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Epithelial-mesenchymal transition - activating transcription factors - multifunctional regulators in cancer

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Abstract

The process of epithelial to mesenchymal transition (EMT), first noted during embryogenesis, has also been reported in tumor formation and leads to the development of metastatic growth. It is a naturally occurring process that drives the transformation of adhesive, non-mobile epithelial like cells into mobile cells with a mesenchymal phenotype that have ability to migrate to distant anatomical sites. Activating complex network of embryonic signaling pathways, including Wnt, Notch, hedgehog and transforming growth factor-β pathways, lead to the upregulation of EMT activating transcription factors, crucial for normal tissue development and maintenance. However, deregulation of tightly regulated pathways affecting the process of EMT has been recently investigated in various human cancers. Given the critical role of EMT in metastatic tumor formation, better understanding of the mechanistic regulation provides new opportunities for the development of potential therapeutic targets of clinical importance.

Key words: Epithelial-to-mesenchymal transition; Metastatic growth; Embryonic signaling pathways; Transcription factors; Cancer

Core tip: This review article discusses the mechanistic regulation of embryonic signaling pathways with a special focus on epithelial mesenchymal transition (EMT)-activating transcription factors in cancer progression *via* modulating the process of EMT. Deciphering this mechanism may lead to the design of cancer therapies by altering the balance between the process of EMT/mesenchymal epithelial transition in cancer stem cells and thereby clinically treat the cancer.

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INTRODUCTION

Epithelial-mesenchymal transition is naturally occurring and a vital process during embryogenesis, adult tissue repair and maintenance. It is characterized by specific gene expression pattern changes, loss of adherent tight junctions that keep epithelial cells in contact with their neighbors, gain of mesenchymal phenotype, including fibroblastoid morphology, and increased mobility potential to distant locations.

Based on the biological context, epithelial mesenchymal transition (EMT) is classified into three types[1]. Type 1 EMT is associated with the transition of epithelial cells to motile mesenchymal cells during implantation, embryo formation, gastrulation, neural crest delamination, in the development of placenta, somites, heart valves, urogenital tract and secondary palate, as well as during branching morphogenesis of different organs. Primary mesenchymal cells act as progenitors and *via* the process of mesenchymal epithelial transition generate secondary epithelia in mesodermal and endodermal organs. Type 2 EMT describes the events in wound healing, tissue regeneration and organ fibrosis where tissue fibroblasts

are generated from epithelial or endothelial cells. Type 3 EMT has been described in epithelial cancer cells where the cells dedifferentiate and acquire a mesenchymal phenotype. These cells invade and metastasize through the circulation and generate a metastatic lesion at distant tissues or organs by MET[2]. The three types of EMT program are considerably distinct biological processes but tumor cells take over the developmental pathways for metastatic dissemination through well conserved or similar genetic controls and biological mechanisms. Besides acquiring a mesenchymal property, cancer cells with EMT phenotype exhibit more aggressive behavior, including resistance to drugs, stresses and apoptosis, inhibition of senescence, immune evasion and acquisition of stem cell-like features. All these dramatic changes in tumor cells allow them to infiltrate surrounding tissues, metastasize at distant locations and promote cancer progression.

Growing evidences in the last few years document the key role of EMT-activating transcription factors (ATFs) in oncogenic transformation. They override cancer safeguard programs against cancer like apoptosis, senescence, regulate cancer cell stemness, determine resistance to chemotherapy and promote tumor angiogenesis.

EMT-ACTIVATING TRANSCRIPTION FACTORS

Recent research documents the involvement of EMT in the induction of cellular traits associated with the metastatic progression of cancer. EMT is characterized by the downregulation of epithelial markers/tight junction components, desmosomes, cytokeratins and gain of mesenchymal markers like reorganization of the cytoskeleton (*e.g.,* switch from cytokeratins to vimentin), and the synthesis of extracellular matrix components and metalloproteases[3]. Loss-of-function mutations and promoter hypermethylation could downregulate E-cadherin expression and function in a number of carcinomas, but modulation of EMT during embryogenesis and cancer progression mostly involves the participation of EMT-ATFs. In addition to a mesenchymal switch, these factors control the entire EMT program and endow cancer cells with stem-like characteristics. These migrating cancer stem cells are not only important in the genesis of primary tumors, but also enhance metastasis and possibly the root cause of tumoral chemoresistance and recurrence[4].

