Tissue Barriers 2, e28127; April 2014; © 2014 Landes Bioscience

The role of angiomotin phosphorylation in the Hippo pathway during preimplantation mouse development

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Keywords: Amot, Amotl2, Merlin/Nf2, Lats, F-actin, Tead4, Yap, Taz, blastocyst

Abbreviations: AJs, Adherens junctions; Amot, angiomotin; Amotl1, angiomotin-like 1; Amotl2, angiomotin-like 2; Cdx2, caudal type homeobox 2; ChIP-seq, chromatin immunoprecipitation followed by high-throughput sequencing; F-actin, filamentous actin; Gata3, GATA binding protein 3; ICM, inner cell mass; Lats, large tumor suppressor; Mob, Mps1 binder; Mst, mammalian STE20-like protein kinase; Nf2, Neurofibromatosis type 2; Sav, Salvador; Taz, transcriptional coactivator with PDZ binding motif; TE, trophectoderm; TJs, tight junctions; Wwtr1, WW domain containing transcription regulator 1; Yap, Yes-associated protein

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Submitted: 01/11/14

Revised: 02/01/14

Accepted: 02/05/14

Published Online: 02/21/14

Citation: Hirate Y, Sasaki H. The role of angiomotin phosphorylation in the Hippo pathway during preimplantation mouse development. Tissue Barriers 2014; 2:e28127; http://dx.doi.org/10.4161/tisb.28127

Extra View to: Hirate Y, Hirahara S, Inoue K, Suzuki A, Alarcon VB, Akimoto K, Hirai T, Hara T, Adachi M, Chida K, et al. Polarity-dependent distribution of angiomotin localizes Hippo signaling in preimplantation embryos. Curr Biol 2013; 23:1181-94; PMID:23791731; http://dx.doi.org/10.1016/j.cub.2013.05.014

The Hippo signaling pathway regulates a number of cellular events, including the control of cell fates in preimplantation mouse embryos. The inner and outer cells of the embryo show high and low levels of Hippo signaling, respectively. This position-dependent Hippo signaling promotes the specification of distinct cell fates. In a recent paper, we identified the molecular mechanism that controls Hippo signaling in preimplantation embryos. The junction-associated scaffold protein angiomotin (Amot) plays a key role in this mechanism. At the adherens junctions of the inner cells, Amot activates the Hippo pathway by recruiting and activating the protein kinase large tumor suppressor (Lats). In contrast, Amot at the apical membrane of the outer cells suppresses Hippo signaling by interacting with F-actin. The phosphorylation of Amot inhibits its interaction with F-actin and activates Hippo signaling. We propose that Amot acts as a molecular switch for the Hippo pathway and links F-actin with Lats activity.

Introduction

The Hippo pathway plays a central role in coordinated tissue formation and organogenesis.[1-3] The components of the Hippo pathway were originally identified in the fruit fly *Drosophila melanogaster* using a genetic screen designed to identify genes that regulate cell proliferation.[4] Over the course of the last decade, studies in *Drosophila* and mammals have revealed an evolutionarily conserved core Hippo pathway and divergent upstream inputs (**Fig. 1A**). The core Hippo pathway involves mammalian STE20-like protein kinase 1/2 (Mst1/2), serine/threonine kinases homologous to *Drosophila* Hippo and their cofactor Salvador 1 (Sav1), second messenger serine/threonine kinases Lats1/2 and their cofactor Mps1 binder (Mob1), and transcriptional co-activators Yes-associated protein (Yap1) and transcriptional coactivator with PDZ binding motif (Taz)/WW domain containing transcription regulator 1 (Wwtr1) that interact with TEA domain family transcription factors (Tead1–4) in the nucleus.[1] Upon activation of the Hippo pathway, Lats1/2 phosphorylates Yap (herein referring to Yap1 and Taz/Wwtr1 collectively). Phosphorylated Yap (p-Yap) is retained in the cytoplasm by interacting with 14–3-3 protein and is degraded by the ubiquitin-proteasome system.[5-8] Thus, the activation of the Hippo pathway results in a loss of the Tead-Yap complex in the nucleus and the repression of the target genes. In contrast, lack of Hippo signaling allows Yap to accumulate in the nucleus, leading to the expression of target genes. In this commentary, we outline the findings of our recent paper[9] that examined the role of the junction-associated scaffold protein angiomotin (Amot) in preimplantation mouse embryos. Together with additional data, we propose a model by which Amot plays a central role in both the activation of the Hippo pathway at adherens junctions (AJs) and its apical F-actin-mediated suppression.

