

# EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness

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**Abstract** Cancer is a complex multistep process involving genetic and epigenetic changes that eventually result in the activation of oncogenic pathways and/or inactivation of tumor suppressor signals. During cancer progression, cancer cells acquire a number of hallmarks that promote tumor growth and invasion. A crucial mechanism by which carcinoma cells enhance their invasive capacity is the dissolution of intercellular adhesions and the acquisition of a more motile mesenchymal phenotype as part of an epithelial-to-mesenchymal transition (EMT). Although many transcription factors can trigger it, the full molecular reprogramming occurring during an EMT is mainly orchestrated by three major groups of transcription factors:

the ZEB, Snail and Twist families. Upregulated expression of these EMT-activating transcription factors (EMT-ATFs) promotes tumor invasiveness in cell lines and xenograft mice models and has been associated with poor clinical prognosis in human cancers. Evidence accumulated in the last few years indicates that EMT-ATFs also regulate an expanding set of cancer cell capabilities beyond tumor invasion. Thus, EMT-ATFs have been shown to cooperate in oncogenic transformation, regulate cancer cell stemness, override safeguard programs against cancer like apoptosis and senescence, determine resistance to chemotherapy and promote tumor angiogenesis. This article reviews the expanding portfolio of functions played by EMT-ATFs in cancer progression.

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### Abbreviations

bHLH	Basic helix loop helix
CSC	Cancer stem cells
EMT	Epithelial-to-mesenchymal transition
EMT-ATF	EMT-activating transcription factors
HNSCC	Head and neck squamous cell carcinoma
MET	Mesenchymal-to-epithelial transition
NSCLC	Non-small cell lung carcinoma

### Introduction

Cancer is a multipronged process that requires the acquisition by malignant cells of a number of capabilities—referred as hallmarks of cancer [1]—that ensure their survival and proliferation and, therefore, the growth of the tumor. Cancer cells in a solid tumor can detach from the main mass to invade the surrounding stroma, enter the circulation and eventually metastasize to distant organs. In most cases, invading cancer cells lose their polarity and intercellular adhesions and acquire a more motile phenotype as part of an epithelial-to-mesenchymal transition (EMT) [2–5]. While EMT was identified in the context of embryogenesis almost three decades ago, its underlying molecular mechanisms have only begun to be understood more recently with the discovery of its role in tumor invasiveness.

A hallmark of EMT is the functional loss of the adherens junction protein E-cadherin. Although transcriptional control of E-cadherin during EMT seems to be prevalent, recent studies point to the existence of intricate relationships between epigenetic, transcriptional and translational mechanisms. Although E-cadherin expression is inhibited by a number of transcription factors, only a small set are known to regulate it directly. The main groups of transcription factors that bind to the E-cadherin promoter and directly repress its transcription—which will be referred to hereafter as EMT-activating transcription factors, EMT-ATFs—are the ZEB (ZEB1, ZEB2) and Snail (Snail1, Snail2, Snail3) families of zinc finger proteins and the Twist family of bHLH factors (Twist1, Twist2) [2, 3]. While E12/E47 and Tbx3 also bind to the E-cadherin promoter [6, 7], the ability of Gooseoid and HMGA2 to repress E-cadherin expression and induce an EMT seems to be mediated by other EMT-ATFs [8, 9]. Most of these transcription factors were originally identified as regulators of embryogenesis and cell differentiation and only later recognized for their role in cancer progression. Still, the

upstream regulatory signals and downstream targets of EMT-ATFs in cancer largely concur with those during embryogenesis [2, 3].

In addition to their now classical function as promoters of tumor invasiveness, over the last few years EMT-ATFs have gained new relevance in light of their role regulating several other hallmarks of cancer. EMT endows cells not only with greater ability to migrate but also with stem cell characteristics that play a role in tumorigenesis and resistance to chemotherapy. Recent evidence shows that EMT-ATFs also participate in early stages of cancer development cooperating in oncogenic transformation, overriding safeguard programs against cancer like apoptosis and senescence or promoting tumor angiogenesis. The expanding portfolio of functions of EMT-ATFs in cancer set them not only as important diagnostic and prognostic biomarkers but also as potential therapeutic targets. This article reviews the roles of EMT-ATFs in cancer and is organized as follows: The next section summarizes the EMT process in cancer. The following section reviews the main regulatory pathways and mechanisms of action of EMT-ATFs. Later sections describe the regulation of tumor invasiveness and metastasis by EMT-ATFs and overview their newly assigned roles in earlier stages of cancer progression. The final section concludes and presents EMT-ATFs as multifunctional regulators of the hallmarks of cancer.