Molecular reprogramming occurring during EMT is triggered and orchestrated by various EMT-ATFs, including the Snail family of zinc-finger transcription factors: Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc); the two-handed zinc-finger factors of d-crystallin/E2 box factor (dEF1) family proteins dEF1/zinc-finger E-box-binding homeobox (ZEB)1 and Smad-interacting protein (SIP)1/ZEB2; the basic helix-loop-helix factors (Twist1 and Twist2); E12/E47 and Tbx3[5-8]. These factors act as molecular switches, respond to the known signaling pathways and regulate the EMT program. These transcription factors recognize the E-box DNA sequences in the promoter region of E-cadherin, recruit cofactors and histone deacetylases and thereby repress its expression[9].

The expression of other epithelial molecules, including claudins, occludins and mucin1, are suppressed by Snail while the genes associated with the mesenchymal and invasive phenotype are induced. Snail has been linked with tumor grade, metastasis, recurrence and poor prognosis, and suppresses tumor suppressor Raf kinase inhibitory protein (RKIP), an inhibitor of nuclear factor kappa B (NF-κB)[10,11]. Snail and Twist further cooperate in inducing the expression of ZEB1 and act as key regulators in the process of EMT. These oncogenic factors when overexpressed in neoplastic cells make them more aggressive and promote the development of metastatic properties. Studies in cell lines and xenograft mice models verify EMT-ATFs' functions in cancer and set them not only as important diagnostic and prognostic biomarkers, but also as potential therapeutic targets. In view of the expanding portfolio of EMT-ATFs as multifunctional regulators in the hallmarks of cancer, it will be important to review their mechanisms of actions.

REGULATORY MECHANISMS OF EMT-ATFS

ZEB1 and ZEB2

The ZEB family comprises of zinc finger/homeodomain proteins-ZEB1 and ZEB2, well conserved among species, interacts with other transcriptional regulators and their activities are modulated by post-translational modifications like SUMOylation by Pc2 or acetylation by p300/pCAF and phosphorylation. These proteins trigger an EMT by repression of epithelial markers and activation of mesenchymal properties.

Growth and steroid hormones; hypoxia inducible factor-1α (HIF-1α) in hypoxic conditions; inflammatory cytokines; ligands [*e.g.,* fibroblast growth factor (FGF), insulin growth factor-1, platelet derived growth factor receptor]; downstream signals frequently activated in tumors like Ras-ERK2-Fra1, NF-κB and JAK/STAT3; classical signaling pathways-transforming growth factor β (TGFβ)/smad; Wnt and Notch directly activate the expression of ZEB factors. They are also regulated by miR-200a-microRNA that inhibits ZEB factors in a reciprocal negative feedback loop and finally induces the emergence of mesenchymal-epithelial transition phenotype in various cancers, extensively reviewed by Sánchez-Tilló *et al*[12].

Snail, Slug and Smuc

All three members of the Snail family, Snail, Slug and Smuc, share the SNAG domain at N terminal region and zinc finger cluster at C terminal region that binds to E-boxes in the regulatory regions of target genes. Snail-mediated histone modifications (deacetylation, methylation and demethylation) cooperate to repress E-cadherin but this mechanism is still not completely known. Post-transcriptional modifications alter protein stability and in-

