Interactome analysis reveals endocytosis and membrane recycling of EpCAM during differentiation of embryonic stem cells and carcinoma cells

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Highlights

Proteomic screen identifies various interaction clusters of EpCAM

EpCAM is subject to retrograde transport and recycling to the plasma membrane

Endocytosis regulates EpCAM levels in mesodermal differentiation and EMT
Interactome analysis reveals endocytosis and membrane recycling of EpCAM during differentiation of embryonic stem cells and carcinoma cells

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SUMMARY

Transmembrane epithelial cell adhesion molecule (EpCAM) is expressed in epithelia, carcinoma, teratoma, and embryonic stem cells (ESCs). EpCAM displays spatiotemporal patterning during embryogenesis, tissue morphogenesis, cell differentiation, and epithelial-to-mesenchymal transition (EMT) in carcinomas. Potential interactors of EpCAM were identified in murine F9 teratoma cells using a stable isotope labeling with amino acids in cell culture-based proteomic approach (n = 77, enrichment factor >3, p value < 0.05). Kyoto Encyclopedia of Genes and Genomes and gene ontology terms revealed interactions with regulators of endosomal trafficking and membrane recycling, which were further validated for Rab5, Rab7, and Rab11. Endocytosis and membrane recycling of EpCAM were confirmed in mF9 cells, E14TG2α ESC, and Kyse30 carcinoma cells. Reduction of EpCAM during mesodermal differentiation and TGFβ-induced EMT correlated with enhanced endocytosis and block or reduction of recycling in ESCs and esophageal carcinoma cells. Hence, endocytosis and membrane recycling are means of regulation of EpCAM protein levels during differentiation of ESC and EMT induction in carcinoma cells.

INTRODUCTION

Epithelial cell adhesion molecule (EpCAM) was initially identified as an antigen expressed on colon carcinoma cells that induced a humoral response in mice (Herlyn et al., 1979; Koprowski et al., 1979). EpCAM was later described to be strongly and frequently expressed in the majority of carcinomas (Baeuerle and Gires, 2007; Went et al., 2004), in pluripotent embryonic stem cells (ESCs) (Gonzalez et al., 2009; Lu et al., 2010, 2013; Ng et al., 2009), and hepatic progenitors (Dolle et al., 2015; Schmelzer et al., 2006, 2007). EpCAM is composed of an extracellular domain, a single type I transmembrane domain, and a short intracellular domain (Balzar et al., 1999). Functional implications of this transmembrane glycoprotein range from cell adhesion and junction (Ladwein et al., 2005; Litvinov et al., 1994a, 1994b; Wu et al., 2013), migration and morphogenesis (Gaiser et al., 2012; Maghzal et al., 2010, 2013; Slanchev et al., 2009), tissue integrity (Gaston et al., 2021; Guerra et al., 2012; Kozan et al., 2015; Lei et al., 2012; Salomon et al., 2017; Sivagnanam et al., 2008), proliferation (Munz et al., 2004; Osta et al., 2004), and signal transduction (Chaves-Perez et al., 2013; Maetzel et al., 2009) to differentiation and stem cell pluripotency (Gonzalez et al., 2009; Huang et al., 2011; Lu et al., 2010, 2013; Ng et al., 2009; Sarrach et al., 2018). Induction of EpCAM-dependent proliferation and differentiation has been linked to regulated intramembrane proteolysis (RIP) of the molecule by alpha- and beta-sheddases and the gamma-secretase complex to generate an intracellular signaling moiety termed EpICD (EpCAM intracellular domain). The resulting EpICD domain can translocate into the nucleus and control the transcription of genes with functions in proliferation (cyclin D, c-Myc) and stem cell pluripotency (Oct3/4, Nanog, Sox2, and Klf4) (Chaves-Perez et al., 2013; Hsu et al., 2016; Huang et al., 2011; Kuan et al., 2017; Lu et al., 2010, 2011; Maetzel et al., 2009). The functions of EpICD can be controlled at various levels, including its initial cleavage and nuclear translocation (Denzel et al., 2009) and its degradation by the proteasome (Huang et al., 2019).

Orthologs of human EpCAM (hEpCAM) have been identified in silico in 52 different species including placental mammals, marsupials, fishes, reptiles, amphibians, and birds (Hachmeister et al., 2013).
Figure 1. SiLCAM- and mass spectrometry-based identification of potential interactors of murine EpCAM

(A) YFP (upper panels) and EpCAM-YFP (lower panels) were visualized by confocal laser scanning microscopy in stable mF9 cell transfectants expressing YFP (top panels) or EpCAM-YFP (lower panels) in the absence of any additional treatment. Nucleic DNA was visualized with Hoechst 33,342 (left panels). Shown are representative images. Scale bar represents 20 µm.
Sequence homologies suggest that EpCAM is a highly conserved protein throughout the animal kingdom. Accordingly, murine and human EpCAMs are 80% identical at the amino acid level (Bergsagel et al., 1992), and murine EpCAM was also reported to be subject to RIP, with similar cleavage patterns and proteases involved (Hachmeister et al., 2013; Tsaktanis et al., 2015). During physiological and pathological differentiation in ESCs and in carcinoma cells, respectively, EpCAM exhibits dynamic changes in expression levels and membrane localization. Upon differentiation, ESCs entirely downregulate EpCAM in the majority of cells (Gonzalez et al., 2009; Lu et al., 2010; Ng et al., 2009), a phenomenon equally observed in EpCAM-positive liver progenitor cells upon final differentiation to hepatocytes (Dolle et al., 2014; Schmelzer and Reid, 2008; Schmelzer et al., 2007). This tight regulation results in the formation of cellular patterning with EpCAM-negative mesodermal cells and EpCAM-positive endodermal cells in differentiating ESC and in the developing embryo (Guerra et al., 2012; Lei et al., 2012; McLaughlin et al., 1999, 2001; Nagao et al., 2009; Sarrach et al., 2018). An important function of EpCAM during embryogenesis and tissue morphogenesis lies in the regulation of cell-cell interactions through the modulation of adherens junctions and tight junctions and the cortical RhoA zone (Gaston et al., 2021; Guerra et al., 2012; Lei et al., 2012; Salomon et al., 2017). Genetic engineering of ESC demonstrated a requirement for an early spatiotemporal EpCAM patterning, the disturbance of which resulted in impaired differentiation along mesodermal and endodermal lineages (Sarrach et al., 2018). Epigenetic regulation of EpCAM at the transcriptional level was observed in 2D and 3D models of ESC differentiation, early embryonic stages, and in single-cell RNA sequencing of early murine gastrulation (Lu et al., 2010; Sarrach et al., 2018; Scialdone et al., 2016). These regulatory mechanisms include chromatin remodeling and histone modifications in the EPCAM promoter (Lu et al., 2010; Sarrach et al., 2018). Additional post-translational regulation of EpCAM availability at the plasma membrane is anticipated, considering a reportedly high protein stability of plasma membrane-localized EpCAM (half-life of 21 h) (Munz et al., 2008) and a delay in mRNA downregulation compared to protein loss (Sarrach et al., 2018). Similarly, in primary tumors and during metastasization, carcinoma cells are characterized by substantial molecular heterogeneity and undergo phenotypic changes along the epithelial-to-mesenchymal transition (EMT) (Thiery et al., 2009; Thiery and Lim, 2013; Ye and Weinberg, 2015), which are associated with frequent loss of EpCAM in circulating and disseminated tumor cells (Brown et al., 2021; Gires et al., 2020; Gorges et al., 2012; Keller et al., 2019; Liu et al., 2019). Besides transcriptional downregulation and RIP-mediated degradation of the protein at the plasma membrane, endocytosis and lysosomal degradation may account for the loss of EpCAM at the plasma membrane.

In the present study, we have performed a proteomic interactome screen to identify potential binding partners of EpCAM in mouse teratoma cells with the aim to delineate pathways involved in regulation of EpCAM protein dynamics. We describe an association of EpCAM with numerous proteins involved in vesicle and membrane trafficking and demonstrate endocytosis and membrane recycling of EpCAM under physiological conditions during differentiation of ESC and upon EMT induction in carcinoma cells.

RESULTS

Identification of murine EpCAM interaction clusters with vesicle transport, mitochondrial, and nuclear transport proteins

The murine teratoma cell line mF9 was stably transfected with expression plasmids for murine EpCAM in fusion with yellow fluorescent protein (YFP) at the C-terminus and with YFP, as a control. EpCAM-YFP was correctly localized at the plasma membrane, whereas YFP was homogeneously expressed throughout the cell (Figure 1A). Stable isotope labeling with amino acids in cell culture (SILAC) was performed with EpCAM-YFP and YFP mF9 cell lines upon labeling with $^{13}$C$_6$-$^{15}$N$_2$-L-lysine and $^{13}$C$_6$-$^{15}$N$_2$-L-arginine.
(Lys-8 and Arg-10; heavy; H) and normal amino acids (Lys-0 and Arg-0; light; L), respectively. Differentially labeled lysates were subjected to immunoprecipitation of YFP and associated proteins using GFP-Trap® agarose beads before identification through liquid chromatography tandem mass spectrometry (LC-MS/MS) and quantification of enrichment ratios from three biological repeats (Figure 1B). Potential interaction partners of EpCAM-YFP were selected based on (1) >3-fold enrichment versus YFP-associated proteins, (2) at least two unique peptides for quantification, and (3) p values ≤ 0.05 in all n = 3 independent biological repeats. A total of 78 proteins complied with these selection criteria including EpCAM (highest H/L ratio of 61.49; p value = 0.018). Table 1 summarizes all proteins considered significant with Ensemble ID, EpCAM-YFP/YFP ratios (H/L), numbers of unique peptides, gene and protein names, cellular localization, function or protein family, and p values. All additional raw data resulting from the screen are included in Data S1.

