Endosomal regulation of contact inhibition through the AMOT:YAP pathway

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INTRODUCTION

Regulation of signals that govern cell proliferation is required for the maintenance of tissue homeostasis. Proliferation may be controlled by several parameters, including growth factors, tissue architecture, and cell–cell contact (Polyak et al., 1994; Faust et al., 2005; McClatchey and Yap, 2012; Alkasalias et al., 2014). Contact inhibition of the cell cycle has been recognized for decades (Eagle and Levine, 1967), and loss of contact-mediated growth control is one of the hallmarks of cancer (Hanahan and Weinberg, 2011). Physical contact between cells has been shown to mediate contact inhibition, and adhesion junctions and proteins required for establishing polarity all regulate contact-mediated cell proliferation (Li and Mrsny, 2000; Perrais et al., 2007; Chen et al., 2010; Kim et al., 2011; Archibald et al., 2014). However, our understanding of how signals generated at the plasma membrane are communicated to the nucleus to limit proliferation remains incomplete.

The Hippo pathway is conserved from Drosophila to mammals and regulates organ size, tissue homeostasis, and contact-mediated cell growth (Camuso et al., 2007; Dong et al., 2007; Zhao et al., 2007; Gumbiner and Kim, 2014). The Hippo pathway was originally identified as a kinase cascade with the downstream effector the oncprotein Yki/YAP (Drosophila/mammals; Justice et al., 1995; Harvey et al., 2003; Huang et al., 2005; Lai et al., 2005; Callus et al., 2006; Zhang et al., 2008; Boggiano and Fehon, 2012). Nonphosphorylated YAP translocates to the nucleus and acts as a transcriptional coactivator (Dong et al., 2007; Wu et al., 2008; Zhao et al., 2008); this transcriptional complex in turn regulates expression of genes involved in cell proliferation and antiapoptosis (Basu et al., 2003; Huang et al., 2005; Overholtzer et al., 2006). Polarity proteins such

ABSTRACT

Contact-mediated inhibition of cell proliferation is an essential part of organ growth control; the transcription coactivator Yes-associated protein (YAP) plays a pivotal role in this process. In addition to phosphorylation-dependent regulation of YAP, the integral membrane protein angiomotin (AMOT) and AMOT family members control YAP through direct binding. Here we report that regulation of YAP activity occurs at the endosomal membrane through a dynamic interaction of AMOT with an endosomal integral membrane protein, endotubin (EDTB). EDTB interacts with both AMOT and occludin and preferentially associates with occludin in confluent cells but with AMOT family members in subconfluent cells. EDTB competes with YAP for binding to AMOT proteins in subconfluent cells. Overexpression of the cytoplasmic domain or full-length EDTB induces translocation of YAP to the nucleus, an overgrowth phenotype, and growth in soft agar. This increase in proliferation is dependent upon YAP activity and is complemented by overexpression of p130-AMOT. Furthermore, overexpression of EDTB inhibits the AMOT:YAP interaction. EDTB and AMOT have a greater association in subconfluent cells compared with confluent cells, and this association is regulated at the endosomal membrane. These data provide a link between the trafficking of tight junction proteins through endosomes and contact-inhibition-regulated cell growth.
as Crumbs, adherens proteins such as E-cadherin, mechanotransduction, and growth factors, all through upstream regulation of the kinase cascade, regulate the Hippo pathway (Faust et al., 2005; Grzeschik et al., 2010; Ling et al., 2010; Dupont et al., 2011; Kim et al., 2011; Halder et al., 2012). However, an additional mode of regulation of YAP is through angiomotin (AMOT) family members (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011). AMOT regulation is phosphorylation independent, in that YAP associates with AMOT at the membrane, regulating its availability to translocate to the nucleus (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011). AMOT has been localized to the tight junction (TJ) and endosomes, and overexpression of AMOT rescues loss of contact inhibition induced by YAP activation (Bratt et al., 2005; Heller et al., 2010; Zhao et al., 2011; Mana-Capelli et al., 2014). However, how contact inhibition regulates this interaction and YAP activity is unknown.

Endocytic membrane trafficking is essential for maintenance of TJ integrity (ivanov et al., 2005; Morimoto et al., 2005; Terai et al., 2006; Yamamura et al., 2008; Marchiando et al., 2010) and epithelial polarity, and disruption of membrane trafficking induces a loss of polarity and an overgrowth phenotype (Lu andilder, 2005). Endotubin (EDTB; also known as MAMDC4, AEGP) is an integral membrane protein that localizes to specialized apical endosomes (Wilson et al., 1987, 2000; Wilson and Colton, 1997; Gokay and Wilson, 2000). Although EDTB does not localize to tight junctions at steady state, it regulates their assembly and maintenance (Wilson et al., 1987; McCarter et al., 2010). Here we report that EDTB regulates YAP through modulation of the AMOT:YAP interaction at endosomal membranes, providing a mechanism for contact-mediated inhibition through trafficking between endosomes and tight junctions.

