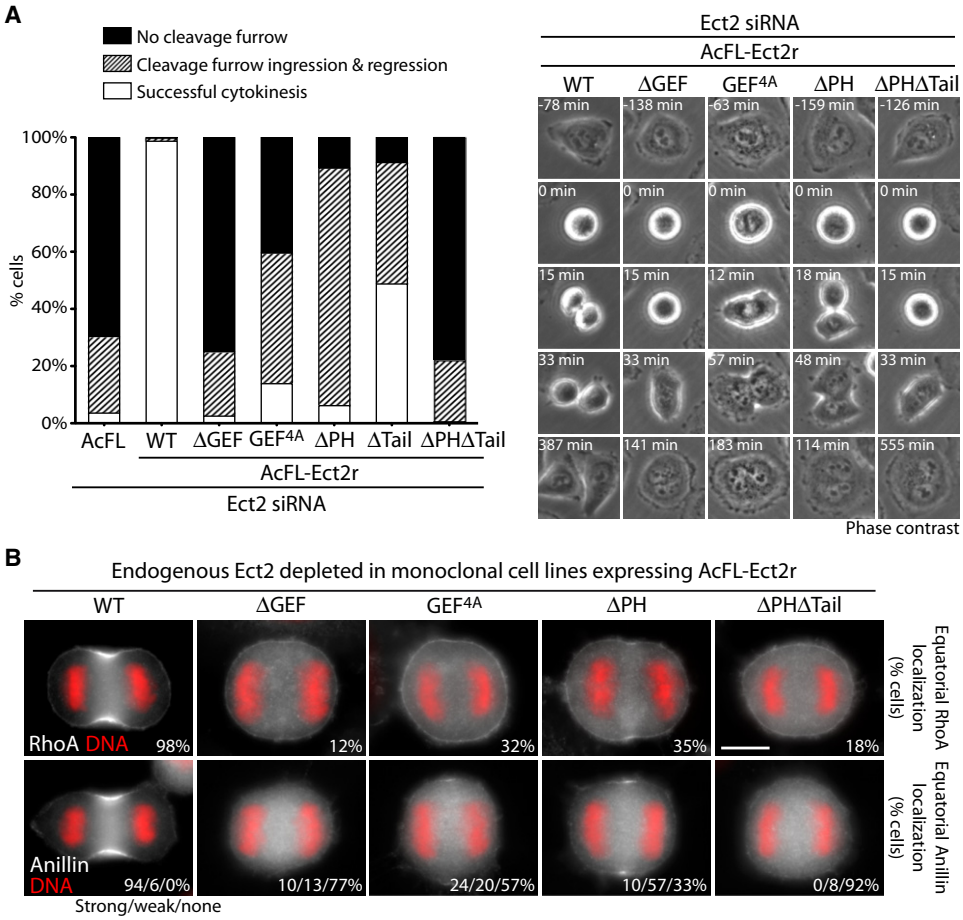

Figure 5. Cleavage Furrow Formation and Equatorial RhoA Concentration Require GEF Activity and Membrane Targeting of Ect2

(A) Quantification of cytokinetic phenotypes in monoclonal cell lines expressing the indicated transgenes using time-lapse microscopy after transfection with Ect2 siRNA (n > 288 cells each) (graph, left panel). Mononucleate cells entering mitosis were scored from 18 to 38 hr posttransfection. Representative cell division phenotypes of selected cell lines are shown as time-lapse series (right panel). Time point t = 0 min was set to the metaphase-to-anaphase transition.

(B) IF analysis of anaphase cells expressing the indicated transgenes 32–36 hr after transfection with Ect2 siRNA (n > 50 anaphase cells each). Cells were fixed in trichloroacetic acid or methanol and stained with anti-RhoA or anti-anillin antibodies, respectively.

equatorial membrane despite retaining exchange activity in vitro. The corollary of these findings is that RhoA activation and contractile ring formation during anaphase requires targeting of Ect2's exchange activity to the equatorial membrane.