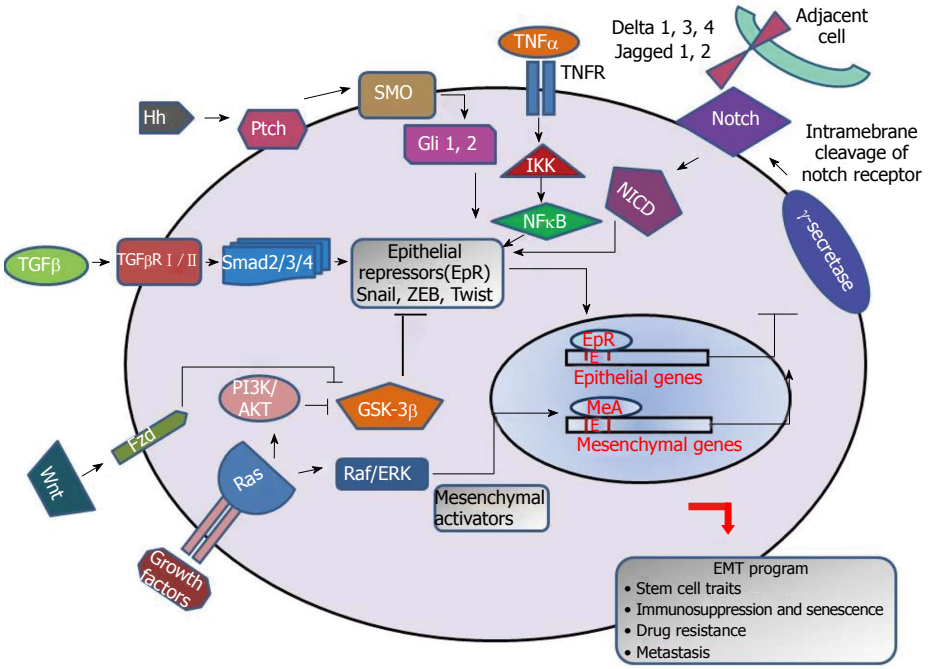

Figure 1 Schematic illustration of embryonic signaling pathways mediating epithelial-to-mesenchymal transition. Wnt, Notch, hedgehog, transforming growth factor β (TGFβ) along with other growth factors of cytokines transduce signal cascades, modulate the expression of epithelial-to-mesenchymal transition (EMT) regulators and allow them to translocate to nucleus. They act as epithelial repressors (EpR) and/or mesenchymal activators (MeA) and bind with E box of promoter regions of epithelial genes and mesenchymal genes respectively. These complexes have an inductive effect on EMT program by repressing epithelial genes and activating mesenchymal genes. IKK: IκB kinase; NF-κB: Nuclear factor kappa B; SMO: Smoothened; TNF-α: Tumor necrosis factor-α; PI3K: Phosphatidylinositol 3 kinase; GSK3β: Glycogen synthase kinase 3β; Fzd: Frizzled.

tracellular localization of Snail1 and Snail2 and modulate their transcriptional activities. Signals, including TGFβ, Notch, tumor necrosis factor-α (TNF-α), EGF, FGF, Wnt, Shh, SCF/c-kit, hypoxia and estrogens, regulate Snail proteins, not only during development but also in cancer cells[3].

Twist1 and Twist2

Basic/bHLH domain in Twist1 and Twist2 mediates their binding to DNA and homo/ hetero-dimerization. Twist box at the C terminal end is involved in both transcriptional activation and repression[13,14]. Binding of Twist factors to other transcriptional regulators, post-translational modifications and choice of partner for dimerization regulate the expression of target genes. Twist1 interaction with components of the NuRD complex, polycomb repressor complexes PRC1 and PRC2 at the E-cadherin promoter is required for E-cadherin repression. In addition, binding of Twist1 to the H4K20 methyltransferase SET8 represses E-cadherin and activates N-cadherin[15,16].

Homo- or heterodimerization of Twist proteins depends on the availability of E12 where Twist/E12 heterodimers can both activate or repress transcription. Post-translational modification like phosphorylation of the bHLH domain of Twist not only alters dimerization partner choice but also binding affinity for DNA[13]. Classical EMT-inducing pathways, such as TGFβ, Wnt, hypoxia and ligand binding activation of receptor tyrosine kinases and inflammatory cytokines receptors, activate Twist factors and have significant implications in tumor invasion and angiogenesis[14,17].

Pathways implicated in modulating the EMT/MET program and tumor progression are further reviewed with a special focus on the involvement of transcription factors in the complex network of signaling pathways.

SIGNALING PATHWAYS INDUCING EMT IN CANCER

The Hh, Notch, Wnt and TGF-β signaling pathways interact through cross talk and serve to increase cellular diversity to extracellular stimuli, elicit EMT response by mobilizing embryonic transcription factors, reprogramming the epithelial cell to acquire both progenitor-like, pro-motility and mesenchymal features (Figure 1). Identification and understanding the interlinked cross-talk in cancer cells which provide tumor cells with an additional mechanism to evade chemotherapy may allow designing more effective anti-tumor combinational therapies.