Interaction clusters within the interactome were analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database and the R-package igraph and were formed based on edge-betweeness clustering. This revealed major clusters associated with the Ras-superfamily small G-proteins of the Rab family, flotillins, integrins alpha 2 and beta 1 (collagen 1 receptor), prohibitins 1 and 2, and nuclear import/export proteins Xpo5 and CSE1L (Figure 1C).

To validate results from the SILAC screen, we selected high- and low-ranking interactors for confirmatory Co-IPs in mF9 cell lysates. Interactions of EpCAM-YFP with prohibitin 1 and 2, which were characterized by high enrichments scores (27.45 and 30.80, respectively), and calnexin, which was characterized by a lower Co-IPs in mF9 cell lysates. Interactions of EpCAM-YFP with prohibitin 1 and 2, which were characterized by slow-down the recycling of certain receptors, which may further help visualizing EpCAM protein in

To validate results from the SILAC screen, we selected high- and low-ranking interactors for confirmatory Co-IPs in mF9 cell lysates. Interactions of EpCAM-YFP with prohibitin 1 and 2, which were characterized by high enrichments scores (27.45 and 30.80, respectively), and calnexin, which was characterized by a lower enrichments score (4.68), were assessed. Co-precipitation of prohibitin 1, prohibitin 2, and calnexin with EpCAM-YFP but not YFP confirmed protein-protein interactions of EpCAM-YFP with two top-ranking and one low-ranking potential interactors (Figure 2A). Since EpCAM is also strongly expressed in pluripotent ESCs (Gonzalez et al., 2009), interaction of EpCAM-YFP with prohibitin 1 and 2 and calnexin was assessed, and enrichment was confirmed in E14TG2α ESCs (Figure 2B).

Classifications according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontology (GO) terms “Biological processes”, “Cellular components”, and “Molecular functions” were conducted to categorize all n = 77 potential interactors of EpCAM identified via SILAC-MS (Tables S1–S3). KEGG pathways and GO terms with the highest protein counts and false discovery rates (FDRs) below 0.05 were related to membrane protein trafficking, endoplasmic reticulum and Golgi apparatus, and mitochondria. The identified terms comprised potential interactors such as flotillin, VAMP8, VAPA and B and numerous Rab proteins that are central effector molecules in endocytosis (Figures 2C and 2D). Potential interactors (n = 59/77) including EpCAM were ranked according to their enrichment scores and implemented in a chord diagram showing the top 15 GO terms in “Cellular Components”, into which the potential interactors feed. GO terms confirmed the involvement of potential EpCAM interactors in protein trafficking and endocytosis processes, endoplasmic reticulum, Golgi apparatus, and mitochondria (Figure 2E).

**EpCAM is present in acidified intracellular vesicles**

Three major functional areas were identified from the classification of potential EpCAM interactors: interactions with components of the endoplasmatic reticulum, mitochondria, and vesicle-mediated intracellular transport. As EpCAM is a transmembrane protein, an association with components of the anterograde transport including the endoplasmatic reticulum is expected and was not further addressed. Prohibitin 1 and 2 are integral components of the inner mitochondrial membrane, suggesting a potential localization and function of EpCAM in mitochondria. Biochemical and imaging-based assessment of a possible localization of EpCAM in or at mitochondria was inconclusive and was therefore discontinued.

Potential EpCAM interactors that are specifically involved in vesicle-mediated transport and are components of an interaction network are depicted in Figure 3A. Rab proteins 1b, 5a, 5c, 7, 8a, 10, 11b, and 14 were part of this interaction network and have central roles in intracellular transport of cargo molecules. Based on the dynamic spatiotemporal regulation of EpCAM expression at the plasma membrane under normal and pathological conditions, we further concentrated on a retrograde transport of EpCAM and degradation in intracellular vesicles. In order to investigate internalization of EpCAM, mF9 cells stably transfected with EpCAM-YFP were treated with bafilomycin A1, an inhibitor of acidification and protein degradation in lysosomes (Yoshimori et al., 1991). Thereby, a potential endocytosis of EpCAM can occur but further degradation in lysosomes is prevented. Additionally, Bafilomycin A1 has been reported to slow-down the recycling of certain receptors, which may further help visualizing EpCAM protein in
Table 1. Putative mEpCAM-YFP interacting proteins identified through comparative quantitative SILAC proteomics of mEPCAM-YFP and YFP

| Ensembl ID             | Mean ratio EpCAM-YFP/YFP | Unique peptides | Gene     | Name                        | Localization       | Function/Family                       | p value |
|------------------------|--------------------------|-----------------|----------|-----------------------------|--------------------|----------------------------------------|---------|
| Ensembl: ENSMUSP00000061935 | 61.49                   | 15            | EPCAM    | mEpCAM                     | Cell membrane      | Cell adhesion molecule                 | 0.018   |
| Ensembl: ENSMUSP0000004375 | 30.80                   | 17            | PHB      | Prohibitin-2                | Mitochondria, cytosol, nucleus | Transcription co-regulator, mitochondrial chaperone | 0.01    |
| Ensembl: ENSMUSP00000030903 | 28.16                   | 15            | ATAD3A   | ATPase family AAA domain-containing protein 3 | Mitochondria | AAA domain ATPase | 0.0002  |
| Ensembl: ENSMUSP000000119603 | 27.45                   | 17            | PHB1     | PHB2                        | Cytosol, nucleus   | Transcription co-regulator             | 0.0045  |
| Ensembl: ENSMUSP00000034138 | 19.48                   | 14            | DNAJA2   | DNAJ homolog subfamily A member 2 | Cytosol, membrane | Co-chaperone of Hsc70                 | 0.012   |
| Ensembl: ENSMUSP00000022962 | 19.17                   | 4             | TTC35    | ER membrane protein complex subunit 2 (Emc2) | ER, cytosol, nucleus, mitochondria | ERAD, ER-mitochondria tethering | 0.034   |
| Ensembl: ENSMUSP00000020673 | 18.91                   | 19            | VDAC1    | Voltage-dependent anion-selective channel 1 | Outer mitochondrial/cell membranes | Ion channel                           | 0.032   |
| Ensembl: ENSMUSP00000069432 | 18.25                   | 61            | GCN1     | elf-2-alpha kinase activator | Cytosol, membrane | Chaperone of uncharged tRNAs          | 0.017   |
| Ensembl: ENSMUSP00000034326 | 17.20                   | 8             | ATP13A1  | Manganese-transporting ATPase 13A1 | ER membrane       | Cation-transporter                     | 0.025   |
| Ensembl: ENSMUSP00000044714 | 17.15                   | 9             | C330027C09 | Cancerous inhibitor of PP2A | Membrane, cytosol | Cadherin-mediated cell adhesion       | 0.024   |
| Ensembl: ENSMUSP00000059501 | 16.41                   | 3             | VAMP8    | Vesicle-associated membrane protein 8 | Lysosome/endosome/cell membranes | SNARE involved in autophagy          | 0.027   |
| Ensembl: ENSMUSP00000098349 | 16.14                   | 6             | FLOT2    | Flotillin-2                  | Cell membrane, endosome | Formation of caveolae                 | 0.029   |
| Ensembl: ENSMUSP00000079752 | 15.36                   | 6             | LRP22    | Low-density lipoprotein receptor-related 2 | Membrane          | HDL endocytosis                         | 0.016   |
| Ensembl: ENSMUSP00000051293 | 15.09                   | 6             | CLPTM1   | Cleft lip and palate transmembrane protein 1 | Membrane          | T cell differentiation                  | 0.013   |
| Ensembl: ENSMUSP00000073462 | 14.61                   | 5             | MON2     | Mon2                        | Cytosol           | Golgi-ER trafficking                   | 0.038   |
| Ensembl: ENSMUSP00000036198 | 14.53                   | 21            | AI314180 | Proteasome-associated protein ECM29 homolog | Cytosol           | Proteasome assembly                    | 0.005   |
| Ensembl: ENSMUSP00000030538 | 14.28                   | 8             | DDOST    | Dolichyl-diphosphooligosaccharide protein glycosyltransferase 4KBo subunit | ER | Essential subunit of the N-oligosaccharyl transferase complex | 0.044   |
| Ensembl: ENSMUSP0000001569 | 13.62                   | 6             | FLOT1    | Flotillin-1                  | Cell membrane, endosome | Formation of caveolae                 | 0.008   |
| Ensembl: ENSMUSP00000099470 | 12.25                   | 2             | CDS2     | Phosphatidate cytidylyltransferase 2 | Mitochondrial inner membrane | CDP-diacylglycerol provider          | 0.043   |