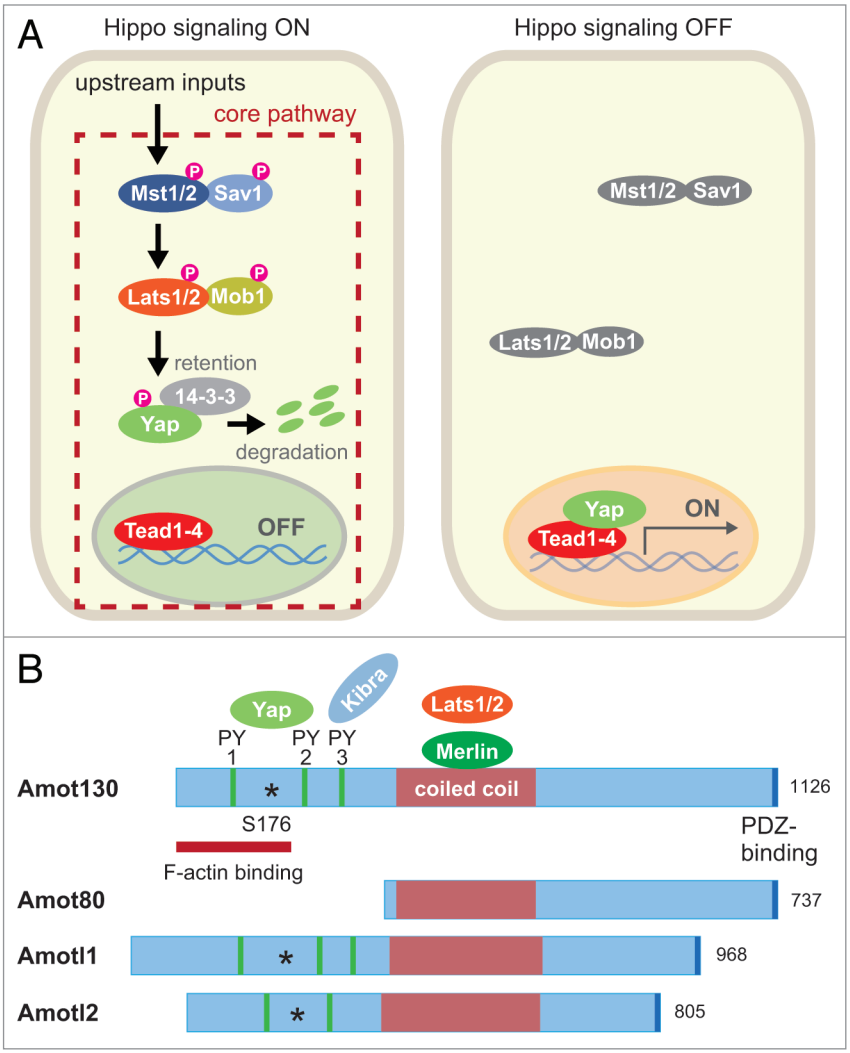

Figure 1. Schematic representation of the Hippo pathway. (**A**) Input from divergent upstream pathways activates the core Hippo pathway. Activated Lats kinase phosphorylates Yap, leading to its cytoplasmic retention and degradation. Without nuclear Yap, the expression of Tead target genes is suppressed. In contrast, when Hippo signaling is inactive, Yap enters the nucleus, complexes with Tead, and activates the transcription of target genes. (**B**) The domain structures of Amot family proteins and Amot-interacting proteins. The numbers at the right of each scheme indicate the numbers of amino acid residues of the proteins. Amot has two isoforms: p130 (Amot130) and p80 (Amot80). Amot80 lacks an N-terminal domain. The N-terminal domain of Amot 130 contains three PY motifs. Yap interacts with the first two PY motifs,[23-25,50] and Kibra interacts with the third PY motif.[9] The third PY motif is not present in Amotl2. The Amot N-terminal domain also interacts with F-actin. A Lats phosphorylation site (asterisk, S176) is present within the essential region of the Amot F-actin binding domain.[34] Merlin interacts with the coiled-coil domain of Amot.[21] The interaction of Amot with Lats requires both the N-terminal and coiled-coil domains, but the exact interaction motifs remain unknown.[9,22]

The Role of the Hippo Pathway in Preimplantation Cell Fate Specification

During mouse development, the first cell fate specification event takes place between the morula and blastocyst stage, prior to the implantation of the embryo into the uterus (preimplantation) (Fig. 2A). The morula is an aggregate of 16–32 cells. The outer cells of the morula acquire apicobasal polarity and differentiate into a cyst-like epithelial tissue in the blastocyst called the trophectoderm (TE). The TE is required for implantation and subsequently forms the placental tissues. The inner cells are nonpolar cells that form the inner cell mass (ICM), an aggregate of cells attached to one side of the TE. The ICM gives rise to the embryo proper and several extraembryonic tissues.

The specification of TE and ICM cell fates is regulated by the Hippo pathway (**Fig. 2B**).[9,10] In the outer cells, Hippo signaling is quiescent, allowing Yap to accumulate in the nucleus. Nuclear Yap complexes with Tead4, converting it into a transcriptional activator. The Tead4-Yap complex activates the expression of TE-specific transcription factors, including Cdx2 and Gata3, promoting TE differentiation.[10-15] In the inner cells, Hippo signaling is active, inhibiting the nuclear accumulation of Yap. The absence of nuclear Yap renders Tead4 inactive, thereby preventing the expression of TE-specific transcription factors. Likely through an autoactivation mechanism,[16] this allows the high-level expression of pluripotency-related transcription factors Oct3/4, Nanog, and Sox2, which promote the differentiation of cells into the ICM.[10,15,17]

The Requirement of Amot Family Proteins in Hippo Signaling

Amot is a junction-associated scaffold protein involved in Hippo signaling. Amot plays a key role in the regulation of the mammalian Hippo pathway at AJs but is absent in *Drosophila*. This fact may reflect a difference in the organization of the intercellular junctions of these species. Amot contains three PY motifs in the N-terminal region, a coiled-coil domain in the central region, and a PDZ-binding motif at the C-terminus (**Fig. 1B**). There are two Amot-related proteins, Amotl1 and Amotl2.[18] The domain structure is largely conserved among the Amot family of proteins. Through these domains and motifs, Amot interacts with many proteins involved in cell polarity[19] and junction formation,[19,20] and with the Hippo pathway components Merlin,[21] Kibra,[9] Lats,[9,22] and Yap.[23-25] In addition, the N-terminal region of Amot exhibits an F-actin binding/bundling activity.[26]

Amot is localized throughout the plasma membrane at the AJs (junctional Amot) and restricted to the apical domain, including tight junctions (apical Amot),