Centralspindlin Directs the Concentration of Ect2 at the Equatorial Plasma Membrane

Consistent with its key role in cleavage furrow formation, the association of Ect2 with the plasma membrane is subject to tight spatiotemporal control. Ect2 accumulates at the plasma membrane only after anaphase onset and then becomes concentrated at the cell equator (Figure 6A; Movie S2) (17/18 cells). The transiently expressed Ect2CT fragment that contains both membrane interaction domains but lacks the BRCT repeats required for spindle midzone targeting localizes all around the cell periphery (Figure 2B). This suggests that the primary determinant for the spatial distribution of Ect2 at the membrane is unlikely to be a polarized distribution of membrane lipids. Recruitment of Ect2 to the spindle midzone, which underlies the equatorial cortex, could direct the concentration of the protein at the equatorial membrane. To test this hypothesis, we depleted the MgcRacGAP and Mklp1 subunits of centralspindlin, the protein complex that recruits Ect2 to the spindle midzone, in a monoclonal cell line expressing AcFL-Ect2r and H2B-mCherry (Figure S3). Depletion of MgcRacGAP and Mklp1 abolished the localization of Ect2 to the spindle midzone and prevented cleavage furrow ingression (Figure 6A; Movie S2). Although Ect2 accumulated at the membrane after anaphase onset, the protein was distributed all around the cell periphery and no longer concentrated at the equator (Figure 6A; Movie S2) (n > 8 cells each). Similar results were obtained following acute inactivation of Plk1 by addition of the small molecule inhibitor BI 2536 (Lénárt et al., 2007) (Figure 6A; Movie S2) (97/99 cells). Phosphorylation of MgcRacGAP by Plk1 is required for complex formation between centralspindlin and Ect2 (Burkard et al., 2009; Wolfe et al., 2009). Ect2 proteins lacking the nucleotide exchange function accumulate at the equatorial membrane despite absence of furrowing (Figure 4C). Thus, the failure of Ect2 to accumulate at the equatorial membrane in the absence of Plk1 activity and centralspindlin is unlikely to be an