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| Ensembl ID                  | Mean ratio EpCAM-YFP/YFP | Unique peptides | Gene       | Name                                                                 | Localization                  | Function/Family                                      | p value |
|----------------------------|--------------------------|-----------------|------------|----------------------------------------------------------------------|-------------------------------|-----------------------------------------------------|---------|
| Ensembl: ENSMUSP00000072669 | 12.08                    | 3               | ARMC10     | Armadillo repeat-containing protein 10                               | ER                            | Suppressor of p53 transcriptional activity           | 0.005   |
| Ensembl: ENSMUSP00000097093 | 11.26                    | 3               | BTAF1      | TATA-binding protein-associated factor 172                          | Nucleus                       | ATPase                                              | 0.026   |
| Ensembl: ENSMUSP00000033131 | 10.72                    | 3               | 2400001E08Rik | Regulator complex protein LAMTOR1                                     | Endo/lysosome, cell membrane  | Amino acid sensing and mTORC1 activation            | 0.024   |
| Ensembl: ENSMUSP00000055206 | 10.54                    | 14              | DNAJC13    | DNAJ heat shock protein family (Hsp40) member C13                   | Cytosol, endosome, lysosome   | Chaperone, endosome organization                    | 0.021   |
| Ensembl: ENSMUSP00000064155 | 10.53                    | 11              | RIF1       | Telomere-associated protein Rif1                                     | Nucleus                       | DNA damage checkpoint                               | 0.032   |
| Ensembl: ENSMUSP0000021335 | 10.20                    | 4               | SCFD1      | Sec1 family domain-containing protein 1                             | Cytosol, ER membrane, Golgi apparatus | SNARE-pin assembly, ER transport                  | 0.03    |
| Ensembl: ENSMUSP00000432113 | 10.16                    | 2               | USMG5      | Up-regulated during skeletal muscle growth protein 5                | Mitochondrial membrane        | Maintenance of ATP synthase in mitochondria         | 0.00015 |
| Ensembl: ENSMUSP0000033509  | 10.00                    | 2               | EBP        | Emopamil binding protein                                            | ER and nuclear membrane       | Sterol isomerase                                    | 0.032   |
| Ensembl: ENSMUSP00000127504 | 9.23                     | 23              | L1TD1      | LINE-1 type transposase domain-containing protein 1                | Cytosol                       | RNA-binding                                         | 0.045   |
| Ensembl: ENSMUSP00000030202 | 9.11                     | 5               | GLIPR2     | Golgi-associated plant pathogenesis-related protein 2               | Golgi apparatus membrane     | EMT, ERK regulation, negative regulator of autophagy | 0.00045 |
| Ensembl: ENSMUSP0000030118  | 8.86                     | 17              | DNAJA1     | DnaJ homolog subfamily A member 1                                   | Cytosol, membrane             | Co-chaperone of Hsc70                               | 0.0058  |
| Ensembl: ENSMUSP0000024897  | 8.82                     | 9               | VAPA       | Vesicle-associated membrane protein-associated protein A           | ER, cell membrane             | Activation of RRs signaling, ER morphology and vesicle trafficking | 0.029   |
| Ensembl: ENSMUSP0000023486  | 8.77                     | 18              | TFRC       | Transferrin receptor protein 1                                      | Cell membrane                 | Iron uptake                                         | 0.028   |
| Ensembl: ENSMUSP0000043488  | 8.64                     | 9               | XPOT       | Exportin-T                                                          | Cytosol, nucleus              | Nuclear export of tRNAs                             | 0.015   |
| Ensembl: ENSMUSP0000055777  | 7.96                     | 5               | MLEC       | Malectin                                                             | ER membrane                   | N-glycosylation, Glc2-N-glycan binding protein      | 0.04    |
| Ensembl: ENSMUSP0000034400  | 7.57                     | 6               | CYB5B      | Cytochrome b5 type B                                                | Mitochondrion outer membrane | Electron carrier                                    | 0.018   |
| Ensembl: ENSMUSP0000030797  | 7.34                     | 5               | VAMP3      | Vesicle-associated membrane protein 3                               | Lysosome/endoosome/cell membranes | SNARE involved in endosome to trans-Golgi network | 0.01    |

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| Ensembl ID       | Mean ratio EpCAM-YFP/YFP | Unique peptides | Gene     | Name                          | Localization                        | Function/Family                                                                 | p value |
|------------------|--------------------------|----------------|----------|-------------------------------|-------------------------------------|--------------------------------------------------------------------------------|---------|
| Ensembl: ENSMUSP00000050336 | 7.34                     | 11             | CKAP4    | Cytoskeleton-associated protein 4 | Cytosol, ER, cell membrane          | Anchoring of ER to microtubules Dickkopf1 receptor involved in tumor progression | 0.031   |
| Ensembl: ENSMUSP00000072154 | 7.17                     | 2              | YES1     | Tyrosine protein kinase Yes    | Cell membrane, cytosol              | Non-receptor tyrosine kinase involved in cell growth, survival, apoptosis, cell-cell adhesion and differentiation | 0.0005  |
| Ensembl: ENSMUSP00000046714 | 6.66                     | 4              | SLC7A1   | High affinity cationic amino acid transporter 1 | Cell membrane                       | Amino acid transport                                                        | 0.044   |
| Ensembl: ENSMUSP00000005651 | 6.53                     | 6              | POR      | NADPH-cytochrome p450 reductase | ER membrane                         | Electron transfer from NADP to cyt P450                                        | 0.019   |
| Ensembl: ENSMUSP00000125504 | 6.36                     | 3              | ATP5J2   | ATP synthase subunit f         | Mitochondrion                        | Mitochondrial membrane ATP synthase                                           | 0.009   |
| Ensembl: ENSMUSP00000002790 | 6.10                     | 37             | CSE1L    | Exportin-2                    | Nucleus, cytosol                     | Export receptor for importin-alpha                                           | 0.014   |
| Ensembl: ENSMUSP00000044533 | 6.01                     | 5              | SCAND3   | Scan domain-containing protein 3 | Mitochondria, nucleoplasm           | Unknown                                                                         | 0.015   |
| Ensembl: ENSMUSP00000066238 | 5.94                     | 2              | RAP1B    | Ras-related protein 1b         | Cell membrane, cytosol              | GTPase involved in endothelial cell polarity and barrier function            | 0.0015  |
| Ensembl: ENSMUSP0000007959 | 5.86                     | 2              | RHOA     | Transforming protein RhoA      | Cell membrane, cytosol              | Focal adhesion assembly and signaling                                         | 0.019   |
| Ensembl: ENSMUSP00000025804 | 5.74                     | 5              | RAB1B    | Ras-related protein Rab-1B     | ER, Golgi apparatus, mitochondria   | Intracellular membrane trafficking and vesicular transport between ER and Golgi | 0.01    |
| Ensembl: ENSMUSP00000032946 | 5.72                     | 5              | RAB6A    | Ras-related protein Rab-6A     | Golgi apparatus                      | Intracellular membrane trafficking from Golgi to ER                          | 0.028   |
| Ensembl: ENSMUSP00000028238 | 5.64                     | 8              | RAB14    | Ras-related protein Rab-14     | Endosome, Golgi apparatus           | Intracellular membrane trafficking from Golgi to ER Regulation of endocytic transport of ADAM10, N-cadherin/CHD2 shedding and cell-cell adhesion | 0.02    |
| Ensembl: ENSMUSP00000043508 | 5.62                     | 15             | TNPO1    | Transportin 1                  | Cytosol, nucleus                     | Nuclear protein import                                                        | 0.031   |

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| Ensembl ID            | Mean ratio | EpCAM-YFP/YFP Unique peptides | Gene                  | Localization | Function/Family                                                                 | p value |
|----------------------|------------|-------------------------------|-----------------------|--------------|---------------------------------------------------------------------------------|---------|
| ENSMUSP00000020637   | 4.68       | 18                             | CANX                  | ER           | Calnexin                                                                          | 0.02    |
| ENSMUSP000000110021  | 4.63       | 11                             | RAB11B                | Recycling endosome membrane | Intracellular membrane trafficking and endocytic recycling | 0.0175  |
| ENSMUSP00000021001   | 4.59       | 2                              | RAB10                 | Golgi apparatus, endosome, cytoplasmic vesicles | Intracellular trafficking from Golgi to cell membrane | 0.003   |
| ENSMUSP00000020637   | 4.68       | 18                             | CANX                  | ER           | Calnexin                                                                          | 0.02    |
| ENSMUSP000000010000  | 4.63       | 11                             | RAB11B                | Recycling endosome membrane | Intracellular membrane trafficking and endocytic recycling | 0.0175  |
| ENSMUSP00000021001   | 4.59       | 2                              | RAB10                 | Golgi apparatus, endosome, cytoplasmic vesicles | Intracellular trafficking from Golgi to cell membrane | 0.003   |
| ENSMUSP0000000106656 | 4.55       | 2                              | GN2B                  | Cytosol      | Guanine nucleotide-binding protein subunit 2                                   | 0.0175  |
| ENSMUSP0000000107729 | 4.47       | 11                             | ITGA6                 | Cell membrane | Integrin alpha-6                                                                | 0.03    |
| ENSMUSP00000030398   | 4.37       | 7                              | SLC2A1                | Cell membrane | Solute carrier family 2, facilitated glucose transporter member 1               | 0.015   |
| ENSMUSP000000097303  | 4.37       | 13                             | CKAP5                 | Cytosol      | Cytoskeleton-associated protein 5                                                | 0.018   |
| ENSMUSP00000029815   | 4.18       | 5                              | CISO2                 | ER, mitochondria | CDGSH iron-sulfur domain-containing protein 2                                  | 0.0074  |
| ENSMUSP00000130194   | 4.17       | 17                             | SLC3A2                | Cell membrane | Solute carrier family 3 member 2                                                | 0.012   |