RESULTS

YAP localizes to intracellular puncta and colocalizes with a specialized endosomal marker

Contact inhibition requires communication between the plasma membrane/junctions and the nucleus, and YAP has been localized to lateral membranes, the nucleus, and intracellular puncta (Gilbert et al., 2011; Mana-Capelli et al., 2014). EDTB affects tight junction assembly but does not localize to lateral membranes at steady state (McCarter et al., 2010); it resides on specialized apical endosomes and does not colocalize with classical early endosomal markers such as EEA1 (Wilson et al., 1987, 2000). In addition, EDTB does not colocalize with transferrin receptor or Rab11 but does label with internalized tracer and colocalizes with Rab14 (Wilson and Colton, 1997; Gokay and Wilson, 2000; Kitt et al., 2008). To determine whether YAP is present on EDTB endosomes, we colabeled for endogenous EDTB and YAP. We find that there is colocalization of these markers on some intracellular puncta with a Pearson’s r = 0.31. Of interest, in contrast to the findings of Gilbert et al. (2011), labeling of EAA1 and YAP results in very little colocalization (Figure 1A, Pearson’s r = 0.18), suggesting that, in Madin–Darby canine kidney (MDCK) cells, YAP resides on the specialized endosomal compartment marked by EDTB.

Expression of EDTB full-length or cytoplasmic domain results in translocation of YAP to the nucleus and loss of growth control

It is possible that cycling of junctional proteins or YAP regulators to endosomes could regulate YAP activity. We generated stable MDCK cell lines expressing full-length endotubin (ET-FL), a green fluorescent protein (GFP) fusion protein containing only the cytoplasmic domain of EDTB (GFP-CD; Supplemental Figure S1A; McCarter et al., 2010) or GFP. These constructs are expressed at 1.2–2 times the level of endogenous EDTB (Supplemental Figure S1, B–E). At steady state, expression of ET-FL or GFP-CD does not affect the distribution of tight junction proteins (McCarter et al., 2010). Labeling of confluent cells expressing full-length endotubin (Figure 1, B and C) or GFP-CD (Figure 1D) showed extensive localization of YAP in the nucleus. However, although there is no change in the protein or phosphorylation level of the Hippo pathway kinase Mst1/2 (Supplemental Figure S1F), there is an increase in YAP protein levels (Supplemental Figure S1G) and YAP phosphorylation (Supplemental Figure S1H) in cells expressing both ET-FL and GFP-CD. However, because the ratio of total and phosphorylated YAP is unchanged, there is more unphosphorylated (potentially active) YAP in the cells overexpressing ET-FL or GFP-CD.

We next tested whether the increased nuclear YAP resulted in increased cell proliferation. Phospho–histone H3 (pHH3) labeling shows that proliferation is increased twofold in cells expressing ET-FL or GFP-CD (Figure 2A). Furthermore, when these cells are grown to confluence, expression of GFP-CD results in the formation of many small, multilayered foci (Figure 2B). Transmission electron microscopy of the GFP-CD foci indicates that the cells retain an epithelial morphology, including apical microvilli (Figure 2B), and quantification shows that foci formation increases more than fivefold (Figure 2C). Formation of foci could indicate a loss of epithelial polarity. To assess polarity, we labeled control and GFP-CD cells grown on permeable supports with antibodies against the apical marker gp135/podocalyxin and the basolateral marker E-cadherin. As shown in Figure 2D, GFP-CD cells exhibit apical gp135 and basolateral E-cadherin expression. In addition, foci are also polarized and sometimes form polyplike structures that retain polarity (Supplemental Figure S2E).

Because results obtained using GFP-CD were often more pronounced than those obtained using full-length endotubin, most experiments used overexpression of GFP-CD, as it provides greater mechanistic insight into the domains important for the observed effects. In fact, this approach has been used successfully to define the function of other integral membrane proteins (Wodarz et al., 1993; Delmas et al., 1999). Specifically, overexpression of the cytoplasmic domain of Crumbs defined its role in epithelial polarity and establishment of apical membranes (Wodarz et al., 1993, 1995), and expression of the Crumbs cytoplasmic domain drives proliferation through activation of Yorkie (Ling et al., 2010; Robinson et al., 2010).

Changes induced in cells expressing GFP-CD are not the result of epithelial-to-mesenchymal transition

Migration assays indicate that GFP-CD cells have increased rates of migration (Supplemental Figure S2A), and GFP-CD cells plated on type II collagen grow as spindle-shaped cells when subconfluent (Supplemental Figure S2B). However, they never invade the collagen matrix. Changes in morphology and migration of epithelial cells can be the result of reprogramming to a mesenchymal phenotype, with loss of E-cadherin and up-regulation of the intermediate filament protein vimentin and epithelial-to-mesenchymal transition (EMT) transcription factors (Thiery et al., 2009), and forced expression of YAP results in EMT in the epithelial cell line MCF10A (Overholtzer et al., 2006). However, analysis of protein levels of EMT markers shows that E-cadherin levels are maintained and there is no increase in vimentin (Supplemental Figure S2C). Furthermore, quantitative PCR shows that transcription factors and markers associated with EMT are decreased (Supplemental Figure S2D). Surprisingly, the levels of the transcription factor Snail are increased without a significant reduction in the proteins levels of E-cadherin and only a small decrease in E-cadherin message or an increase in Zeb
transcription factors, all direct targets of Snail (Dave et al., 2011). We interpret this to mean that the expression of GFP-CD is not causing EMT.