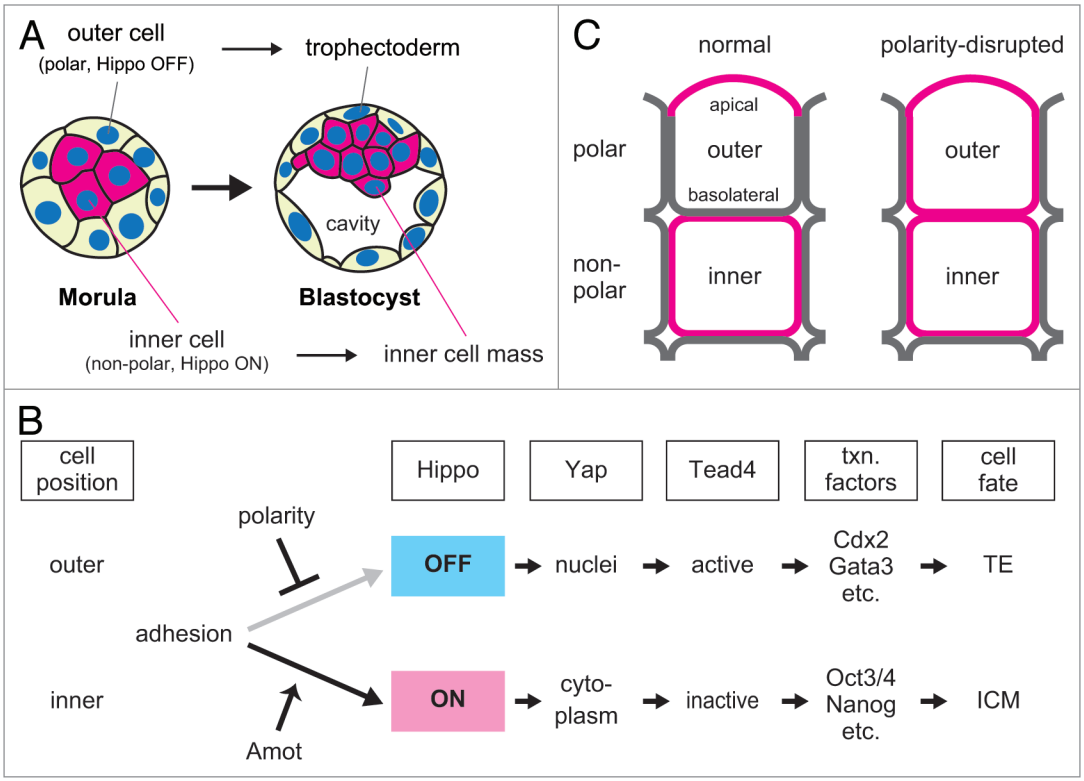

Figure 2. Differential Hippo signaling specifies distinct cell fates in the preimplantion mouse embryo. (**A**) At the morula stage, mouse embryos consist of inner and outer cells. The outer cells are specified to become the trophectoderm (TE), whereas the inner cells are specified to become the inner cell mass (ICM) at the blastocyst stage. (**B**) In the inner cells, cell adhesion activates Hippo signaling in an Amot-dependent manner, allowing the inner cells to express ICM-specific transcription factors and adopt ICM fate. In the outer cells, Hippo signaling is suppressed by cell polarity, which specifies the outer cells to become the TE. (**C**) The distribution of Amot (shown in red) in normal and polarity-disrupted embryos.

in the nonpolar inner and polarized outer cells, respectively (**Fig. 2C**). In preimplantation embryos, Amot is required for the activation of the Hippo pathway in the inner cells (**Fig. 2B**). In *Amot* mutant (KO) embryos, the inner cells show reduced Hippo signaling, leading to the nuclear accumulation of Yap and the induction of TE genes.[9] However, the mutant phenotype subsides after the blastocyst stage (> 32 cells), allowing the mutant embryos to develop until postimplantation stages.[27] To attenuate Hippo signaling completely, a simultaneous knockdown of *Amotl2* in *Amot* KO embryos is required, indicating a redundant role of Amotl2 in Hippo signaling.[9,17] Amotl2 is not expressed in the inner cells of wildtype embryos; thus, we initially assumed that Amotl2 was not involved in the activation of Hippo in the inner cells. However, Amotl2 is ectopically induced in the inner cells of *Amot* KO embryos.[9] Because Tead4 was found to bind in close proximity to the *Amotl2* gene in a chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) analysis of trophoblast stem cells,[14] we predict that the impairment of Hippo signaling in the inner cells of *Amot* KO embryos allows nuclear accumulation of Yap, which in turn complexes with Tead4 and induces *Amotl2* expression.