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| Ensembl ID                  | Mean ratio EpCAM-YFP/YFP | Unique peptides | Gene       | Name                  | Localization                      | Function/Family                                                                 | p value |
|-----------------------------|---------------------------|-----------------|------------|-----------------------|-----------------------------------|--------------------------------------------------------------------------------|---------|
| Ensembl: ENSMUSP00000124480 | 4.17                      | 4               | CYB5       | Cytochrome b5         | ER                                | Electron carrier                  | 0.0085  |
| Ensembl: ENSMUSP00000107323 | 4.13                      | 4               | CTNND1     | Catenin delta-1       | Cytosol, nucleus, cell membrane   | WNT signaling                      | 0.0289  |
|                            |                           |                 |            |                       |                                   | Cadherin-mediated adhesion EGF-R/PDGFR-CSF-1R/ERBB2 signaling                  |         |
| Ensembl: ENSMUSP0000009003  | 4.13                      | 3               | RALA       | Ras-related protein Ral-a | Cell membrane                     | Multifunctional GTPase involved in GTP-dependent exocytosis and signaling     | 0.0013  |
| Ensembl: ENSMUSP000017975   | 4.13                      | 3               | RAB5A      | Ras-related protein Rab-5a | Cell membrane, endosome, cytosol | Intracellular trafficking from cell membrane to early endosomes               | 0.036   |
| Ensembl: ENSMUSP000021471   | 4.12                      | 2               | TMX1       | Thioredoxin-related transmembrane protein 1 | ER membrane                      | Redox-reaction                     | 0.001   |
| Ensembl: ENSMUSP000033500   | 4.12                      | 6               | ERAS       | Embryonic Ras         | Cell membrane                     | GTPase involved in embryonic stem cell teratogenesis                         | 0.0289  |
| Ensembl: ENSMUSP000096232   | 4.08                      | 4               | RAB8A      | Ras-related protein Rab8a | Cell membrane, Golgi apparatus, endosome | Intracellular trafficking, exocytosis, polarized vesicular trafficking | 0.043   |
| Ensembl: ENSMUSP000087457   | 4.04                      | 9               | ITGB1      | Integrin beta-1       | Cell membrane                     | Integrin-mediated adhesion interacts with Integrin alpha-6 as a receptor for laminin | 0.0039  |
| Ensembl: ENSMUSP0000000001  | 3.85                      | 3               | GNAI3      | Guanine nucleotide-binding protein G 8K subunit alpha | Cytosol, cell membrane | G-protein coupled signaling | 0.029   |
| Ensembl: ENSMUSP000032476   | 3.59                      | 10              | SLC2A3     | Solute carrier family 2, facilitated glucose transporter member 3 | Cell membrane | Glucose transporter | 0.0038  |
| Ensembl: ENSMUSP000127808   | 3.24                      | 7               | RP24-421P3.2 | Unknown predicted protein | unknown | unknown | 0.012   |
| Ensembl: ENSMUSP000096073   | 3.24                      | 28              | CNOT1      | CCR4-NOT transcription complex subunit 1 | Cytosol, P-body, nucleus | mRNA deadenylation | 0.034   |
| Ensembl: ENSMUSP000025250   | 3.06                      | 8               | BAG6       | Bcl2-associated anthanogene 6 | Cytosol, nucleus | Chaperone | 0.0039  |

Criteria: EpCAM-YFP/YFP ratio >3, p value < 0.05.
intracellular vesicles (Johnson et al., 1993). Treatment with bafilomycin A1 (10 nM) resulted in accumulation of EpCAM-YFP in intracellular vesicles suggesting that EpCAM is endocytosed and degraded in acidic intracellular vesicles (Figure 3B). As described earlier, EpCAM is post-translationally processed via RIP yielding a soluble intracellular domain EpICD via the intermediate of a membrane-tethered C-terminal fragment (Maetzel et al., 2009) (see scheme in Figure S1A). EpICD-YFP was stably expressed in mF9 cells and localized in the cytoplasm and nucleus of control-treated cells (DMSO; Figure 3B). Neither the treatment of EpICD-YFP nor of control YFP-expressing cells resulted in any apparent accumulation in intracellular vesicles (Figure 3B). Next, a pre-cleaved version of EpCAM composed of a 15-amino acids short membrane-proximal portion of the extracellular domain, the transmembrane domain, and the intracellular domain fused to YFP corresponding to the EpCAM C-terminal fragment (mEp-CTF; Figure S1A) was stably expressed in mF9 cells. Cleavage of mEp-CTF by the gamma-secretase complex was inhibited using DAPT, resulting in a stabilization of mEp-CTF at the plasma membrane (Huang et al., 2019). Additional treatment of the cells with bafilomycin A1 resulted in an accumulation of mEp-CTF in intracellular vesicles, demonstrating that membrane tethering is a prerequisite for the endocytosis and degradation of EpCAM in acidified vesicles and suggesting that the internalization motif of EpCAM is located within its C-terminal fragment (Figure S1B).

Quantification of YFP fluorescence by flow cytometry, as a measure of the expression level of EpCAM-YFP, Ep-CTF-YFP, EpICD-YFP, or YFP, exhibited a significant 2.14-fold increase in YFP intensities after bafilomycin A1 treatment of EpCAM-YFP cells but no effect on EpICD-YFP and YFP expression levels (Figure 3C). Quantification of YFP intensities of Ep-CTF-YFP cells showed a 2.8-fold increase of DAPT and bafilomycin A1 treatment of EpCAM-YFP and YFP stable transfectants of mF9 and E14TG2a murine embryonic stem cells (B). Phb1, Phb2, and Caln levels were comparable in whole-cell lysates of EpCAM-YFP and YFP stable transfectants of mF9 and E14TG2a. Amounts of EpCAM-YFP and YFP following immunoprecipitation with GFP-Trap® agarose beads were controlled with GFP-specific antibodies and were comparable. Shown are each one representative immunoblot from three independent experiments.

Endocytosis and membrane recycling of EpCAM
To assess the presence of EpCAM in early, late, and recycling endosomes, EpCAM-YFP-expressing mF9 cells were transiently transfected with mCherry-tagged versions of Rab5, Rab7, and Rab11. Localization of EpCAM-YFP within intracellular vesicles was confirmed by laser scanning confocal fluorescence microscopy (Figure 4A). Localization of EpCAM-YFP in lysosomes/acidic compartments was confirmed by co-staining with lysotracker (Figures 4A and 4B). Co-localization of EpCAM-YFP with mCherry-tagged versions of Rab5, Rab7, and Rab11 and with lysosomes was quantified using Manders’ coefficients in bafilomycin A1-treated mF9 cells compared to DMSO-treated cells. Accumulation of EpCAM-YFP in intracellular vesicles upon bafilomycin A1 treatment was associated with increased Manders’ coefficients, representing an increased fraction of all three Rab proteins and lysosomes overlapping with EpCAM-YFP (Figure 4B). Co-localization of EpCAM with Rab5, Rab7, and Rab11 was confirmed by immunofluorescence staining of endogenous proteins (Figure S2). Interaction of EpCAM with Rab5, Rab7 and Rab11 was further investigated through co-immunoprecipitation experiments in stably EpCAM-YFP transfected mF9 cells. EpCAM-YFP but not YFP co-precipitated with Rab5, Rab7, and Rab11 following immunoprecipitations of whole-cell lysates with GFP-Trap® agarose beads (Figure 4C).