To examine other proliferation pathways, such as mitogen-activated protein kinase (MAPK) and AKT, that could drive increased proliferation seen with overexpression of ET-FL or GFP-CD, we analyzed their activation. As shown in Supplemental Figure S3, A and B, neither MAPK nor AKT signaling is activated after overexpression of the full-length or cytoplasmic domain of EDTB. In contrast, there is an increase in the cell cycle regulatory protein cyclin D1 when either full-length or cytoplasmic domain of EDTB is overexpressed (Supplemental Figure S3, A and B), consistent with increased activity of YAP (Camargo et al., 2007; Cao et al., 2008).

AMOT and EDTB colocalize on endosomes

An additional mechanism of control of YAP activation and nuclear localization is YAP association with the scaffold proteins p130-AMOT and p100-AMOTL2 (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011). AMOT isoforms localize to junctions and intracellular puncta that have been identified as endosomal membranes (Bratt et al., 2005; Wells et al., 2006; Heller et al., 2010). As with YAP, immunofluorescence of endogenous EDTB and AMOT or AMOTL2 shows extensive colocalization of these proteins on intracellular puncta (AMOT:EDTB, Pearson’s $r = 0.65$ and AMOTL2:EDTB, Pearson’s $r = 0.79$), suggesting an association on specialized early endosomes. Furthermore, labeling for endogenous AMOT and YAP also shows colocalization on intracellular puncta (Pearson’s $r = 0.56$; Figure 3A). However, as with YAP:EEA1, there is limited colocalization of AMOT with EEA1 (Pearson’s $r = 0.18$).

EDTB modulates the interaction between AMOT family members and YAP

AMOT is synthesized as several isoforms due to different translational start sites (Figure 3B), in addition to being a member of a multigene family (Bratt et al., 2002). Interaction between YAP and AMOT occurs with only the p130 form of AMOT, as well as the long form of AMOTL1 and AMOTL2.
(Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011). To test for an interaction between EDTB and AMOT, we used the EDTB cytoplasmic domain fused to glutathione S-transferase (GST). We find that this domain of EDTB brings down p130-AMOT (Figure 3C). In addition, GST-CD also interacts with the highly related AMOTL2 protein (Figure 3C). Consistent with the GST pull-down results, co-immunoprecipitation from cells expressing ET-FL and HA-p130AMOT confirms the interaction of EDTB and AMOT (Figure 3D).

Of importance, EDTB competes with YAP for binding to both AMOT and AMOTL2, as expression of GFP-CD results in decreased AMOT:YAP and AMOTL2:YAP association (Figure 3, E and F). To assess the requirement for YAP in GFP-CD-induced proliferation, we knocked down YAP in MDCK cells expressing GFP-CD. In these cells, YAP protein levels are reduced >90%, and the proliferation of cells expressing GFP-CD is greatly reduced (Figure 4, A and B), suggesting that GFP-CD-induced proliferation is mediated through the YAP pathway. Control cells also had slightly decreased proliferation (0.74-fold) as measured by PHH3 labeling (p < 0.1).

YAP knockdown decreases cell growth of several carcinoma and transformed cell lines (Zhao et al., 2008; Zhi et al., 2012; Huang et al., 2013; Xie et al., 2013) when measured over several days, and it may be that these cells have greater sensitivity to YAP activity at steady state. We next reasoned that, if EDTB competes with YAP for binding to AMOT, overexpression of p130-AMOT would rescue the GFP-CD-induced increase in proliferation. As shown in Figure 4C, p130-AMOT overexpression in GFP-CD cells complements the increased proliferation phenotype. Conversely, knockdown of EDTB should result in decreased proliferation and a corresponding increase in YAP:AMOT localization in MDCK cells due to the greater availability of AMOT to bind YAP. In fact, when EDTB expression is reduced using short hairpin RNA (shRNA), there is both a reduction in the proliferation rate (0.68-fold, p < 0.05) and an increase in the colocalization of YAP and AMOTL2 (Figure 4, D–F).