The role of Amot appears to be context dependent. Although a recent study showed that Amot functions as a transcriptional cofactor of Tead-Yap in the nuclei of adult liver cells and HEK293 cells,[28] it does not play such a role in preimplantation embryos. In the absence of Amot, embryos express the Tead4 target gene *Cdx2* and form TE.

Activation of Hippo Signaling by Phosphorylated Junctional Amot

The cell adhesion molecules of the AJ are cadherins. Homodimerization of E-cadherin reportedly activates the Hippo pathway in cultured cells.[29] The primary cell adhesion molecule in preimplantation embryos, E-cadherin, is involved in the activation of the Hippo pathway.[9,10,30] The cytoplasmic domain of E-cadherin interacts with the adaptor proteins α-, β-, and p120-catenin. α-Catenin interacts with a FERM domain-containing Hippo component, Merlin (encoded by *Nf2*),[31] which interacts with Amot.[21] Probably through these interactions, Amot interacts with the E-cadherin/catenin complex.[9] Indeed, Merlin is also essential for the activation of the Hippo pathway in preimplantation embryos.[32] In the inner cells, both Merlin and Amot are present at the AJs, permitting Hippo signaling to occur. The conserved serine residue at position 176 of mouse Amot (S176; corresponding to S175 in human AMOT) is phosphorylated by Lats.[9,33-35] In preimplantation embryos, phosphorylated Amot (p-Amot) is present exclusively at the AJs (**Fig. 3B**). The phosphomimetic form of mouse Amot (Amot-S176E) shows increased interaction with Lats2 and constitutively activates Hippo