Endocytosis of endogenous EpCAM in untreated mF9 cells was addressed with an antibody-based internalization and membrane recycling assay (see STAR Methods). The assay relies on labeling of EpCAM on the plasma membrane with an EpCAM Alexa 488-conjugated antibody and subsequent quenching of the remaining cell surface fluorescence after internalization of EpCAM with an anti-Alexa 488 antibody (Figure 4D, steps 1–3). Samples were assessed every 5 min and demonstrated increasing endocytosis of
Figure 3. EpCAM localizes to intracellular vesicles

(A) Spherical network of SILAC potential interactors of EpCAM found in the GO BP term “vesicle-mediated transport”. Degree of interactions encodes node size. Color shows the enrichment ratio from low (blue) to high (red). Arrow indicates direction of interaction.
EpCAM that reached a plateau at approx. 20 min with a mean percentage of endocytosed molecules of 8.25% (Figure 4E, left panel). Additionally, membrane localization of stained endogenous EpCAM, quenching, and endocytosis were confirmed by confocal imaging in samples of mF9 cells subjected to the internalization assay (Figure S3A). Membrane recycling of EpCAM was assessed by allowing cells to recycle EpCAM to the membrane after initial internalization, followed by quenching of the remaining cell surface fluorescence with anti-Alexa 488 antibody. The amount of recycled EpCAM was calculated as a percentage of endocytosed EpCAM (Figure 4D, steps 4 and 5). A recycling rate of 59.93% was determined for endogenous EpCAM in mF9 cells (Figure 4E, right panel). Hence, EpCAM localizes in Rab5-, Rab7-, and Rab11-associated early, late, and recycling endosomes and is subject to endocytosis and membrane recycling.

Enhanced EpCAM endocytosis in mesodermal differentiation of ESC and EMT of carcinoma cells

The expression of EpCAM is tightly regulated and relates to the tissue of origin. Both, in vitro and in vivo, EpCAM expression is repressed in pluripotent ESCs undergoing mesodermal differentiation while it is retained in endodermal tissue (Sarrach et al., 2018). In mouse embryonic development, loss of EpCAM is observed at the single-cell level during initial steps of gastrulation with the emergence of EpCAM-low/negative early mesodermal progenitors (Sarrach et al., 2018; Scialdone et al., 2016).

To address whether endocytosis is instrumental in the withdrawal of EpCAM from the plasma membrane in differentiating cells, murine pluripotent ESCs were subjected to a guided mesodermal differentiation protocol (See STAR Methods). EpCAM expression at the plasma membrane was analyzed by flow cytometry under pluripotency (day 0) and upon finalization of the mesodermal differentiation protocol (day 5). EpCAM expression at the plasma membrane was reduced to 57.16% on average, which translates to a reduction in mean fluorescence intensity ratio from an average of 51.68 to 29.72 (Figure 5A, left panel and Figure 5B). In parallel, internalization and membrane recycling of EpCAM were monitored according to the protocol depicted in Figure 4C. Membrane localization, quenching, and endocytosis were confirmed by confocal imaging in E14TG2a ESC (Figure S3B). Upon guided mesodermal differentiation, endocytosis of EpCAM increased from an average of 10.68% under pluripotency to 19.38% following mesodermal differentiation (Figure 5A, middle panel). Recycling of EpCAM to the plasma membrane was entirely blocked from 24.81% under pluripotency to −12.21% following mesodermal differentiation (Figure 5D, right panel). Negative recycling values most likely reflected residual ongoing internalization of EpCAM during recycling steps. Mesodermal differentiation was confirmed by the loss of pluripotency markers Sox2, Oct3/4, and Nanog and the induction of the cardiomyocyte marker α-cardiac actin (α-CAA) and the mesodermal marker vimentin (Figure 5C).

Next, EMT was induced in the EpCAM-positive esophageal carcinoma cell line Kyse30 upon treatment with TGFβ. EpCAM expression was reduced following TGFβ treatment with the MFI-R decreasing from an average of 251.75 to 144.25 (Figure 5D, left panel). In parallel, endocytosis rates were increased from an average of 8.3%–15.05%, and recycling rates were moderately decreased from an average of 57.98%–48.9% (Figure 5D, middle and right panels). EMT induction via TGFβ was confirmed by the enhanced expression of EMT transcription factors ZEB1, ZEB2, SNAI1 (Snail), and SNAI2 (Slug) (Figure 5E).

Hence, EpCAM downregulation during mesodermal differentiation in ESCs and during EMT in carcinoma cells is linked to enhanced endocytosis and a block or reduction of its recycling to the plasma membrane.

DISCUSSION

EpCAM displays tissue selectivity with dynamic changes in expression strength and patterning. Spatiotemporal cell-specific expression of EpCAM is best exemplified in differentiating ESCs and in malignant cells during cancer progression. Pluripotent ESCs express high levels of EpCAM that are specifically suppressed during the differentiation to mesodermal lineages, whereas endodermal cells and epithelia retain EpCAM.
**A**

|          | DMSO | Baflomycin |
|----------|------|------------|
| EpCAM-YFP| ![Image](image1) | ![Image](image2) |
| Rab5-mCherry | ![Image](image3) | ![Image](image4) |
| Rab7-mCherry | ![Image](image5) | ![Image](image6) |
| Rab11-mCherry | ![Image](image7) | ![Image](image8) |
| Lysotracker | ![Image](image9) | ![Image](image10) |
| **Merge** | ![Image](image11) | ![Image](image12) |

**B**

- **Rab 5**
  - Manders' coefficient (Rab5:EpCAM)
  - Control vs. Baflomycin

- **Rab 7**
  - Manders' coefficient (Rab7:EpCAM)
  - Control vs. Baflomycin

- **Rab 11**
  - Manders' coefficient (Rab11:EpCAM)
  - Control vs. Baflomycin

**C**

- **mF9**
  - IB: Rab5
  - IP: YFP
  - IB: Rab7
  - IP: YFP
  - IB: Rab11
  - IP: YFP

**D**

1. Surface staining of EpCAM at 4°C for 1h
2. Endocytosis of labeled EpCAM at 37°C for 30 min
3. Quenching of surface signal at 4°C for 1h
4. Recycling of endocytosed EpCAM back to surface at 37°C for 30 min
5. Re-quenching of surface signal at 4°C for 1h

**Endocytosis assay**

**Endocytosis - recycling assay**

**E**

- **Endocytosed EpCAM (%)**
  - Time (min)
  - One-way ANOVA: p < 0.0001

- **Recycled EpCAM (%)**
  - Time (min)
  - One-way ANOVA: p < 0.0001
Figure 4. Endocytosis of EpCAM in mF9 teratoma cells

(A) mF9 cells stably transfected with EpCAM-YFP and transiently transfected with m-Cherry-tagged Rab5, Rab7, and Rab11 were visualized by laser scanning confocal microscopy. Lysosomes were detected with Lysotracker (Red DND-99 Ex577/Em590 nm). Cells were treated with either DMSO or 10 nM bafilomycin A1 (10 nM) as indicated. Scale bars represent: Rab5 5 μm, Rab7 and Rab11 20 μm (DMSO) and 5 μm (Bafilomycin); Lysotracker: 20 μm. Brightness and contrast of both channels were adjusted linearly.

(B) Co-localizations of EpCAM-YFP with the indicated m-Cherry-tagged Rab proteins and lysosomes were assessed as indicated in STAR Methods. Shown are Manders’ coefficients representing the fraction of m-Cherry-tagged Rab protein or lysotracker overlapping with EpCAM-YFP as dot plots with mean and SD from each n = 7 and n = 2–3 independent imaging areas for bafilomycin A1-treated cells and DMSO-treated cells, respectively.

(C) Whole-cell lysates from mF9 cells stably transfected with EpCAM-YFP or YFP were immunoprecipitated with GFP-Trap agarose beads. Immunoprecipitated proteins were separated by SDS-PAGE and detected with anti-YFP, anti-Rab5, anti-Rab7, and anti-Rab11 antibodies in combination with HRP-conjugated secondary antibodies. Additionally, whole-cell lysates were separated by SDS-PAGE, and Rab5, Rab7, and Rab11 proteins were detected with specific antibodies in combination with HRP-conjugated secondary antibodies. Shown are representative results from three independent experiments.

(D) Schematic representation of the endocytosis and membrane recycling assay.

(E) Kinetics of EpCAM endocytosis (left panel) and membrane recycling (right panel). Shown are mean percentages of EpCAM endocytosis and membrane recycling with SD over a time of 30 min from three independent experiments. One-way ANOVA with Dunnet’s multiple tests. **** p value < 0.0001.

expression (Gonzalez et al., 2009; Lu et al., 2010; Ng et al., 2009; Sarrach et al., 2018). Earliest stages of mesodermal commitment are accompanied by a complete loss of human EpCAM to generate EpCAM+/CD66+ mesodermal progenitors with the potency to differentiate into hematopoietic, endothelial, mesenchymal, muscle and cardiomyocyte cells (Evseenko et al., 2010). In mice, EpCAM expression is repressed at mesodermal commitment are accompanied by a complete loss of human EpCAM to generate EpCAM expression (Gonzalez et al., 2009; Lu et al., 2010; Ng et al., 2009; Sarrach et al., 2018). Earliest stages of gastrulation in mesodermal progenitors starting at day E7.0 (Sarrach et al., 2018), which is accompanied by a gain of mesodermal markers such as vimentin in human and mouse (Evseenko et al., 2010; Sarrach et al., 2018). Primary carcinomas, metastases, and cancer stem cells express high levels of EpCAM too (Baeuerle and Gires, 2007; Gires et al., 2009; Went et al., 2004), whereas circulating and disseminated tumor cells (CTCs/DTCs) display heterogeneous EpCAM expression with frequent loss during EMT (Brown et al., 2021; Gires and Stoecklein, 2014; Gorges et al., 2012; Keller et al., 2019; Liu et al., 2019). Single-cell analyses of carcinomas of the oral cavity have disclosed a high level of molecular heterogeneity and have identified a subset of cells of primary tumors in a state of partial EMT with a gradual loss of epithelial differentiation (Puram et al., 2017, 2018). EpCAM expression was identified as the major characteristic of retained epithelial differentiation of carcinoma cells (Puram et al., 2017), and loss of EpCAM expression at the edges of tumor areas was frequently accompanied by expression of the mesenchymal marker vimentin (Baumeister et al., 2018). Hence, partial EMT is a central feature of tumor progression that is controlled by tumor-intrinsic programs and cues from the tumor microenvironment (Aggarwal et al., 2021; Ciriello and Magnani, 2021).