**Cell confluence affects the EDTB:AMOT association**

To test whether EDTB could regulate YAP via the AMOT pathway, we examined AMOT:EDTB interaction in subconfluent and confluent cultures of MDCK cells. If EDTB regulates the AMOT:YAP interaction during contact inhibition, there should be greater EDTB:AMOT interaction in subconfluent cells, freeing YAP to translocate to the nucleus. There is no change in the amount of EDTB, YAP, AMOT, or AMOTL2 in cells, regardless of confluence (Figure 5A). This contrasts with previous reports, in which levels of the YAP were found to be either up or down under confluent conditions (Zhao et al., 2007, 2010). This may relate to cell-type differences, as experiments in these studies were performed in fibroblasts. After communoprecipitation, we find increased EDTB:AMOT and EDTB:AMOTL2 complexes in subconfluent cells (Figure 5B). In addition, there is an increase in AMOT:YAP association in confluent cultures (Figure 5B), consistent with AMOT sequestering YAP to prevent proliferation. Analysis of the localization of EDTB and AMOT or AMOTL2 on endosomes in subconfluent and confluent cultures shows decreased colocalization of EDTB:AMOT and EDTB:AMOTL2 in confluent cultures (Figure 5, C and D).

To further characterize the relationship between contact inhibition of proliferation and junction assembly, we next tested whether EDTB interacts with the tight junction protein occludin and whether that interaction depends on cell confluence. To test this, we performed communoprecipitation experiments with occludin, followed by immunoblotting for EDTB in subconfluent and confluent cells. Immunoblotting analysis shows that EDTB associates with occludin in confluent, but not subconfluent, cells (Figure 5E).

**Disruption of EDTB results in growth in soft agar**

Growth in soft agar is a hallmark of transformation and loss of growth control. When cells expressing GFP or GFP-CD are plated in soft agar, control cells form occasional small, irregular colonies (Figure 6A). However, cells expressing GFP-CD form large, branching structures and generate more colonies than control cells (Figure 6A). In addition, in GFP-CD cultures, after growth in soft agar for 3 wk, we observe cells dispersing from the original colony and the formation of additional foci. This was never observed in control cultures.

**EDTB is overexpressed in early-stage liver cancer**

In addition to its known role in the control of organ size and contact inhibition, YAP expression is increased in the majority of liver hepatocellular carcinomas (LHGs; Bai et al., 2012). To determine whether EDTB expression is altered in these cancers, we examined the levels of EDTB mRNA expression (RNASeqV2 rsem values) using data available from the Cancer Genome Database (tcga-data.nci.nih.gov). Using 48 matched tumor and normal pairs, we found a two-fold or greater increase in EDTB expression in 17% of patients (Supplemental Table S1). Of interest, within this group, 75% are stage I tumors. The rsem expression values for EDTB in these tumor samples (N = 105) range from 7 to 6579. Binning these samples into three groups based on these expression values shows a trend in which the majority of stage I tumors are medium to high expressing, and the percentage decreases with increasing stage (Figure 6B). Examination of only EDTB high-expressing tumors (rsem >450) indicates that 44% of stage I tumors are high expressing, and 54% of all high-expressing tumors are stage I (Supplemental Table S2). This may indicate that dysregulation of EDTB promotes early events in the development of hepatic cancer.

**DISCUSSION**

Contact inhibition of cell proliferation is essential for regulation of organ size and to prevent the overproliferation that characterizes cancer (Hanahan and Weinberg, 2011). In transformed cells, overexpression of occludin restores assembly of the tight junction and cell-cell contact–mediated growth control, suggesting that the formation

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**FIGURE 2:** EDTB overexpression results in loss of growth control. (A) Representative cells expressing ET-FL or GFP-CD labeled with PHH3. Proliferation is increased with overexpression of GFP-CD or ET-FL. Error bars, SEM of percentage of cells that are PHH3 positive; ***p < 0.005, **p < 0.01. (B) GFP-CD cells form multiple small, multilayered foci (arrows) when grown on Transwell filters, whereas control cells remain as a monolayer with limited number of foci. Transmission electron microscopy (TEM) of control and GFP-CD cells grown on filters shows that control cells grow as a monolayer but GFP-CD cells form multilayered foci. Foci of GFP-CD–expressing cells maintain epithelial characteristics, such as apical microvilli (arrowheads). Scale bars, 100 μm (phase), 1 μm (TEM). (C) Quantification of the multilayer area shows an increase in foci formation in cells expressing GFP-CD. Error bars, SEM; ***p < 0.005. (D) Immunofluorescence labeling of apical (p130, podocalyxin) and basolateral (E-cadherin) markers shows that the polarized distribution of these proteins is maintained with expression of GFP-CD. The foci (arrowheads) also maintain this polarized expression pattern. Scale bar, 20 μm.
FIGURE 3: EDTB, AMOT, and YAP all localize to endosomes, and EDTB regulates AMOT interaction with YAP. (A) Localization of endogenous AMOT, AMOTL2, YAP, EDTB, and EEA1 in MDCK cells grown on coverslips. EDTB colocalizes with AMOT (Pearson’s r = 0.65) and AMOTL2 (Pearson’s r = 0.79) on intracellular puncta (arrows), but there is
EDTB is a member of the MAMDC family. The extracellular domain of EDTB contains several meprin-A5 protein–protein tyrosine phosphatase μ (MAM) domains (Speelman et al., 1995), which are believed to mediate protein interactions and adhesion (Zondag et al., 1995; Gao and Garbers, 1998), whereas the short (40 amino acids) cytoplasmic tail contains apical endosomal sorting motifs (Gokay et al., 2001). Diet1, a MAMDC protein related to EDTB, affects bile acid synthesis through regulation of fibroblast growth and maintenance of tight junctions are important components of contact inhibition (Chen et al., 2000; Li and Mrsny, 2000). Adherens junctions are also an important component of contact inhibition, and E-cadherin regulates the YAP pathway through the regulation of phosphorylation of Mst/Lats (Kim et al., 2011). We do not see any change in the upstream phosphorylation of YAP regulators with EDTB overexpression, suggesting that EDTB regulates YAP through modulation of the interaction of YAP with AMOT at the endosomes.