TGFβ pathway

TGFβ signaling has been implicated in the process of EMT, embryogenesis and cancer pathogenesis. During early stages of tumor growth, TGFβ acts as a tumor suppressor and induces growth arrest and apoptosis. In later stages of tumor progression, TGFβ and its receptors along with other receptor tyrosine kinases (RTKs) regulate transcription by Smad dependent/independent TGFβ receptor signaling pathways, initiate cancer growth and metastasis. TβR Ⅰ and TβR Ⅱ, serine-threonine kinase receptors after binding with TGFβ ligand, activate Smad2 and Smad3. Activated Smad2/3 forms complexes with Smad4, translocated into the nucleus, and interact with various transcription factors/coactivators to regulate target gene transcription[18]. Many EMT promoting transcription factors like Snail (Snail1 and Snail2/Slug), Twist (basic helix- loop- helix), six family homeobox and ZEB (ZEB1 and ZEB2/SIP1) are induced by TGFβ. These transcription factors interact with Smads and result in the formation of EMT promoting Smad complexes. This is followed by suppression of transcription of E-cadherin, occludin and claudin, and promotion of

tumor growth[19,20]. TGFβ signaling activates Smad independent pathways-phosphatidylinositol 3 kinase (PI3K), Akt, mitogen activated protein kinase and small GTPases of the Rho family, which function along with Smad dependent pathways to modulate the transcription of EMT regulators-Snail, Twist and ZEB. TGFβ also collaborate with other signaling pathways: Notch, Wnt/βcatenin, NFκB, RTKs, PDGF/PDGF receptor autocrine loop to facilitate an EMT like switch, allow efficient cell migration and invasion of metastatic cancer cells[21-25].

Notch pathway

Four Notch receptors (Notch1-4) bind with the five ligands (Jagged1, 2 and Delta like1, 3, 4), initiate Notch signaling between adjacent cells, followed by intramembrane cleavage of Notch receptor by γ-secretase, release of Notch intracellular domain and translocation to the nucleus to generate a transcription factor complex with transcriptional regulators CSL (RBPJk), mastermind like 1 and histone acetyltransferase p300/CBP. Notch target genes *Myc*, cell cycle regulator p21, hairy/enhancer of split (HES) and the HES related repressor (HERP, HRT and HEY) families are activated but insufficient to induce EMT and hence are coordinated with other signaling pathways. TGFβ increases Notch activity through Smad3, upregulate Jagged1 and HEY1 and thereby Slug expression, and suppress E-cadherin. The Wnt1 transformed cells enhance expression of the Delta like1, 3 and 4 ligands, needed for tumorigenic phenotype. Notch controls Snail expression either directly or by indirect mechanisms which operate *via* increased expression of lysyl oxidase (LOX) by recruiting HIF-1α to LOX promoter. This stabilizes the Snail protein, induces EMT and cancer metastasis[2].

Wnt/βcatenin pathway

Wnt signaling can be either canonical/β-catenin or non-canonical. Activation of canonical signaling is initiated by binding of Wnt ligands (Wnt1 and Wnt3a) to seven transmembrane domain receptor Frizzled (Fzd) and the lipoprotein receptor related protein complexes. Activation of Wnt signaling leads to the inhibition of destruction complex which consists of adenomatous polyposis coli, glycogen synthase kinase 3β (GSK3β), Axin and casein kinase-1α. This inhibition of destruction complex stabilizes β-catenin and allows its accumulation/ translocation to the nucleus followed by its interaction with the lymphoid enhancer factor/T cell factor complex leading to targeted gene transcription. Fifty percent of breast carcinomas show β-catenin accumulation within the nucleus or cytoplasm and it is correlated with a poor prognosis. EMT program is activated by induced expression of intracellular protein Axin2 that stabilizes Snail and by blocking the activity of GSK3β[26,27]. In addition to Wnt/β-catenin canonical pathway, Wnt ligands can activate EGFR signaling through Fzd, whereas EGFR can activate β-catenin, a downstream effector of the Wnt pathway, *via* the RTKPI3K/Akt pathway, and plays a critical role in cell proliferation and oncogenesis[28]. Induction of Wnt signaling, EMT and stem cell-like properties, including the $CD44^{high}/CD24^{low}$ signature by silencing or loss of the Wnt antagonist secreted frizzled-related protein 1, has been reported in numerous types of human cancer, including colon, breast, melanoma and prostate carcinomas.