However, most studies concentrated on the expression of EpCAM at the transcriptional level via epigenetic changes (Lu et al., 2010; Sarrach et al., 2018). The present data shed light on a network of proteins involved in post-translational regulation of EpCAM and its availability at the plasma membrane. Numerous potential EpCAM interactors identified in the present study are instrumental in vesicle-mediated membrane trafficking, including several members of the GTPase family of Rab proteins that are active throughout anterograde and retrograde trafficking of membrane proteins (Figure 6), and are essential in the regulation of cell polarity and migration. Recently, Gaston et al. have shown a central role for EpCAM in remodeling of membranous areas exhibiting enhanced RhoA activity in migrating epithelial cells. Cell polarization, stress fiber formation, and myosin-II activity depend on an EpCAM-mediated spatial distribution of RhoA at the single-cell level through common endosomal trafficking and recycling (Gaston et al., 2021). Our findings further support this reported interaction of EpCAM with RhoA.

EpCAM interactor Rab5c is instrumental in Wnt11-dependent regulation of E-cadherin endocytosis in zebrafish gastrulation to influence mesoenoderm cohesion (Ulrich et al., 2005). Proper development of epithelia (derived from endoderm) in zebrafish depends on EpCAM through an interaction with E-cadherin and the formation of adherens junctions (Slanchev et al., 2009). Furthermore, Rab5c is involved in recycling of integrin beta-1, a potential interactor of EpCAM, which is important for invasiveness of breast cancer cells (Onodera et al., 2012). Regulation of the connection of EpCAM, integrin beta-1, and E-cadherin to Rab5c and additional Rab molecules could represent a means of cooperatively controlling the turnover of these proteins to regulate cell adhesion, segregation, and motility in ESC differentiation and metastasis formation.
Figure 5. Endocytosis of EpCAM during mesodermal differentiation of ESC

(A) Left: EpCAM expression in E14TG2α ESC under pluripotency (day 0, D0) and following mesodermal differentiation (see STAR Methods) was analyzed with Alexa-488-labeled specific antibody. Shown are scatter dot plots with means and SD of n = 3 independent experiments performed in duplicates. Middle and right: EpCAM endocytosis (middle) and membrane recycling (right) was assessed in E14TG2α ESC under pluripotency (day 0, D0) and following mesodermal differentiation. Shown are scatter dot plots with means and SD of n = 3 independent experiments performed in duplicates. Student’s t test; ** 0.01, *** 0.001, **** 0.0001.

(B) EpCAM expression in E14TG2α ESC under pluripotency (day 0, D0) and following mesodermal differentiation was analyzed with Alexa-488-labeled specific antibody. Shown are representative examples of unstained pluripotent ESCs and stained pluripotent (D0) and mesodermally differentiated ESCs (D5) in gated dot plots from n = 3 independent experiments performed in duplicates.

(C) Expression of pluripotency markers Sox2, Oct3/4 and Nanog, and mesodermal markers α-CAA and vimentin was quantified by qRT-PCR in E14TG2α ESC under pluripotency (day 0, D0) and following mesodermal differentiation. Shown are scatter dot plots with means and SD of n = 3 independent experiments performed in triplicates. Student’s t test is indicated. ** 0.01; **** 0.0001.

(D) Left: EpCAM expression in Kyse30 carcinoma cells under control (Ctrl.) and following TGFβ treatment (TGFβ) (see STAR Methods) was analyzed with Alexa-488-labeled specific antibody. Shown are scatter dot plots with means and SD of n = 3 independent experiments performed in duplicates. Middle and right: EpCAM endocytosis (middle) and membrane recycling (right) was assessed in Kyse30 cells under control (Ctrl.) and following TGFβ treatment (TGFβ). Shown are scatter dot plots with means and SD of n = 3 independent experiments performed in duplicates. Student’s t test; ** 0.01, **** 0.0001.

(E) The mRNA expression of EMT transcription factors ZEB1/2, SNAI1/2, and TWIST was quantified by qRT-PCR in Kyse30 cells under control (Ctrl.) and following TGFβ treatment (TGFβ). Shown are scatter dot plots with means and SD of n = 3 independent experiments performed in triplicates. Student’s t test; **** 0.0001, n.s. not significant.
Accordingly, reduction of hEpCAM was observed in carcinoma cells that adopted a migratory and invasive phenotype (Driemel et al., 2014; Tsaktanis et al., 2015). Despite a distinctive expression pattern of EpCAM during EMT in normal differentiation with an exclusion in mesodermal cells, the actual role of EpCAM in EMT in cancer remains controversially discussed. Both, activating and inhibitory functions of EpCAM in EMT have been described (Gao et al., 2015; Pan et al., 2018; Sankpal et al., 2009, 2011, 2017). Here, we demonstrate a partial loss of EpCAM expression upon mesodermal differentiation of ESC and following induction of EMT by TGFβ in esophageal carcinoma cells. These changes were accompanied by increased endocytosis and reduced recycling of EpCAM. Recently, Wu et al. reported on a matriptase-dependent di-basic cleavage of EpCAM that destabilizes its interaction with the tight junction protein Claudin-7, resulting in endocytosis and lysosomal degradation of EpCAM (Wu et al., 2017). EpCAM interactor Rab14 might additionally be involved in EpCAM turnover through targeting of the ADAM10 protease to the plasma membrane (Linford et al., 2012). ADAM10 is a reported interaction partner of EpCAM (Le Naour et al., 2006) that contributes to the initial cleavage of EpCAM during regulated intramembrane proteolysis (RIP) (Maetzel et al., 2009). We conclude that endocytosis and membrane recycling of EpCAM are post-translational means for the regulation of its availability
and, thereby, its function at the plasma membrane during normal and pathologic differentiation (Figure 6).

Lastly, interactions with a wide variety of mitochondrial and nuclear import/export proteins suggest an alternative localization of EpCAM in mitochondria and the nucleus. In fact, top three ranking potential interactors were mitochondrial proteins (prohibitin 1/2 and ATAD3A), and thirteen out of seventy-seven proteins interacting with EpCAM are located in mitochondrial membranes. Although experimental proof is currently lacking, it is tempting to speculate that EpCAM might play a role at the interface of mitochondria and the ER, as was reported for the amyloid precursor protein (APP). APP is present in mitochondria-associated membranes (MAMs), where it becomes cleaved by BACE-1 and the gamma-secretase complex (Del Prete et al., 2017). A connection of EpCAM with the endoplasmic reticulum (ER) in form of an interaction with the ER aminopeptidase 2 (ERAP2) has been reported (Gadalla et al., 2013), although the actual function of this interaction remained unexplored. Proteins involved in nuclear import and export of cargos such as TNPO1 and CSE1L may hint toward a possible nuclear translocation of EpCAM. Co-precipitation of EpCAM with TNPO1 and CSE1L was confirmed in mF9 cells, and EpCAM could be detected in nuclear extracts and by confocal imaging of mF9 and Kyse30 cells (data not shown). However, based on technical drawbacks regarding the contamination of nuclear fraction with membranous components and a current lack of function, final conclusions on a nuclear localization of EpCAM are not feasible.

In summary, the present results provide an insight into the regulation of EpCAM expression during mesodermal differentiation and EMT. Additionally, the findings are a valuable platform for future studies on alternative localizations and functions of EpCAM, which might explain its numerous roles in various cell types.