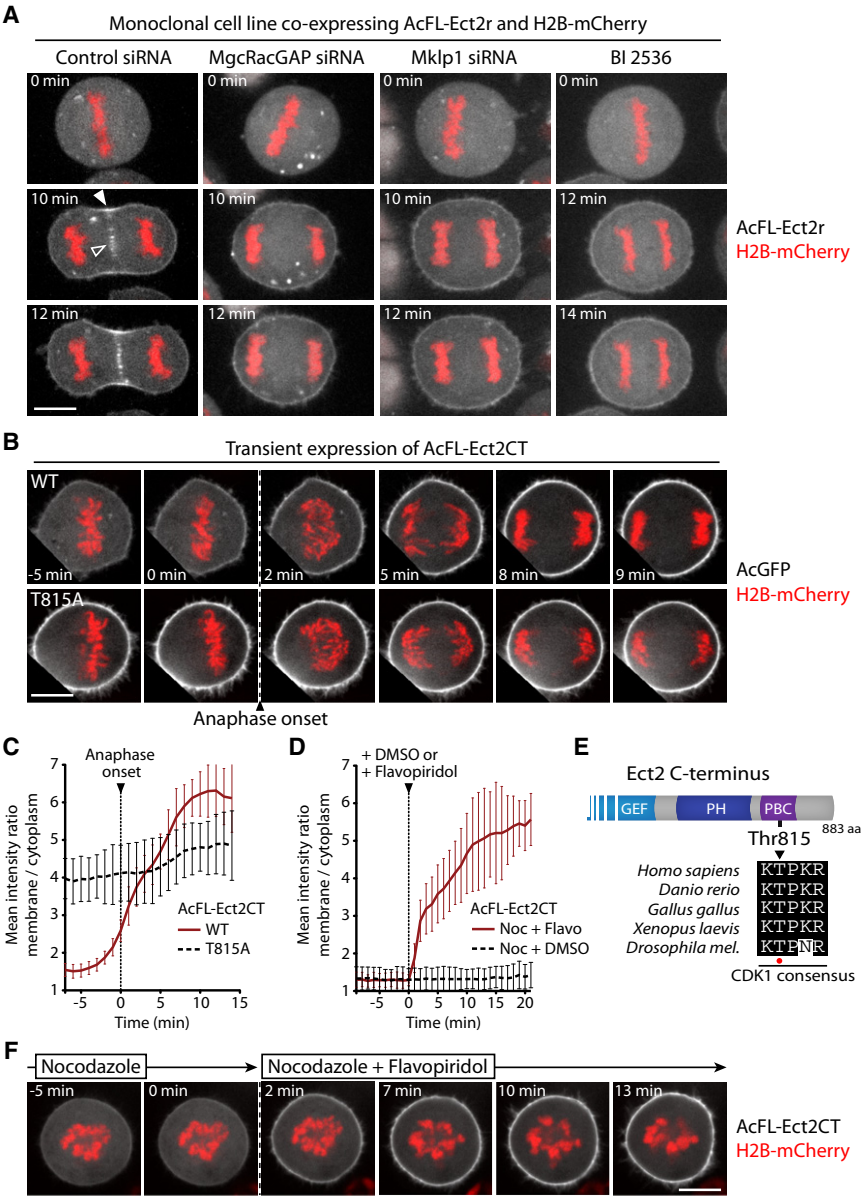

Figure 6. Spatial and Temporal Control of Ect2 Localization to the Equatorial Membrane during Anaphase

(A) Confocal live-cell imaging of a monoclonal cell line stably expressing AcFL-Ect2r (white) and H2B-mCherry (red). Cells were recorded 24 hr after transfection with control siRNA, MgcRacGAP siRNA, or Mklp1 siRNA. Alternatively, cells were released from a metaphase arrest and treated with 250 nM BI 2536 20 min after the release prior to recording (right panel). Time point t = 0 min was set to the metaphase-to-anaphase transition. The open and filled arrowheads indicate localization to the spindle midzone and equatorial cell membrane, respectively. For immunoblot analysis of protein extracts following depletion see Figure S3. Scale bars in this panel and the following panels represent 10 μm. For full sequence, see Movie S2.

(B) Confocal live-cell imaging of transiently expressed AcFL-Ect2CT-WT or AcFL-Ect2CT-T815A (white) (see Figure S1A for constructs) in cells stably expressing H2B-mCherry (red). Time point t = 0 min was set to the metaphase-to-anaphase transition. Anaphase onset is indicated by a dashed line. For full sequence, see Movie S3.

(C) Quantification of the localization of AcFL-Ect2CT-WT (red solid line) and AcFL-Ect2CT-T815A (black dashed line) during mitosis based on time-lapse series as shown in (B). Graph displays the ratio of mean intensity at the plasma membrane to mean intensity in the cytoplasm. Time point t = 0 min was set to the metaphase-to-anaphase transition. Anaphase onset is indicated by a dashed line. Error bars represent the standard deviation of the analysis of eight cells for each construct.

(D) Quantification of the localization of AcFL-Ect2CT in nocodazole-arrested cells treated with either solvent control dimethyl sulfoxide (DMSO) (black dashed line) or 15 μM flavopiridol (red solid line) at t = 0 min. Quantification is based on time-lapse series as shown in (F). Graph displays the ratio of mean intensity at the plasma membrane to mean intensity in the cytoplasm. The time point of DMSO and flavopiridol addition is indicated by a dashed line. Error bars represent the standard deviation of the analysis of 8 cells for each condition.

See also Figure S3.

(E) Sequence alignment of the region surrounding position threonine 815 in human Ect2. Conserved residues are colored in black. Thr815 is embedded within the polybasic cluster of Ect2 and is part of a highly conserved bona fide CDK1 consensus sequence.

(F) Confocal live-cell imaging of transiently expressed AcFL-Ect2CT (white) in cells stably expressing H2B-mCherry (red). Cells were arrested in mitosis by addition of 165 nM nocodazole and subsequently treated with 15 μM flavopiridol. The time of flavopiridol addition was set to t = 0 min and is indicated by a dashed line. For full sequence, see Movie S4.

indirect consequence of the absence of furrow formation. Our experiments suggest that concentration of Ect2 at the equatorial membrane during anaphase requires the interaction of Ect2 with the spindle midzone component centralspindlin.

Inactivation of CDK1 at Anaphase Onset Controls the Association of Ect2 with the Plasma Membrane

Whereas Ect2 remains cytoplasmic until the metaphase-to-anaphase transition, a fraction of Ect2 protein translocates to the plasma membrane and becomes concentrated at the equator upon anaphase onset (Figure 6A; Movie S2). In contrast to the equatorial enrichment, the anaphase-specific surge of Ect2 membrane localization does not require the association of the protein with the spindle midzone (Figure 6A). This suggests that two distinct mechanisms govern the spatial and temporal control of Ect2 membrane targeting.