### EMT in cancer

E-cadherin mediates homotypic intercellular adhesion and interacts with intracellular proteins to establish and coordinate the morphology, polarity and function of epithelial cells [10, 11]. The downregulation of E-cadherin is inherent to EMT, but EMT also entails the downregulation of other epithelial specification genes like components of tight and gap junctions, desmosomes, cytokeratins, etc. [2, 3]. In parallel, there is an induction of mesenchymal markers (e.g., N-cadherin, cadherin-11), reorganization of the cytoskeleton (e.g., switch from cytokeratins to vimentin), and the synthesis of extracellular matrix components and metalloproteases [2, 3].

Many signals unleashing an EMT during embryogenesis are also active in cancer. Thus, stimuli such as TGF $\beta$ , FGF, EGF, IGF, HGF, PDGF, estrogens, Wnt, Shh, inflammatory cytokines or hypoxia as well as oncogenes like Ras<sup>V12</sup>, ErbB2 or mutant p53 may be involved in EMT during cancer progression (reviewed in [3]). Triggering and maintenance of the mesenchymal state requires cooperation between several of these pathways through autocrine signaling loops [12].

These growth factors, inflammatory, hypoxic and oncogenic signals, along with an increasing number of microRNAs, converge in the induction of EMT-ATFs either at the transcriptional level or by increasing their protein or mRNA stability. E-cadherin expression and function could be downregulated by loss-of-function mutations, but modulation of EMT during embryogenesis and cancer progression mostly involves the participation of EMT-ATFs providing a high degree of functional redundancy and plasticity. E-cadherin has also been found to be silenced by promoter hypermethylation in a number of carcinomas. Some of the signals triggering EMT activate or repress the expression of non-coding microRNAs (miRs), which in turn modulate tumor invasion and metastasis by regulating EMT-ATFs transcripts (see below), targeting E-cadherin itself, or altering the expression of small GTPases or extracellular matrix receptors (reviewed in [13]).

Downregulation of E-cadherin is a critical initial step in EMT, not only because of the disruption of adherens junctions but also because loss of E-cadherin reinforces the EMT process by inducing the expression of Twist1 and ZEB1 in a feed-forward loop [194]. EMT-ATFs do not simply repress E-cadherin but are able to orchestrate the entire EMT program, inhibiting and activating a wide array of epithelial and mesenchymal genes, respectively [14–21].

Expression of EMT markers by primary human tumors correlates with enhanced invasiveness and poorer clinical prognosis. Cancer cells at the tumor invasive edge that have transitioned through an EMT secrete cytokines and proteases that promote angiogenesis, remodel the peritumoral extracellular matrix, and activate non-neoplastic stromal cells. In turn, stromal cells release factors that reinforce the EMT in cancer cells and foster survival, growth, and invasiveness of the tumor, thus creating a reciprocal influence between the tumor and its microenvironment [22, 23]. EMT is required for cancer cells to shift from a collective type of invasion—where cells retain E-cadherin and some intercellular adhesions—to an individual mesenchymal type of invasion, a change that depends on TGF $\beta$  signals at the tumor–host interface [24, 25]. While both models of invasion allow cancer cells to reach the lymphatic circulation, optimal hematogenous dissemination only occurs in EMT-mediated individual invasion [22, 25].

In addition to the mesenchymal switch, the molecular reprogramming encompassed by the EMT also endows cancer cells with stem-like characteristics [12, 26]. Many of the signals controlling normal stem cell homeostasis are inducers of EMT and seem to contribute to the generation and maintenance of cancer stem cells (CSCs). In an influential article, Brabletz and colleagues [27] proposed a model of tumor progression where only cancer cells at the

tumor–host interface, the “migrating CSCs”, undergo EMT and acquire mesenchymal and stem-like characteristics and therefore migratory and self-renewal capacities. CSCs are thought to be important in the genesis of primary tumors and metastasis and could also be at the root of tumoral chemoresistance and recurrence [28].