FIGURE 4: YAP activity is required for GFP-CD–induced increase in proliferation. (A) MDCK cells expressing GFP or GFP-CD were infected with YAP shRNA lentivirus. Lysates were analyzed by Western blot for expression of YAP. YAP protein levels are decreased >90% in both the control and GFP-CD–expressing cells. (B) Changes in proliferation were determined by PHH3 labeling. Proliferation of GFP-CD cells is significantly decreased with YAP knockdown. Error bars, SEM; ***p < 0.005. YAP knockdown in control cells also resulted in decreased proliferation (0.74-fold of that of parent lines). (C) MDCK cells expressing GFP-CD were transfected with p130AMOT. Overexpression of p130-AMOT complements the GFP-CD increased-proliferation phenotype. Error bars, SEM; *p < 0.05. (D) Endogenous EDTB was knocked down in MDCK cells using two independent shRNAs, individually or together. Lysates were analyzed by Western blot to verify the reduction in EDTB expression. (E) Proliferation is decreased when EDTB expression is reduced. Error bars, SEM; *p < 0.05. (F) Pearson r of AMOTL2:YAP colocalization in EDTB knockdown cells. Knockdown of endotubin results in a significant increase in AMOTL2:YAP colocalization. Four to six images were quantified for each condition. Error bars, SEM; ***p < 0.005.

EDTB and maintenance of tight junctions are important components of contact inhibition (Chen et al., 2000; Li and Mrsny, 2000). Adherens junctions are also an important component of contact inhibition, and E-cadherin regulates the YAP pathway through the regulation of phosphorylation of Mst/Lats (Kim et al., 2011). We do not see any change in the upstream phosphorylation of YAP regulators with EDTB overexpression, suggesting that EDTB regulates YAP through modulation of the interaction of YAP with AMOT at the endosomes.

limited colocalization with EEA1 (Pearson’s r for AMOT/EEA1 is 0.18). AMOT and YAP also colocalize on intracellular puncta (Pearson’s r = 0.56). Scale bars, 5 μm (top), 1 μm (insets). (B) AMOT and AMOTL2 domain structure. p80-AMOT and p60-AMOTL2 isoforms result from an internal start site. The amino-terminal domains of p130-AMOT and p100-AMOTL2 interact with YAP through the PPxY motif. (C) MDCK lysates collected at 90% confluence were used to assess the physical interaction of EDTB and AMOT/AMOTL2. Pull-down using the cytoplasmic domain of EDTB fused to GST (GST-CD) analyzed by Western blot shows interaction with AMOT and AMOTL2 relative to the GST-only control. AMOT input is sometimes not detected, indicating concentration by pull down. (D) Coimmunoprecipitation of ET-FL and HA-p130-AMOT. HEK293 cells were transfected with ET-FL and HA-p130AMOT. Lysates were immunoprecipitated with EDTB or HA antibodies. Western blot analysis shows coimmunoprecipitation of EDTB and AMOT. (E) Competition of EDTB with YAP for binding to AMOT and AMOTL2. MDCK cells expressing GFP-CD or control plasmid were immunoprecipitated with antibody against YAP and analyzed by Western blot for AMOT or AMOTL2. In the presence of GFP-CD, there is a significant decrease in the amount of AMOT and AMOTL2 associated with YAP compared with control. (F) Quantification of E. Error bars, SEM from three independent experiments. **p < 0.01, ***p < 0.005.
factor (FGF) trafficking. Of interest, Diet1, like EDTB, is localized to a specialized endosomal compartment, where it colocalizes with FGF15/19 (Vergnes et al., 2013). This interaction is critical in maintaining signaling from the small intestine to the liver (Vergnes et al., 2013). Based on this together with the results reported here, it appears that the MAMDC family proteins play a role in signal transduction through modulation of protein trafficking.