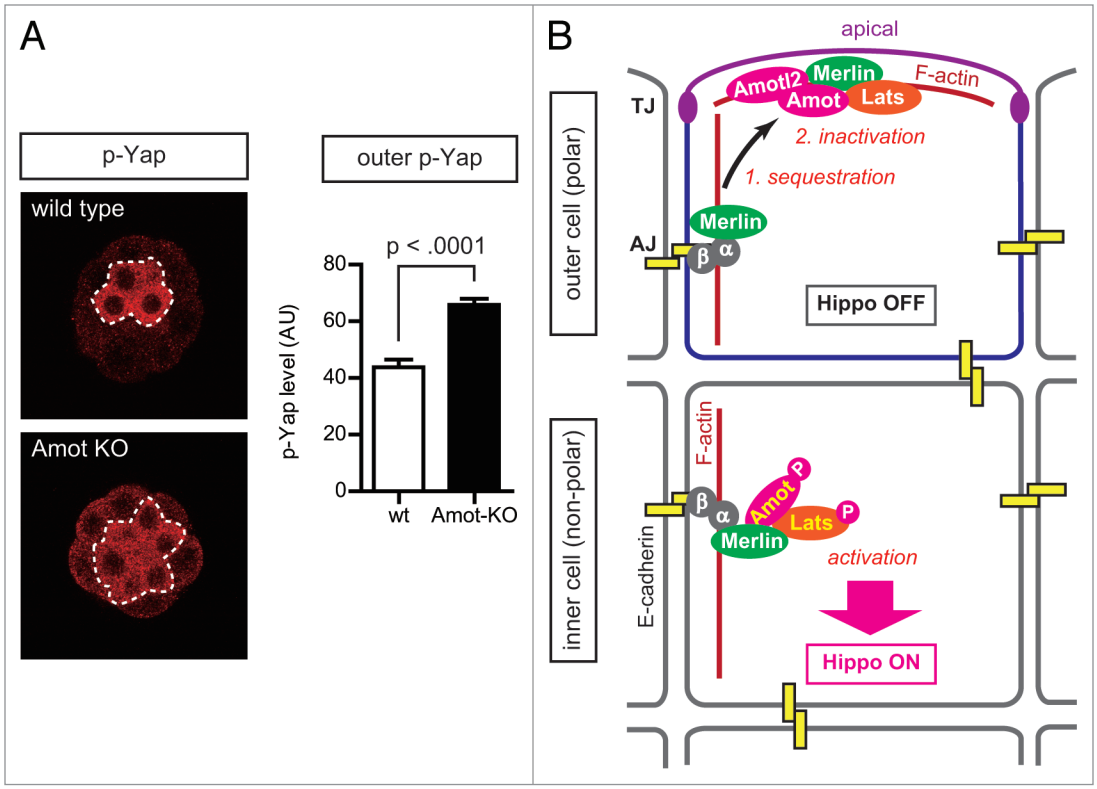

Figure 3. The molecular basis of differential Hippo signaling in preimplantation embryos. (**A**) *Amot* KO enhances Hippo signaling in the outer cells as shown by an increased level of phosphorylated Yap (p-Yap). The inner cells are encircled by dashed lines in the left panels. The graph shows the mean level of p-Yap in the outer cells of wildtype (wt) (n = 4) and *Amot* KO (n = 5) embryos. Error bars show the standard error of the mean. These data suggest that Hippo signaling is inhibited at the apical membrane. (**B**) A model of Hippo pathway regulation in preimplantation embryos. In the inner cells, phosphorylated Amot makes an active complex with Merlin–Lats at the AJs, activating Hippo signaling. In the outer cells, (1) cell polarity factors sequester Amot from the basolateral AJs, thereby attenuating E-cadherin-mediated Hippo signaling. (2) Nonphosphorylated apical Amot makes an inactive complex with Merlin–Lats, which binds cortical F-actin, suppressing Lats activity.

signaling, indicating that the phosphorylation of Amot is a key mechanism that switches on the Hippo pathway (**Fig. 3B**).

Cell Polarity Controls Hippo Signaling Through Amot

Although AJs are present in both the inner and outer cells, the outer cells show a significantly lower level of Hippo signaling than the inner cells. Differences in cell polarization play a role in establishing this difference. As described previously, only the outer cells have apicobasal cell polarity, and this polarization is regulated by the Par-aPKC system.[9,36-38] Disruption of cell polarity by the knockdown of *partitioning defective 6* β (*Pard6b*) results in the aberrant activation of Hippo signaling, a reduction in Cdx2 expression in the outer cells, and the disruption of TE epithelialization at a later stage.[9,36] The disruption of cell polarity by inhibiting other Par-aPKC regulators also results in the aberrant activation of Hippo signaling in the outer cells.[9]