To investigate the temporal regulation of Ect2 membrane association in the absence of the ability of Ect2 to bind to the midzone, we transiently transfected the carboxy-terminal Ect2CT fragment (Figure 2A) into cells stably expressing H2B-mCherry. Time-lapse imaging revealed that Ect2CT remained largely cytoplasmic with only a weak accumulation at the

membrane (1.5-fold) in early mitosis and prior to anaphase onset (Figure 6B; Movie S3). Strikingly, at the time of sister chromatid segregation the cytoplasmic pool of Ect2CT rapidly and quantitatively translocated to the plasma membrane (Figure 6B; Movie S3). Image analysis revealed that the translocation of Ect2CT to the membrane started 3 min before anaphase onset and was completed 10 min after sister chromatid splitting (Figure 6C). This rapid switch in protein localization at the metaphase-to-anaphase transition raised the possibility that changes in post-translational modification could control the ability of Ect2's PH domain and PBC to interact with the plasma membrane. The loss of CDK1 target phosphorylation at the metaphase-to-anaphase transition triggers the onset of cytokinesis and mitotic exit (reviewed in Barr and Gruneberg, 2007; Wurzenberger and Gerlich, 2011). Thus, phosphorylation by CDK1 represents a candidate mechanism for inhibiting the membrane association of Ect2. Kinase assays have previously identified Thr815 as the major CDK1/cyclin B phosphorylation site within the carboxy-terminal half of Ect2 in vitro (Niiya et al., 2006). Phosphorylation of Thr815 has subsequently been confirmed in vivo by global phosphoproteomic analysis of mitotic cell extracts (Dephoure et al., 2008). Thr815 is embedded in Ect2's polybasic cluster and is the phosphoacceptor residue of a highly conserved CDK1 consensus sequence (Figure 6E). We therefore replaced Thr815 by alanine in Ect2CT and tracked the distribution of the mutant protein through cell division. In stark contrast to the wild-type counterpart, Ect2CT-T815A was strongly enriched at the plasma membrane as soon as cells entered mitosis and no longer displayed the surge in membrane association at anaphase onset (Figures 6B and 6C; Movie S3). To further examine the control of Ect2 membrane localization by CDK1, cells expressing Ect2CT were arrested in mitosis using the spindle poison nocodazole and then treated with the CDK inhibitor flavopiridol. While Ect2CT remained largely cytoplasmic in mitotically arrested cells, acute chemical inhibition of CDK1 by addition of flavopiridol triggered the rapid translocation of the protein to the plasma membrane (Figures 6D and 6F; Movie S4). These data suggest that the activation state of CDK1 acts a switch to temporally control the association of Ect2 with the plasma membrane during mitosis.

DISCUSSION

Over the last three decades, classical micromanipulation experiments and functional studies have established that the mitotic spindle plays a pivotal role in the formation and positioning of the cleavage furrow in animal cells (reviewed in D'Avino et al., 2005; von Dassow, 2009). Our work in human cells has identified the mechanistic basis and spatiotemporal control of an important step in the delivery of the cytokinetic signal to the plasma membrane. We propose that targeting of the RhoGEF Ect2 to the equatorial membrane at anaphase onset activates RhoA and leads to the formation of a cleavage furrow (see Graphical Abstract).

Key to our ability to scrutinize the association of Ect2 with the plasma membrane was the development of a genetic complementation system that allowed us to track the protein at native expression level through cell division in live cells. The interaction of Ect2 with the plasma membrane during cytokinesis requires the protein's PH domain and, unusually for a DH-type GEF (Rossman et al., 2005), a C-terminal polybasic cluster. Ect2's guanine nucleotide exchange activity and its ability to interact with the plasma membrane are mechanistically separable properties that are both required for RhoA activation and cleavage furrow formation. Ect2 variants that lack both membrane association domains but retain exchange activity localize to the spindle midzone, yet are unable to support cleavage furrow formation. This result implies that the recruitment of Ect2 to the spindle midzone might not be sufficient for controlling cortical events during cytokinesis. Although never decisively tested, Ect2's GEF function represents an integral part of current models for cleavage furrow formation. Consistent with the identification of a missense mutation in the DH domain of a *pebble* allele in *Drosophila* (Prokopenko et al., 1999), our in vivo and in vitro experiments provide strong evidence for a key role of Ect2's exchange function in controlling RhoA activity and contractile ring formation.