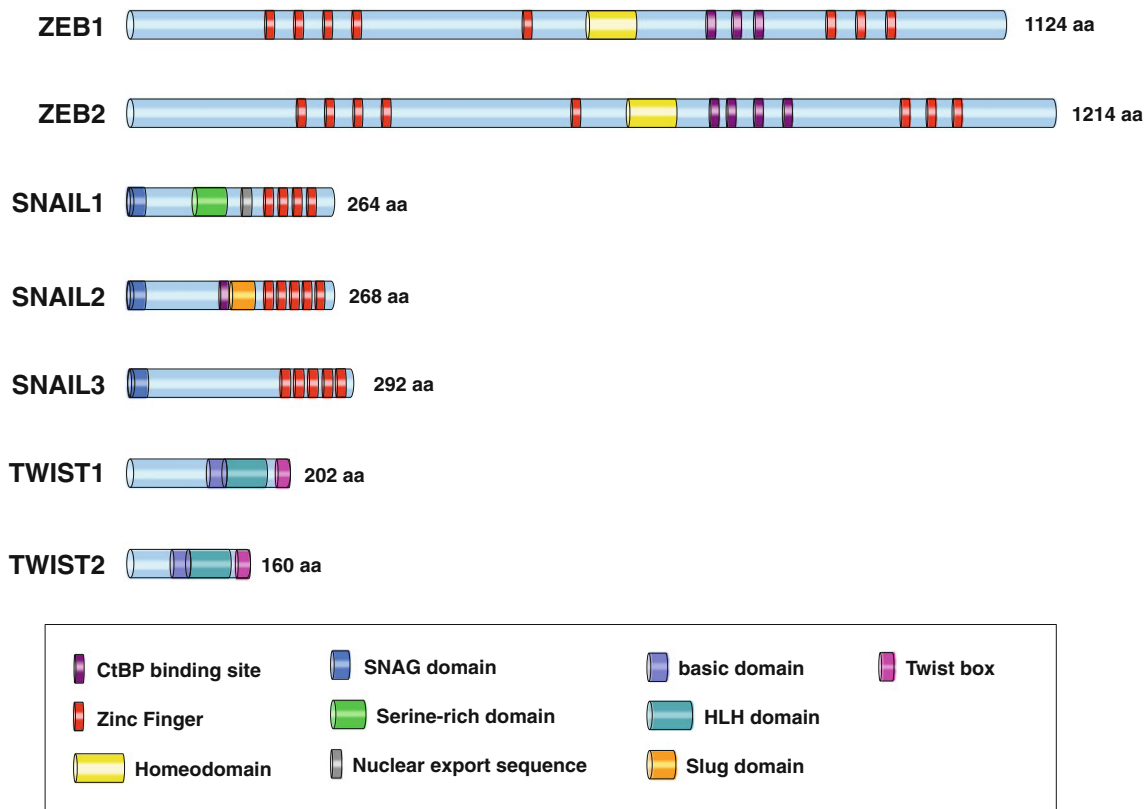
As in embryogenesis, the EMT occurring during cancer progression is a reversible process. At the site of micro-metastasis, epigenetic changes and the absence of EMT-inducing signals in the microenvironment lead to the re-expression of particular sets of microRNAs and the downregulation of EMT-ATFs (see below), allowing cancer cells to regain the epithelial characteristics of the primary tumor, through a mesenchymal-to-epithelial transition (MET), and grow to form a secondary tumor [29, 30]. In fact, recent evidence also indicates that EMT and metastatic dissemination of cancer cells may in fact occur from very early in tumor progression [31].

## Regulation and mechanisms of action of EMT-ATFs

### ZEB1 and ZEB2

The ZEB family comprises zinc finger/homeodomain proteins that are highly conserved across species (see an excellent review in [32]). In higher organisms, the family is constituted by two members: ZEB1 and ZEB2. ZEB factors contain multiple independent domains to interact with other transcriptional regulators (Fig. 1) (reviewed in [32] and [33], see also [34–41]). ZEB proteins bind to CtBP corepressors that in turn recruit histone deacetylases and methyltransferases, polycomb, and coREST [37–43]. Transcriptional repression by ZEB1 is also mediated through recruitment of the histone acetyl transferase Tip60, the SWI/SNF chromatin remodeling ATPase BRG1 and the histone deacetylase SIRT1 [44–46]. Meantime, in addition to CtBP, ZEB2 interacts with the NuRD remodeling and deacetylase repressor complex [47]. On the other hand, ZEB factors can also activate transcription through binding to histone acetyltransferases p300/pCAF ([48–50], reviewed in [33]). ZEB1 and ZEB2 repressor activities are modulated by post-translational modifications. SUMOylation by Pc2 or acetylation by p300/pCAF disrupt ZEB factor binding to CtBP [48–51]. Although its transcriptional significance remains unclear, phosphorylation of ZEB1 varies widely among cell types [52].

ZEB1 and ZEB2 trigger an EMT by repression of epithelial markers and activation of mesenchymal ones ([14–16], [20] and reviewed in [33, 53, 54]). ZEB1 and ZEB2 could also regulate EMT through their repressor effect on miRs (see below). ZEB2 mRNA also functions as a competitive endogenous RNA (ceRNA) sponging miRs



**Fig. 1** Domain structure human EMT-ATFs: human ZEB1 (GenBank reference sequence AAA20602.1), human ZEB2 (AAI27103), human Snail1 (NP\_005976.2), human Snail2 (NP\_003059.1), human

Snail3 (NP\_840101.1), human Twist1 (NP\_000465.1), and human Twist2 (NP\_476527.1)

targeting other transcripts, thus activating their expression [55].