We find that the endosomal protein EDTB both colocalizes and interacts with AMOT family members. EDTB localizes to EEA1-negative early endosomes in a variety of cell types, whereas AMOT and AMOTL2 localize to both the tight junction and endosomes (Wilson and Colton, 1997; Wilson et al., 2000; Wells et al., 2006; Heller et al., 2010; Zhao et al., 2011; Mana-Capelli et al., 2014). Reduced expression of AMOT induces loss of contact inhibition and the formation of foci (Zhao et al., 2011), and AMOT regulates YAP activity by sequestering YAP in the cytoplasm (Chan et al., 2011; Zhao et al., 2011; Mana-Capelli et al., 2014). Although the AMOT:YAP association has traditionally been presumed to occur at the plasma membrane, overexpression of AMOT localizes YAP to the cytosol (Wang et al., 2011). Our results show that AMOT, YAP, and EDTB are located on endosomes, and EDTB competes with YAP for binding to AMOT, likely at the endosomal membrane. Under conditions of increased EDTB, YAP is displaced from p130-AMOT and/ or AMOTL2 and translocates to the nucleus. Our findings of increased colocalization and greater association of EDTB with AMOT and AMOTL2 in subconfluent cells, and conversely an increased interaction of EDTB and occludin in confluent cells, further support the model that endosomes play a role in mediating signaling from this pathway. Although we cannot exclude that this interaction occurs at the junction, it is important to note that EDTB overexpression does not disrupt tight junctions at steady state, and endotubin is not localized to junctions at steady state (Wilson et al., 1987; McCarter et al., 2010).

We find that EDTB message levels are increased in many cases of early-stage liver cancer. This suggests that EDTB may be a predisposing factor in the development of hepatic cancer. This is consistent with another report that identified mutations of EDTB in early events in the development of breast cancer (Sjoblom et al., 2006). Although recent data in transgenic mice argued against the role of AMOT as a suppressor of YAP activity (Yi et al., 2013), contrasting data indicated that AMOT can sequester YAP and prevent its translocation to the nucleus. There are multiple pathways that lead to the activation of YAP, and it is plausible that this activation is context dependent and the regulation of AMOT:YAP interaction via EDTB is important for a subset of cancers.

Recent evidence shows that F-actin competes with YAP for AMOT binding, and disruption of the actin-binding domain on AMOT results in colocalization of AMOT and YAP on endosomes (Mana-Capelli et al., 2014). On the basis of this and the results reported here, we propose a model in which the AMOT:YAP interaction may occur predominantly on endosomes and the availability of EDTB to interact with AMOT is regulated by the presence of tight junction proteins in the endosomes (Figure 7). Thus, in confluent cells, tight junction proteins bind to EDTB, preventing the EDTB:AMOT interaction and allowing AMOT to sequester YAP to prevent nuclear translocation and proliferation.

**FIGURE 5:** Interaction between EDTB and AMOT is regulated by cell density. (A) The expression levels of EDTB, AMOT, AMOTL2, YAP, and occludin in subconfluent and confluent MDCK cultures were analyzed by Western blot analysis. (B) Top, immunoprecipitation of MDCK subconfluent and confluent cultures with EDTB was analyzed by Western blot for AMOTL2. Middle, immunoprecipitation of MDCK subconfluent and confluent cultures with AMOT was analyzed by Western blot for EDTB. Bottom, immunoprecipitation of MDCK subconfluent and confluent cultures with YAP was analyzed by Western blot for AMOT. (C) MDCK cells plated on coverslips were immunolabeled for EDTB and AMOT or AMOTL2. (D) The amount of colocalization between AMOT/EDTB and AMOTL2/EDTB is significantly reduced in confluent cells. The colocalization of AMOT/EDTB and AMOTL2/EDTB is significantly reduced in confluent cells. Average endosome number per image is 475, and eight images were quantified per condition. Error bars, SEM; ***p < 0.005. (E) Immunoprecipitation of MDCK subconfluent and confluent cultures with occludin was analyzed by Western blot for EDTB. The image is spliced from the same gel.
FIGURE 6: Increased expression of EDTB results in growth in soft agar and is associated with early-stage LIHC samples. (A) MDCK cells expressing GFP (control) or GFP-CD were grown in soft agar for 3 wk. GFP-CD cells form long, branching structures (arrows) that produce satellite colonies (arrowheads) after 3 wk in culture. Scale bars, 100 μm (left), 20 μm (right). Right, quantification of colony formation after 14 d in cells expressing GFP-CD. Error bars, SEM; **p < 0.01. (B) Using the Cancer Genome Database, we collected the rsem expression values for EDTB in 105 liver hepatocellular carcinomas. The tumors were binned into three equal groups based on EDTB expression levels. Separating these groups by tumor stage shows that 44% of stage I tumors express EDTB at high levels.