The local concentration of Ect2 at the equatorial membrane, but not membrane association of the protein per se, requires the interaction of Ect2 with the essential midzone component centralspindlin. Thus, the formation of the spindle midzone at anaphase might break the isotropic distribution of Ect2 at the periphery and target the RhoGEF to the equatorial membrane in the cleavage plane. This hypothesis is supported by micromanipulation experiments (Bement et al., 2005) and could account for the important role of centralspindlin and the spindle midzone in the formation and positioning of the cleavage furrow. In addition to acting as a spatial cue for Ect2 membrane localization, binding to centralspindlin might also contribute to the activation of Ect2's C-terminal domains (Kim et al., 2005; Saito et al., 2004; Yüce et al., 2005).

Although centralspindlin and Ect2 colocalize at the spindle midzone, centralspindlin, unlike Ect2, does not accumulate significantly at the equatorial membrane in human cells (K.-C.S. and M.P., unpublished observation). Consistent with recent observations (von Dassow et al., 2009), a direct transfer of the Ect2-centralspindlin complex from the microtubules to the plasma membrane is therefore unlikely to account for the concentration of Ect2 at the equatorial membrane. Nevertheless, the spatial separation of a membrane-associated pool of Ect2 from MgcRacGAP could provide the solution to the enigmatic question of how a complex containing two proteins of opposing activities, a RhoGEF and a RhoGAP (Miller and Bement, 2009), can control the formation of a Rho-dependent contractile zone.

The requirement for centralspindlin and Plk1 offers an alternative hypothesis for how Ect2 accumulates at the equatorial membrane. The exchange of spindle-midzone associated Ect2 with the protein's cytoplasmic pool could create a concentration gradient around the spindle midzone. Coupled with the ability of Ect2 to dynamically associate with the plasma membrane at anaphase, this local concentration gradient could lead to the accumulation of Ect2 at the equatorial membrane flanking the spindle midzone. Two positive feedback mechanisms might subsequently reinforce the equatorial zone of Ect2. First, the interaction of the centralspindlin subunit MgcRacGAP with the contractile ring component anillin, which was observed in *Drosophila*, could bring cortical microtubules closer to the equatorial membrane (D'Avino et al., 2008; Gregory et al., 2008). Second, the contraction of the cleavage furrow itself will lead to the compression of the spindle midzone and thus to the

increase of the local concentration of Ect2 in the cleavage plane. Testing these hypotheses and deciphering the mechanism underlying the concentration of Ect2 at the equatorial membrane will require the tracking of Ect2 and its dynamic properties at different cellular locations in combination with pharmacological, genetic, and physical perturbation experiments. Future work is also required to test the relative contributions of the spindle midzone and the dominant microtubule asters to the positioning of Ect2 at the membrane in large animal cells, such as embryonic blastomeres.

Although the lipids responsible for Ect2 membrane localization in vivo remain to be determined, the membrane association domains of Ect2 can interact with phosphoinositides in vitro. Experiments in different animal cell systems have established that $PI(4,5)P_2$ localizes to the cleavage furrow and that blocking the access to $PI(4,5)P_2$ prevents cell division (reviewed in Atilla-Gokcumen et al., 2010). These findings raise the exciting possibility that the interaction of Ect2 with $PI(4,5)P_2$ in the inner leaflet of the plasma membrane may contribute to the equatorial concentration of Ect2 during cytokinesis.

Our analysis suggests that the association of Ect2 with the plasma membrane is inhibited by CDK1 phosphorylation prior to anaphase onset. Since CDK1 is inactivated at the metaphase-to-anaphase transition (Wurzenberger and Gerlich, 2011), the CDK1-dependent regulation of Ect2 membrane association allows the temporal coordination of chromosome segregation with cleavage furrow formation. This temporal control mechanism is likely to cooperate with the well-established inhibition of spindle midzone-linked cytokinetic processes by CDK1 (Glotzer, 2009).

This study has characterized an important step in the delivery of the cytokinetic signal to the cell periphery (see Graphical Abstract). The targeting of Ect2 to the plasma membrane and its spatiotemporal control could represent conserved principles that underlie the partitioning of sister genomes and the birth of new daughter cells during cytokinesis in animal cells.