**Limitations of the study**

Limitations of the present study must be considered since combination of immunoprecipitation and SILAC approach failed to enrich α- and β-sheffdas, components of the γ-secretase complex, claudins, and intracellular ligands reported for human EpCAM such as FHL2 and β-catenin. Technical limitations related to protein amounts as well as a possibly transient interaction of EpCAM with these proteins may account for the lack of enrichment. Finally, the limited number of different cellular states that can be analyzed via SILAC and issues regarding data analysis based on a potential proline-to-arginine conversion in cells may further impact on how comprehensive the analysis of EpCAM interactors eventually is.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103179.
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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| GFP-Trap® agarose beads | ChromoTek, Planegg-Martinsried, Germany | ChromoTek Cat# gta-20, RRID:AB_2631357 |
| Antibodies          |        |            |
| Anti-GFP            | Santa Cruz, USA | Santa Cruz Biotechnology Cat# sc-9996, RRID:AB_627695 |
| Anti-Prohibitin 1   | Santa Cruz, USA | Santa Cruz Biotechnology Cat# sc-28259, RRID:AB_2164486 |
| Anti-Prohibitin 2   | Santa Cruz, USA | Santa Cruz Biotechnology Cat# sc-67045, RRID:AB_2283865 |
| Anti-Calnexin       | Enzo, USA | Enzo Life Sciences Cat# ADI-SPA-860, RRID:AB_10616095 |
| Anti-Rab5           | Cell Signaling, USA | Cell Signaling Technology Cat# 46449, RRID:AB_2799303 |
| Anti-Rab7           | Cell Signaling, USA | Cell Signaling Technology Cat# 9367, RRID:AB_1904103 |
| Anti-Rab11          | Cell Signaling, USA | Cell Signaling Technology Cat# 5589, RRID:AB_10693925 |
| Anti-murine EpCAM-Alexa-488 | Abcam, Cambridge UK | Cat# ab237384 |
| Anti-human EpCAM-Alexa-488 | Abcam, Cambridge UK | Cat# ab237395 |
| Anti-Alexa-488      | Thermo Fisher Scientific, USA | Thermo Fisher Scientific Cat# A-11094, RRID:AB_221544 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Lysotracker         | Thermo Fisher Scientific, USA | Cat# L7528 |
| Mouse ES cell basal medium | ATCC, LGC Standards GmbH, Germany | Cat# SCRR-2011 |
| Embryonic stem cell grade FBS | Bio&SELL GmbH, Germany | Cat# FBS-E |
| **Critical commercial assays** |        |            |
| PowerUp SYBR Green Master Mix | Applied Biosystems, USA | Cat# A25742 |
| **Experimental models: cell lines** |        |            |
| Murine F9           | ATCC, USA (Prof. Marcus Conrad) | ATCC Cat# CRL-1720, RRID:CVCL_0259 |
| E14TG2a             | ATCC, USA | ATCC Cat# CRL-1821, RRID:CVCL_9108 |
| Kyse30              | DSMZ, Germany | DSMZ Cat# ACC-351, RRID:CVCL_1351 |
| **Oligonucleotides** |        |            |
| Murine GUSB qPCR primers | Metabion, Germany | Forward: CAACCTCTGGTGCTTACC |
|                      |        | Re reverse: GGGTGTAGTGTACGTACAGAC |
| Murine Sox2 qPCR primers | Metabion, Germany | Forward: GACAGCTACGGCGACATGA |
|                      |        | Reverse: GGGTCTTCGGTTGACATCTG |
| Murine Oct3/4 qPCR primers | Metabion, Germany | Forward: CGGAAAGAAAGCAGACTAGC |
|                      |        | Reverse: ATGGCGATGTGACTGATCTG |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Murine Metabion, Germany | Forward: TCTTCCTGGTCCCCACAGTTT | Reverse: GCAAGAATAGTTCTCGGGATCAA |
| Murine α-CAA qPCR primers Metabion, Germany | Forward: CTGGATTCTGGCGATGGTGTA | Reverse: CGGACAATTTCACGTTTACGCA |
| Murine Vimentin qPCR primer Metabion, Germany | Forward: ACCGGAGCTATCTGACCAGC | Reverse: CAAGGATCCAGTTTCCGTTCA |
| Human GAPDH Metabion, Germany | Forward: AAGTCCGAGTCACAAGGATTT | Reverse: TAGTTGAGGTCAATGAAGGG |
| Human ZEB1 Metabion, Germany | Forward: TTACACCTTTGCATACAGAACCC | Reverse: TTACGATTACACCCAGACTGC |
| Human ZEB2 Metabion, Germany | Forward: GGAGACGAGTCCAGCTAGTGT | Reverse: CCACCTCCACACCTCCCTATTTC |
| Human SNAI1 Metabion, Germany | Forward: AGATGAGCATTGGCAGCGAG | Reverse: TGGGAAGCCTAACTACAGCGA |
| Human SNAI2 Metabion, Germany | Forward: CGAACTGGACACACATACAGTG | Reverse: CTGAGGATCTCTGGTTGTGGT |
| Human TWIST1 Metabion, Germany | Forward: GCTTGAGGGTCTGAATCTTGCT | Reverse: GTCCGCAGTCTTACGAGAG |

### Recombinant DNA

| pCAG-3SIP vector | pCAG vector modified to include 3x Stop in all three reading frames, encephalomyocarditis IRES, and globin polyA tail. Kind gift from Prof. Marcus Conrad (HMGU, Munich, Germany) | https://edoc.ub.uni-muenchen.de/9432/1/Seiler_Alexander.pdf |
|------------------|----------------------------------------------------------------------------------|----------------------|
| pCAG-EpCAM-YFP   | Cloned by insertion of PCR amplified EpCAM-YFP into EcoR1 cut pCAG-3SIP vector | N/A                  |
| pCAG-EpCTF-YFP   | Cloned by insertion of PCR amplified Ep-CTF-YFP into EcoR1 cut pCAG-3SIP vector | N/A                  |
| pCAG-EpICD-YFP   | Cloned by insertion of PCR amplified EpICD-YFP into EcoR1 cut pCAG-3SIP vector | N/A                  |
| pCAG-YFP         | Cloned by insertion of PCR amplified YFP into EcoR1 cut pCAG-3SIP vector | N/A                  |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Olivier Gires (Olivier.gires@med.uni-muenchen.de).

Materials availability
This study did not generate new unique reagents.

Data and code availability

Data
Raw, processed, and analyzed data from SILAC experiments are provided as a supplementary Excel file termed Data S1. All results are summarized to include protein accession numbers, numbers of peptides, oxidation, MWs, ratios and normalized ratios of heavy and light amino acid-marked peptides, intensities, significance values (normalized t-test), protein functions and clustering.

Code
This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
Murine F9 (mF9; male) cells were cultured in Dulbecco’s modified Eagle medium (DMEM, high glucose) in the presence of 20% FCS (Biochrom AG, Heidelberg, Germany). Pluripotency of E14TG2a cells (male) was achieved through culture on gelatin-treated culture plates (0.1%, InScreeEx, Braunschweig, Germany) in Mouse ES Cell Basal Medium (ATCC, SCRR-2011, LGC Standards GmbH, Germany) supplemented with 1,000U/mL leukemia inducing factor ESGRO LIF (Merck, Darmstadt, Germany), 10% embryonic stem cell grade FBS (FBS-E, Bio&SELL GmbH, Germany) and 0.1nM 2-mercaptoethanol (Thermo Fischer Scientific, MA, USA). For all experiments including E14TG2a ESC, cells in passages below 60, with a colony-forming morphology, and high expression of pluripotency markers Oct3/4, Sox, and Nanog were used. Kyse30 carcinoma cells (male) were obtained from DSMZ and were confirmed by STR typing using ten markers (AMEL, CSF1PO, D13S317, D16S539, D21S11, D5S818, D7S820, TH01, TPOX, vWA). Kyse30 cells were maintained in RPMI 1640, 10% FCS, 1% penicillin/streptomycin, in a 5% CO2 atmosphere at 37°C.
**METHOD DETAILS**

**Transfections, vectors, flow cytometry**

All expression plasmids are based on the 141pCAG-3SIP vector. Using EcoR1 and Nhe1 sites the following coding sequences were inserted following PCR amplification: yellow fluorescence protein (YFP), full-length murine EpCAM (314 aa) fused to YFP (C-terminal), murine EpCAM C-terminal fragment consisting of the signal peptide of EpCAM (aa 1–23) a short linker (lysin, leucin), a Myc tag, and the C-terminal fragment (aa 251–314) fused to YFP (C-terminal), and the EpCAM intracellular domain (26 aa) fused to YFP (Hachmeister et al., 2013). Transfection of expression vectors was conducted with MATra reagent (Iba, Goettingen, Germany) (mF9) or the Amaxa nucleofector kit (Lonza, Ratingen, Germany) (E14TG2a cells). Stable expression was achieved through selection with puromycin (4μg/mL). Bafilomycin A1 (10 nM; Cell Signaling Technology, Frankfurt, Germany) and DAPT (10μM; Sigma Aldrich, Taufkirchen, Germany) treatment of cells was done for a time of 10-24hrs. Fluorescence of YFP and fusion proteins was assessed in a FACScalibur device in the FLH1 channel (Becton Dickinson, Heidelberg, Germany).

**Co-immunoprecipitation and immunoblot**

For co-immunoprecipitation, 4mg of total protein lysate were subjected to GFP-mediated immunoprecipitation of YFP and fusion proteins using GFP-Trap® agrose beads (ChromoTek, Planegg-Martinsried, Germany). Superior expression of YFP compared to EpCAM-YFP was adjusted through the addition of wild-type mF9 cell lysate to YFP lysates. Precipitated proteins were washed with ice-cold washing buffer containing 0.5% tween x100 in TBS, resuspended in 20μL Laemmli buffer (Laemmli, 1970), separated in a 10%-SDS-PAGE, transferred onto activated PVDF membrane (Millipore, Darmstadt, Germany) and detected with GFP (Santa Cruz, sc-9996; USA), prohibitin-1 (Santa Cruz, sc-28259; USA), prohibitin-2 (Santa Cruz, sc-67045; USA), calnexin (Enzo, ADI-SPA-860; USA), Rab5 (Cell Signaling, E6N8S; USA), Rab7 (Cell Signaling, D95F2; USA) and Rab11 (Cell Signaling, D4F5; USA) specific antibodies in conjunction with HRP-conjugated secondary antibodies and ECL (Millipore, Darmstadt, Germany).