The distribution of Amot is one of the key mechanisms that links cell polarity with Hippo signaling in preimplantation embryos. In the outer cells of wildtype embryos, Amot is restricted to the apical domain and is not present in the basolateral AJs (**Fig. 2C**); however, in embryos with disrupted polarity, Amot is present in all domains of the plasma membrane, including the AJs of the outer cells (**Fig. 2C**). Formation of the Amot–Merlin complex at AJs activates the Hippo pathway in the outer cells of the polarity-disrupted embryos. Therefore, Hippo signaling in wildtype outer cells is normally attenuated by cell polarity factors that sequester Amot from the basolateral AJs (**Fig. 3B**). The mechanism by which cell polarity controls Amot distribution in preimplantation embryos remains to be elucidated. This may involve the interaction of Amot with Par3.[19] Amot is a tight junction (TJ) component in epithelial cells, and it largely colocalizes with the TJ protein ZO-1 in both the outer and nonpolar inner cells of the embryo.[9] Therefore, it is also possible that Amot is regulated in parallel to or through its interaction with ZO-1.

Inhibition of Hippo Signaling by Non-Phosphorylated Apical Amot

In our current model, the Amot–Merlin complex at AJs recruits and activates Lats. This model is consistent with other Hippo signaling models, which also propose that Lats is activated at the plasma membrane.[39,40] Amot and Merlin are not only present at the AJs of the inner cells,

but also at the apical membranes of the outer cells and, notably, Lats is also found at the apical membrane.[32] Colocalization of these molecules at the apical membrane is puzzling, because nearly no Hippo signaling is elicited in the outer cells despite the colocalization of these proteins. Interestingly, the outer cells of *Amot* KO embryos show increased Hippo signaling relative to wildtype embryos (**Fig. 3A**).[9] The knockdown of *Amotl2* in *Amot* KO embryos results in the loss of Hippo signaling, suggesting that the signaling in *Amot* KO embryos is mediated by Amolt2.[9,17] These results suggest that in wildtype embryos, apical Amot inhibits Hippo signaling induced by Amotl2. This is opposite to the role of junctional Amot. Unlike junctional Amot, apical Amot is not phosphorylated, raising the possibility that the function of Amot in Hippo signaling depends on its phosphorylation state.[9]