EXPERIMENTAL PROCEDURES

Plasmids, DNA Transfection, and Generation of Stable Cell Lines

For a detailed description please see Supplemental Experimental Procedures. Briefly, AcGFP-FLAG-tagged variants of Ect2 (Figures S1A and S2A) were inserted into pIRESpuro3 (Clontech). The plasmids were transfected into HeLa Kyoto cells that were grown as described in (Petronczki et al., 2007). For the selection of stable cell lines the medium was supplemented with 0.3 μg/ml puromycin (Sigma).

siRNA transfection and Drug Treatment

Lipofectamine RNAiMax (Invitrogen) was used for siRNA transfection. The following siRNA duplexes were used at a final concentration of 30 nM: control (Thermo Scientific siGENOME Non-Targeting siRNA #1 D-001210-01), Ect2 (Thermo Scientific siGENOME D-006450-02), Mklp1 (Invitrogen Stealth HSS114138), and MgcRacGAP (Invitrogen Stealth HSS120934).

To disrupt f-actin, cells were incubated in 20 μM dihydrocytochalasin B (Sigma) for 20 min prior to fixation. To acutely inhibit CDK1, cells were incubated in 165 nM nocodazole (Sigma) for 3 hr before addition of 15 μM flavopiridol (Sigma). To inhibit Plk1 (Figure 6A; Movie S2), cells were synchronized at metaphase as described (Petronczki et al., 2007) and treated with 250 nM BI 2536 (Lénárt et al., 2007) 20 min after release from metaphase.

Immunofluorescence Microscopy

Cells were fixed for 16 hr in −20°C methanol (Figures 1B, 1D, 3C, 4B, and 5B/anillin), for 10 min at 37°C in 4% formaldehyde (Figures 2B and C) or for 15 min on ice in 10% tricholoroacetic acid (Figure 5B/RhoA) before being processed for immunofluorescence (IF) microscopy as described (Lénárt et al., 2007). Images for Figures 1B, 1D, 2C, 3C, 4B, and 5B were acquired on a Zeiss Axio Imager M1 microscope using a Plan Apochromat 63×/1.4 oil objective lens (Zeiss) equipped with an ORCA-ER camera (Hamamatsu) and controlled by Volocity 5.5.1. software (Improvision). Images in Figures 1D, 2C, and 4B were deconvolved using Volocity's iterative restoration function. Images in Figure 2B were acquired with on a Zeiss LSM Upright710 confocal system controlled by Zen 2009 software using a Zeiss Imager.Z2 microscope and a Plan-Apochromat 63×/1.40 Oil DIC M27 lens.

Antibodies and Dyes

For a list of antibodies and dyes used in this study, please refer to Supplemental Experimental Procedures.

Live-Cell Imaging and Image Quantification

Before recording, the medium was changed to phenol-red-free CO_2-independent medium (Lénárt et al., 2007). For Figures 1C, 4C, 6A, 6B, and 6F and Movies S1–S4, frames were acquired at 37°C using a PerkinElmer ERS Spinning disc system equipped with a Nikon TE2000 microscope, a Plan Fluor 40× 1.3 DIC H lens (Nikon) (optovar set to 1.5×), a CSU22 spinning disc scanner (Yokogawa), a IEEE1394 Digital CCD C4742-80-12AG camera (Hamamatsu) and controlled by Volocity 5.5.1 software (Perkin Elmer). Volocity 5.5.1 software was used for quantification of acquired 16 bit images. Mean AcGFP intensities were measured for each time point by averaging six manually placed circular regions of 9 pixels at the cell periphery and two circular regions of 1,000–2,000 pixels size in the cytoplasm followed by subtraction of the mean background signal outside of the cell (Figures 6C and 6D).

To quantify cytokinetic phenotypes in Figure 5A (graph, left panel), phase contrast images of cells were recorded every 5 min in normal medium using an IncuCyte FLR integrated live-cell imaging system (Essen Bioscience). Images in Figure 5A (right panel) were acquired at 37°C using a Zeiss Axio Observer Z1 microscope controlled by SimplePCI software (Hamamatsu) and equipped with an Orca 03GO1 camera (Hamamatsu) and a Plan-Apochromat 10×/0.45 objective.

Purification of Recombinant Proteins and In Vitro Protein Assays

Please refer to Supplemental Experimental Procedures for a description of protein purification procedures, in vitro protein-lipid interaction experiments, and GEF assay.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, Supplemental Experimental Procedures, and four movies and can be found online at doi:10.1016/j.devcel.2011.11.003.

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