Hedgehog pathway

Hedgehog signaling not only modulates tissue polarity but also maintains stem-cell characteristics. Binding of Hh ligands, like Sonic, Desert and Indian to Patched, a 12-pass transmembrane protein, results in the de-repression of smoothened (SMO) and its translocation to the primary cilium, internalization and activation. The zinc-finger transcription factors GLI-1, -2 and -3 are activated, leading to transcription of GLI target genes[29]. Hh signaling regulates EMT by inducing the expression of a repressor of E-cadherin, Snail1. Decreased E-cadherin expression and induced expression of mesenchymal cell markers including Snail has been observed during tumor development and progression. SHH-Gli1 signals are reported to promote EMT by mediating a complex signaling network, including TGFβ, Ras, Wnt, growth factors, PI3K/AKT, integrins, transmembrane 4 superfamily and S100A4 in pancreatic cancer cells. Colorectal xenografts with high metastatic potential, epithelial morphology and EMT-associated markers are examined to have high GLI-1 expression, while SMO antagonists/inhibitors in pancreatic cancer cell lines block EMT and metastasis[30,31].

TNF-α and NF-κB pathway

TNF-α acts as a tumor promoting factor which signals through two distinct cell surface receptors, TNFR1 and TNFR2. TNF receptor associated factor and receptor interacting protein (RIP) are recruited by TNFR1 associated death domain protein, which in turn recruits IκB kinase (IKK) complex and is activated in a RIP dependent manner. Inhibitory protein IκB gets phosphorylated by IKK complex and promotes its rapid ubiquitination and proteasome mediated degradation, thus releasing NF-κB. Upon translocation of free NF-κB to nucleus, many transcription factors, such as Snail, Slug, Twist, and ZEB1/ZEB2 involved in EMT, are induced and mesenchymal marker vimentin and matrix metalloproteinases (MMPs), such as MMP2 and MMP9, are activated[32,33]. Recently, a circuitry between RKIP, a metastatic suppressor, NF-κB and Snail has been identified where overexpression of Snail in tumors inhibits RKIP and induces EMT[34].

Receptor tyrosine kinase pathway

Growth factors, such as hepatocyte growth factor, epidermal growth factor or FGF, activate extensive cross talk between signaling pathways and play a key role in determining the balance between epithelial and mesenchymal traits in cancer cells. They transduce signals *via* constitutive activation of receptor tyrosine kinases (RTKs) and their downstream signaling effectors, such as MAPK

Table 1 Increased expression of epithelial mesenchymal transition-activating transcription factors inducing various hallmarks of cancer

Hallmarks of cancer	Increased expression of EMT-ATFs	Cancer type	Ref.
Increased migratory potential and invasion	Snail, Slug	TNBCs	[42]
	ZEB1, ZEB2	HNSCCs	[43]
	Snail, Slug	Pancreatic cancer	[44]
	Snail	Ovarian cancer	[45]
	Snail1, Slug	Lung cancer	[46]
	SIP1	Intestinal type gastric cancer	[47]
	Snail	Endometrial cancer	[48]
	Twist1	Melanoma	[49]
	ZEB1	Colon cancer	[50]
	SNAI1 and Twist1	Colorectal adenomas	[51]
Angiogenesis	Snail and ZEB1	Colon cancer	[52]
	Twist1	Breast cancer	[53]
	Snail, Slug, and ZEB1	Breast cancer	[54]
Chemo/radioresistance	Snail, Slug	Breast cancer	[55]
	Twist1	Cervical cancer	[56]
	Snail	Head and neck cancer	[57]
	Snail2	Colorectal cancer	[58]
	Snail	Lung cancer	[59]
Resistance to anoikis	SIP1	Intestinal type gastric cancer	[47]
	Snail	Pancreatic cancer	[60]
	ZEB1, Snail1, Slug	Colorectal cancer	[61,62]

EMT-ATFs: Epithelial mesenchymal transition-activating transcription factors; TNBCs: Triple-negative breast cancers; HNSCCs: Head and neck squamous cell carcinomas; ZEB1: zinc-finger E-box-binding homeobox.