Perspective: The Role of Amot Phosphorylation in F-Actin-Mediated Lats Inhibition

Amot has an F-actin binding domain in its N-terminal region, and the phosphorylation of S176 inhibits its actin binding activity.[9,33-35] A nonphosphorylatable form of Amot (Amot-S176A) binds to F-actin, whereas a phosphomimetic Amot (Amot-S176E) does not. F-actin is known to inhibit Lats activity.[41-44] Because Amot interacts with both Lats and F-actin and both interactions are regulated by phosphorylation of Amot on S176, we would propose a model where Amot phosphorylation acts as a molecular switch of Lats activity (**Fig. 3B**).

Amot forms a complex with Merlin–Lats at both the AJs of the inner cells and the apical domain of the outer cells. Activity of the Amot–Merlin–Lats complex is regulated by the phosphorylation status of Amot. In the outer cells, the nonphosphorylated-Amot (np-Amot)–Merlin–Lats complex localizes to the apical domain. Through the interaction of np-Amot with F-actin, the complex is localized to the apical cortex. In the np-Amot complex, Lats is inactive. In the inner cells, the p-Amot–Merlin–Lats complex does not bind to cortical F-actin and, instead, it localizes to the AJs through its interaction with the E-cadherin/catenin complex. Lats is active in this form of the complex. Although the mechanism by which phosphorylated Amot controls Lats activity remains to be elucidated, it is likely that the phosphorylation status alters the conformation of Amot and/or the protein–protein interactions within the complex to activate/inactivate Lats. Supporting this notion, phosphorylation enhances the interaction between Amot and Lats.[9] It is also possible that F-actin and/or F-actin-interacting proteins have Lats inhibitory activities and the binding of np-Amot to F-actin recruits and inactivates Lats.

Conclusions

Although growing evidence highlights the importance of F-actin in the regulation of the Hippo pathway, the mechanism of F-actin-mediated regulation of the Hippo pathway remains elusive. In this regard, the discovery of phosphorylation-dependent regulation of Amot-F-actin binding should contribute significantly toward our understanding of this mechanism. The regulation of the Hippo pathway by cell polarity also requires further investigation, as cell polarity regulates the Hippo pathway in a context-dependent manner. In preimplantation embryos, cell polarity suppresses Hippo signaling, whereas the disruption of cell polarity in *Drosophila* and cultured mammalian epithelial cells inactivates Hippo signaling.[45-49] The role of Amot in epithelial cells is also context-dependent. Apical Amot in the outer cells of preimplantation embryos promotes the accumulation of nuclear Yap by suppressing Hippo signaling, whereas Amot in cultured epithelial cells inhibits the nuclear accumulation of Yap by tethering Yap to TJs.[23-25] Because Amot phosphorylation is a key event for both Hippo pathway activation and the direct interaction of Amot and Yap,[9,34] the regulatory mechanism of Amot phosphorylation should be further investigated to clarify the opposing context-dependent activities of Amot. Amot plays pivotal roles in junction formation, actin polymerization, cell polarity, and the Hippo pathway. Therefore, elucidating the functions of Amot will improve our understanding of the integration of cell adhesion, cell morphology, and the Hippo pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We apologize for those primary works that are not cited due to the scope of this review and space constraints. The research in the laboratory of HS is supported by Grants-in-Aid for Scientific Research (KAKENHI) from MEXT (21116003) and JSPS (23247036) to HS, and from JSPS (23770265) to YH.

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