MATERIALS AND METHODS
Reagents
Primary antibodies used for this study include rabbit anti-AMOT (H-66), goat anti-AMOTL2 (N-14), goat anti–β-catenin, rabbit anti–cyclin D1 (H-295), and rabbit anti-YAP (H-125), purchased from Santa Cruz Biotechnology (Dallas, TX). Rabbit anti–phospho-Akt (473), rabbit anti–phospho-p44/42 MAPK, rabbit anti–p44/42 MAPK, rabbit anti–YAP, rabbit anti–phospho-YAP (Ser-127), rabbit anti-Mst1/2 (3682), and phospho-Mst1 (3681) were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti–β-actin, rat anti–E-cadherin (clone DECMA-1), and mouse anti–phospho-histone H3 were purchased from Sigma-Aldrich (St. Louis, MO). Mouse anti–E-cadherin was purchased from BD Biosciences (San Jose, CA), mouse anti–hemagglutinin (HA) from Covance (San Diego, CA), rabbit anti–AMOT from Antibody Verify, and rabbit anti–AKT from BD Biosciences. Secondary antibodies used with the LI-COR Odyssey infrared imager include anti-rabbit, -goat, and -mouse purchased from LI-COR (Lincoln, NE). Polyethyleneimine (PEI; Polysciences, Warminster, PA) was prepared as a 1 μg/ul solution in water and sterilized by filtration.

Cell culture
MDCKII cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 1000 U of penicillin, 1 mg/ml streptomycin, 20 mM l-glutamine (Sigma-Aldrich), and nonessential amino acids (Mediatech, Manassas, VA). Generation of cell lines expressing the EDTB and EDTB cytoplasmic domain (GFP-CD) has been described previously (Gokay and Wilson, 2000; McCarter et al., 2010). The GFP-CD cell lines were maintained in DMEM supplemented with G418 (400 μg/ml). Both the EDTB knockdown (ETKD) and the YAP knockdown (YAPKD) cell lines were generated using lentiviral constructs.

ET 1014 shRNA:
Forward primer
cgggaactgctcgctcgcttctatctcgagatagaagacgagggagcagtttttttg
Reverse primer
aattcaaaaaactgtcgctgtccttctactcagatgagacgacgacgagt
ET 3603 shRNA:
Forward primer
cgggacatctgttcatgcggatcgacgcatcggatgaacgagtgttttttgg
Reverse primer
aattcaaaaaacatgctctactgatccccgtgacagttctaggaacagtgt
YAPKD:
Forward primer
cgggagctctgactgcttcatccctcgagggatgtcagagctcagagctttttttgg
Reverse primer
aattcaaaaaagctctgacttctcctcgagggatgtcagagctcagagctcttg

All cultures were selected using puromycin (2 μg/ml) after lentiviral transduction. For controls, we used the pLK0.1 puro construct (Addgene; Stewart et al., 2003).

Transwell filters
Twelve-well Transwell filters (Corning, Corning, NY) were seeded with 250,000 cells/well. Fresh medium was added daily and cells incubated for an additional 4–5 d. For immunolabeling, filters were processed as described later. For multilayer analysis, images were obtained using an Olympus IMT-2 microscope equipped with Hoffman modulation contrast filters, and multilayer area was calculated using ImageJ (National Institutes of Health, Bethesda, MD).

Immunofluorescence labeling and confocal microscopy
For immunofluorescence labeling, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% saponin/phosphate-buffered saline, and incubated in primary antibodies for 2 h at room temperature, followed by incubation with fluorescent secondary antibodies (Molecular Probes, Grand Island, NY; Jackson Labs, Bar Harbor, ME). Images were acquired using an Olympus Fluoview 1100 or 1200 confocal microscope with a 60× (numerical aperture 1.4) oil immersion objective. Excitation wavelengths of 405, 488, 568, and 594 nm were used for simultaneous two- or three-channel recording. Images were processed and merged using Photoshop software (Adobe Systems, San Jose, CA) or ImageJ. For comparison between control and knockdown cells, identical imaging and processing parameters were used. Pearson’s r was calculated from 60× images generated using the Olympus Fluoview confocal microscope and software. The average Pearson’s r was derived from seven images.

Transient transfection
Cells were plated at ~60% confluence 24 h before transfection. PEI was used for transient transfections. The PEI:DNA ratio for the transfections was 3:1, with 4.5 μg of PEI used for transfections in a six-well plate. Cells were incubated with the PEI:DNA solution for 16 h, and cells were fixed or lysed 48 h later.

Immunoblot analysis
For quantification of signaling pathways, lysates were collected at ~90% confluence. Cells were lysed with RIPA (50 mM Tris, 100 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN) supplemented with calyculin A (Sigma-Aldrich) and sodium orthovanadate (Sigma-Aldrich) for 5 min on ice. Cells were scraped, passed through a 27-gauge needle, vortexed, and incubated on ice for 30 min. The insoluble materials were removed by centrifugation, and supernatant was retained for analysis. For immunoblotting, 12–25 μg of protein was placed in LI-COR SDS sample buffer containing 100 μM dithiothreitol and separated on a 7.5% SDS-PAGE gel. Protein was transferred to nitrocellulose, blocked using 5% milk, and incubated with primary antibodies in Tris-buffered saline–Tween/5% milk for 2 h at RT or overnight at 4°C. For phosphospecific antibodies, 5% bovine serum albumin was used as the blocking solution. For all experiments, samples were run in triplicate or quadruplicate, and each experiment was run at least three independent times. Blots were imaged and quantified using the LI-COR Odyssey system.