**Confocal laser scanning microscopy**

Fluorescence was analyzed in mF9 cells stably transfected with YFP, EpCAM-YFP, mCTF-YFP, or mEpiICD-YFP, and transiently transfected with mCherry-tagged versions of Rab5, Rab7, and Rab11 (Addgene; #27679, #55127, #55124). For live cell imaging, cells were plated on Ibidi 8-well glass bottom m-slides (Ibidi, Matrinsried, Germany; # 80827), stained with 10μg/ml Hoechst33342 (Sigma, Germany #94403) and imaged in phenol red-free RPMI1640 (Thermo Fisher Scientific, #11835030). Rab5 (Cell Signaling, E6N8S; USA), Rab7 (Cell Signaling, D95F2; USA), and Rab11 (Cell Signaling, D4F5; USA) were stained with specific antibodies in combination with phycoerythrin-conjugated secondary antibodies. Lysosomes were stained with 19 nM Lysotracker (Thermo Fisher Scientific, L7528; USA) for 30 minutes in the respective culture medium. Fluorescence was visualized immediately using a TCS-SP8 scanning system, a DM-IRB inverted microscope using a 63x oil objective with a NA of 1.4 and LAS AF software (Leica, Nussloch, Germany). To visualize endocytosed EpCAM, mF9, E14TG2a, and Kyse30 cells were plated as described above and subjected to the internalization assay described below, and samples were taken after staining with an anti-murine EpCAM-Alexa-488 antibody (Abcam, Cambridge, UK; ab237384 and #ab237395), after quenching with an anti-Alexa-488 antibody, and after 30 min of internalization. Alexa-488 fluorescence was imaged using a TCS-SP8 scanning system, a DM-IRB inverted microscope using a 63x oil objective with a NA of 1.4 and LAS AF software (Leica, Nussloch, Germany).

Brightness and contrast of microscopic images were adjusted linearly where indicated in figure legends and scale bars were added using Fiji version 1.52i (Schindelin et al., 2012). Colocalization analysis was performed using the JACoP plugin version 2.1.1 (Bolte and Cordelieres, 2006) with the function “M1 & M2 coefficients” and manually adjusted thresholds.

**KEGG and GO-term classification**

Potential EpCAM interactors analysis was performed using STRING (v.11.0) to scrutiny Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms, including biological process (BP), cellular component (CC), and molecular function (MF). Enrichment analysis were applied based on the Fisher’ exact test, considering the whole quantified interaction proteins as background dataset. Benjamini-Hochberg correction for multiple testing was further applied to adjust p-value, and only functional categories and pathways with p value <0.05 were considered significant.
**Mesodermal differentiation and EMT induction**

Guided mesodermal differentiation of pluripotent murine embryonic stem cells was induced as described by Sarrach et al. ([Sarrach et al., 2018](#)) via a modified protocol from Kanke et al. ([Kanke et al., 2014](#)). For this protocol, E14TG2α cells were seeded in 6 well plates (100,000 cells/well) in Mouse ES Cell Basal Medium supplemented with 1000U/mL ESGRO® LIF, 10% stem cell grade FBS and 0.1nM 2-mercaptoethanol 24 hours before start of differentiation. To induce mesodermal differentiation, cells were thoroughly washed with PBS and cultured in differentiation medium w/o LIF (Mouse ES Cell Basal Medium, 10% stem cell grade FBS, 0.1nM 2-mercaptoethanol) with 30μM CHIR99021 (Sigma, St. Louis, MO, USA) and 5 μM Cyclopamine (Selleckchem, Houston, USA) at 37°C and 5% CO₂. After 5 days incubation, cells were harvested with Trypsin for further processing.

Induction of epithelial-to-mesenchymal transition (EMT) in Kyse30 cells was performed as follows: cells were seeded in 6 well plates (100,000 cells/well) in complete medium for 24 hours, after which the medium was removed, cells washed with PBS, and cultured in FCS-free medium for another 24 hours. EMT was induced by adding recombinant TGFβ (Abcam, Cambridge, UK, #ab50036) at 20 ng/mL and cells were harvested 48 hours later for further processing.

**Internalization and membrane recycling**

The assay was performed via a modified protocol from Arjonen et al. ([Arjonen et al., 2012](#)). Murine F9 cells, pluripotent and mesodermally differentiated E14TG2α cells, and control- and TGFβ-treated Kyse30 cells were harvested and washed twice with cold cell staining buffer (PBS with 3% stem cell grade FBS). Cells were then incubated with an anti-EpCAM AF488-conjugated antibody (ab237384, Abcam, Cambridge, UK) 1:50 in 400μL cell staining buffer for 1 hour at 4°C in the dark, after which they were washed 3 times with cell staining buffer. Samples were then divided into 4 parts: 100% labeled control, quenching background control, endocytosis group, and recycling group. The 100% labeled control can be directly analyzed by flow cytometry (Beckman Coulter Cytoflex device, Germany). The quenching background group was incubated with an anti-AF488 antibody (A-11094, Thermo Fisher Scientific, MA, USA) 1:50 in 100μL cell staining buffer for 1 hour at 4°C in the dark to quench cell surface fluorescence. After washing 3 times with cell staining buffer, cells were analyzed by flow cytometry. The endocytosis and recycling groups were suspended in 1mL cell culture medium and incubated at 37°C for 30 mins to allow internalization of cell surface EpCAM. Cells were then washed with cold cell staining buffer and the cell surface fluorescence was quenched by incubation with the anti-AF488 antibody. For analysis of endocytosis in mF9 cells, samples were assessed every 5 mins. After washing 3 times with cold cell staining buffer, the endocytosis group was analyzed by flow cytometry. The recycling group was again incubated at 37°C for 30 mins to allow recycling of internalized EpCAM to the membrane, after which the cell surface fluorescence was again quenched and the cells analyzed by flow cytometry. Recycling rate was measured every 10 mins for mF9 cells. To calculate endocytosis and recycling rates, live cells were gated and Mean Fluorescence Intensities (MFI) were normalized against unstained controls (MFI(sample)-MFI(unstained control)), after which the quenching background was subtracted from all samples (MFI(sample)-MFI(quenching background)). To calculate endocytosis rates, MFI of the samples were normalized to the 100% labeled control (MFI(endocytosis group)/MFI(100% labeled)), while recycling rates were calculated by subtracting the MFI of the endocytosis group and normalizing to the same (MFI(recycling)-MFI(endocytosis))/MFI(endocytosis)).

**Quantitative real-time polymerase chain reaction**

Total mRNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and reverse transcribed with the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Quantitative PCR was performed using the PowerUp SYBR Green Master Mix (Applied Biosystems, MA, USA) in a volume of 10μl using gene-specific primers on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, MA, USA). Samples were normalized to the housekeeping gene Glucuronidase Beta (GUSB) and relative gene expression was calculated using the delta delta Ct (ΔΔCt) formula. Real time primer sequences are shown below.

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**References**

Sarrach et al. (2018)

Kanke et al. (2014)
The general procedure of the SILAC screen implementing mF9 cells expressing EpCAM-YFP or YFP has been described elsewhere (Sarrach et al., 2018). Briefly, mF9 cells stably expressing EpCAM-YFP or YFP proteins were cultured 14 days (representing 3–5 passages) in medium (Silantes, Munich, Germany) containing heavy (lysine-8/arginine-10) and light amino acids (lysine-0/arginine-0), respectively. In three biological repeats, 3mg (exp. #1 and #2) or 7mg (exp.#3) whole cell lysate were incubated with 30 µL GFP-Trap® agarose beads (3 hrs, 4°C, rotation), washed in 700 µl 0.2% tween in PBS. Independent samples (n = 3) from EpCAM-YFP and YFP immunoprecipitants were pooled and proteins recovered upon heating (95°C, 5 min) in Laemmli buffer (Laemmli, 1970). Immunoprecipitated proteins were separated on SDS-PAGE, trypsinized by in-gel digestion, and analyzed via LC-MS/MS on a LTQ Orbitrap XL coupled to an Ultimate 3000 nano-HPLC. SILAC data analysis was performed using the MaxQuant software (Merl et al., 2012). Potential interaction partners were defined as proteins enriched by \( R^3 \)-fold with \( p \) values \( \leq 0.05 \) and \( \geq 2 \) unique peptides in all independent experiments. Two-sided unpaired t-tests were conducted on individual protein intensities for each label and sample (intensities for replicate #3 were adjusted for differences in protein input in the IP). All proteomic data are compiled in supplementary Excel file Data S1.

Statistical analysis was performed in GraphPad Prism 8 and is indicated in figures and figure legends including numbers of independent experiments (n), statistical tests used, and the level of significance. Data are presented as mean with SD where indicated.