or PI3K, affect the expression of EMT regulators, control cytoskeletal organization and confer epithelial cells with an increased rate of proliferation[2]. Activation of Ras mediated by growth factors also cooperates with TGFβ to induce Snail1 expression. Ras-activated MAPK promotes EMT and metastasis *via* increasing Twist1 serine 68 phosphorylation and stabilization in breast tumor cells. Inhibition of the ERK-MAPK pathway has been reported to restore E-cadherin expression in cells with moderate levels of Ras signaling. Despite having ability to destroy epithelial cell polarity and tight junction assembly, these oncogenic pathways whose signaling involves RTKs fail to induce EMT and mesenchymal migratory phenotype. Interplay of multiple signaling pathways has been reported to sufficiently elicit EMT in various carcinomas.

EMT AND CANCER STEMNESS

Cellular traits associated with the metastatic progression of cancer are believed to be induced by EMT. In recent years, multiple research reports are being added up to prove the significant involvement of interlinked network of signaling pathways *via* induction of EMT-ATFs in EMT control programs. The origin of cancer stem cells is controversial and it is not clear whether different cancer stem cells arise from multipotent tissue stem cells or from reprogramming of differentiated cells that revert to a stem cell-like phenotype. Research advancements suggest that invading carcinoma cells which function as migrating CSCs have undergone an EMT process. The acquisition and maintenance of stemness in non-tumorigenic, immortalized human mammary embryonic cells, along with the capacity to form mammospheres, self-renewal and increasing tumorigenecity in xenotransplants by overexpressing EMT-ATFs has been observed[35-37]. Higher expression of EMT-ATFs in colorectal and ovarian cancers correlates with the derepression of stemness gene signature further suggests the origin of CSCs from the dedifferentiation of non-stem cancer cells rather than proliferation of existing CSCs. Experimental evidences in ovarian cancer cells suggest that increased expression of Snail1 and Snail2 mediate chemo and radioresistance by inducing expression of stem-like promoting genes, such as Nanog, kruppel-like factor 4 and T cell factor 4, and by suppressing p53-mediated apoptosis[38,39]. EMT induction and acquired stem-cell like characteristics in pancreatic tumor cells and breast cancer cells are linked with gemcitabine and tamoxifen resistance respectively. Table 1 sites current studies on the connection between the increased expression of EMT-ATFs and various hallmarks of cancer induced by them in human cancer.

Experimental studies in human cancer cell lines explore the possibility of cancer cells transition between tumorigenic and non-tumorigenic states and this balance can be altered by modulating the EMT-ATFs expression. Since the drug resistance to apoptosis exhibited by cancer cells due to induced EMT is critical for the ability of cells to survive the passage from primary tumors to the sites of dissemination, potential reversal of EMT by silencing EMT master regulators could restore the drug sensitivity. This opens up new therapeutic strategies to reverse metastable EMT by stabilizing the non-invasive epithelial phenotype and restore sensitivity to cytotoxic agents and thereby clinically treat the cancer[40,41].

CONCLUSION

Reactivation of an embryonic development program re-

ferred to as EMT is the main cause for the metastatic dissemination of cancer cells from the primary tumor. It is a highly conserved morphogenic process, associated with the loss of epithelial cell markers, apicobasal polarity, cell-cell contacts and gain of mesenchymal phenotypes with increased invasive characteristics. Transcription factors regulating the process of EMT belong to the ZEB, Snail and Twist families and are tightly controlled at transcription, translational, protein stabilization and epigenetic levels. Activation of these factors by complex network of dynamic signaling pathways are implicated in the cancer stem cell property, immune suppression, increased resistance to radio/chemotherapeutic drugs and cancer recurrence. Deciphering the mechanistic regulation between EMT, increased metastatic potential, expanding subpopulation of cancer stem cells and chemoresistance may lead to improvements in cancer therapy. Potential implications of various anticancer agents in altering the levels of master regulators of EMT switch, killing cancer stem cells by sensitizing them to radio/chemotherapeutic drugs, as well as increasing response to DNA damage and reprogramming the sensitivity of tumor cells to apoptosis, may provide a novel therapeutic strategy in the treatment of cancers.

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