Immunoprecipitation of subconfluent and confluent cultures
For analysis of protein expression and protein–protein interaction of subconfluent and confluent cultures, cells were grown in 10-cm dishes. For subconfluent cells, the cultures were lysed at 50–60% confluence. For confluent cultures, cells were grown to 100% confluence, fresh medium was added, and cells were incubated for an additional 24 h. For immunoprecipitation, cells were lysed in an NP-40 buffer (1% NP-40, 25 mM Tris, pH 7.2, 60 mM NaCl, 5 mM MgCl 2, 5% glycerol). Lysates (500 μg of protein) were preclarified for 2 h at 4°C using protein G Dynabeads (Life Technologies, Foster City, CA) and incubated with 2 μg of primary antibody overnight at 4°C, followed by 2-h incubation with protein G Dynabeads at 4°C. Beads were washed with lysis buffer, resuspended in SDS loading buffer, and separated by electrophoresis. As described earlier, experiments were performed in triplicate or quadruplicate and repeated at least three times.

GST pull down
EDTB-C D GST proteins were purified by sonication of bacterial lysates in ice-cold STE buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 8, 1 mM EDTA) supplemented with phenylmethylsulfonyl fluoride/leupeptin and lysozyme and incubated with Glutathione Sepharose 4 Fast Flow beads (GE Healthcare, Piscataway, NJ) for 2 h at 4°C. Lysates from MDCK were prepared using PLC lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 150 mM NaCl, 5% glycerol, 0.5% Triton X-100, 1.5 mM MgCl 2, 1 mM ethylene glycol tetraacetic acid, pH 8.0) supplemented with protease inhibitor cocktail and calyculin A. Lysates were incubated with GST beads for 3 h at 4°C. The GST beads were washed four times with PLC lysis buffer, and proteins were eluted from beads by incubation at 95°C for 5 min in SDS–PAGE loading buffer.

Proliferation assay
We plated 5 × 10 4 cells on coverslips and incubated them for 24 h before fixation and labeling with anti–phospho-histone H3. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and the percentage of phospho-histone H3–labeled cells was determined through direct counting of blinded samples. For the p130-AMOT rescue, 2.5 × 10 4 cells were plated and transfected with p130 AMOT after 24 h. Cells were incubated for an additional 24 h before fixation and labeling. For all proliferation experiments, three images at 40× magnification were collected from each coverslip, and three coverslips were used for each experimental and control condition. All cells in the field of view were counted, and the total number of cells ranged from 100 to 300 for each image (n = 500–1000 cells/condition).

AMOT rescue
MDCK cells expressing GFP-CD were transfected with HA p130-AMOT (Addgene, Cambridge, MA) (Zhao et al., 2011) using PEI. After 48 h, the cells were assessed for phospho-histone H3–positive nuclei as described. For controls, pCDNA3 was transfected into GFP-CD–expressing cells.
Soft agar colony-forming assay

Anchorage-independent growth was determined using a soft agar assay. MDCK control and GFP-CD cells were plated in 0.3% agar over a base of 0.5% agar prepared in DMEM. One thousand cells were seeded into six-well plates, and colony formation was assessed after 14–21 d of culture. Colonies were counted after 14 d in culture from three independent wells, and the experiment was repeated at least five times.

Electron microscopy

MDCK cells stably expressing control or GFP-CD constructs were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and embedded in Spurrs resin (Electron Microscopy Sciences, Hatfield, PA). Sections were examined using a Philips 410STEM at 80 kV. Images were acquired using an AMT-XR40 (Advanced Microscopy Techniques, Woburn, MA) digital camera.

Migration assay

Cells were grown to confluence in a six-well plate (Corning). Confluent monolayers were wounded using a pipette tip to create a small circular area devoid of cells. The plate was marked to allow tracking of individual wounds. The wounds were imaged using an Olympus IMT-2 microscope equipped with Hoffman modulation contrast filters at hourly intervals for 3 h, and wound area was quantified using ImageJ. Wounds to the monolayer were made in separate wells of the six-well plate. Quantification of four wounds for both the control and GFP-CD–overexpressing cells were measured. The experiment was repeated three times.

Quantitative real-time PCR

Total RNA was extracted from GFP-CD or control cells cultured for 7 d on Transwell filters using TRIZOL reagent (Life Technologies) and measurements were averaged for each gene analyzed. RT reactions were run in triplicate using the iScript CDNA synthesis kit (Bio-Rad). Primer sequences were from Wyatt et al. (2007). Data were normalized against the housekeeping gene actin. cDNA was measured using a fluorometer (Turner Biosystems, Madison, WI) with the Quanti-IT OligoGreen ssDNA reagent and kit (Molecular Probes). Equal aliquots were pipetted into each reaction tube. Real-time PCR was performed using the Platinum SYBR Green qPCR Supermix UDG (Invitrogen, Grand Island, NY). In each experiment, triplicate reactions were averaged for each gene analyzed.

Statistical analysis

Statistical comparisons were made using Excel (Microsoft) and a Student’s t test. Data are expressed as the mean ± SEM.

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