ZEB1 and ZEB2 have overlapping, but still distinct, patterns of expression that result from their regulation by classical signaling pathways and an expanding set of microRNAs (see excellent reviews in [53, 54, 311] and below). In most cell types, TGF $\beta$  factors induce both ZEB proteins that in turn modulate TGF $\beta$  signaling in opposing ways with ZEB1 synergizing with R-Smads and ZEB2 repressing their activity. In fact, ZEB factors mediate some TGF $\beta$  functions during development, cell differentiation, and proliferation [48, 49, 56–58]. ZEB1 and ZEB2 are induced by HIF-1 $\alpha$  in hypoxic conditions, inflammatory cytokines, and by ligand-mediated activation (e.g., FGF, IGF-1, PDGFR) of receptor tyrosine kinases [59–66]. ZEB factors are directly activated by downstream signals frequently activated in tumors like Ras-ERK2-Fra1, NF $\kappa$ B, and JAK/STAT3 [61, 67, 68].

Aberrant activation of the canonical Wnt pathway by loss-of-function mutation of APC gene, as occurring in most colorectal carcinomas, induces ZEB1 [69]. ZEB1 expression is also induced by COX2/PGE2, which can activate Wnt signaling by inhibiting GSK3 $\beta$  [70]. The Notch pathway, deregulated in many cancers, also activates

ZEB1 expression [71, 72]. In turn, ZEB1 enhances Notch activity by indirectly increasing Jag1 and its coactivators Maml2/Maml3 [72]. ZEB factors are also upregulated by growth and steroid hormones [73–75]. Hippo/YAP signaling and the tumor suppressor Rb/E2F pathway activate and repress ZEB1 transcription, respectively, without affecting ZEB2 levels [76, 77]. SIRT1 is recruited by ZEB1 to repress the E-cadherin promoter but SIRT1 itself also induces ZEB and Snail factors but not Twist [46]. Interestingly, SIRT1 and miR-200a—a microRNA that inhibits ZEB factors (see below)—are involved in a reciprocal negative feedback loop [78]. ZEB1 is repressed at the promoter level by Grainyhead-like 2, a transcription factor tightly co-regulated with E-cadherin [79]. Finally, ZEB1 and ZEB2 are downstream of Snail and Twist factors (see below).

In addition to transcriptional regulation by these upstream signals, ZEB factors expression is also controlled at the mRNA and protein level. They are regulated by a complex network of microRNAs (see below). Furthermore, YB-1, a protein associated with increased invasiveness in breast carcinomas, activates the mRNA of ZEB2 without affecting ZEB1 [80]. ZEB2 is also controlled at the protein level, and its binding to F-box FBXL14 targets it for ubiquitin-proteasome degradation [81].

### Snail1, Snail2 and Snail3

The Snail family comprises three members: Snail1 (originally identified as Snail), Snail2 (Slug), and Snail3 (Smuc) (Fig. 1). All three contain a single C-terminal zinc finger cluster that bind to E-boxes in the regulatory regions of target genes. At the N-terminal end, they share a SNAG (Snail-Gfi1) domain that mediates binding to Sin3A/HDAC1/HDAC2 and Ajuba-PRMT5-PRC2 and LSD1-coREST complexes [82–85]. Only Snail2 maintains the central CtBP binding motif present in *Drosophila* that has also been implicated in recruitment of HDAC and coREST [86]. A recent report shows the recruitment of NCoR and CtBP1 to the SNAG and SLUG domains of Snail2, respectively, with the former being essential for Snail2-mediated EMT [87]. The precise mechanism by which Snail-mediated histone modifications (deacetylation, H3K27 and H4R3 methylation, H3K4 demethylation) cooperate to repress E-cadherin has not been fully elucidated. Snail1 also represses epithelial specification genes, including E-cadherin, by interacting with Smad3/Smad4 [88]. Overexpression of Snail1 decreases binding of AKT1 to the E-cadherin promoter in favor of AKT2 on which Snail1 depends for its repressor activity [89]. Conversely, activation of mesenchymal genes by Snail factors seems to be, at least in part, indirect, via E-cadherin downregulation.

Snail1 and Snail2 transcriptional activities are also modulated by post-transcriptional modifications that alter their protein stability and intracellular localization. Thus, binding of Snail1 to Sin3a is strengthened by PKA and CK2 phosphorylation [90]. In a similar manner, phosphorylation of Snail2 modulates its ability to transcriptionally repress E-cadherin [87]. Phosphorylation of Snail1 by GSK3 $\beta$  functionally inactivates it through both CRM1-dependent export to the cytoplasm and SCF/ $\beta$ -TrCP ubiquitin-mediated proteasome degradation [91]. Interaction of Snail1 with LOXL2 and LOXL3 prevents GSK3 $\beta$ -induced degradation [92], which is, however, fostered by phosphorylation by CK1 $\epsilon$  [93]. Snail factors are also targeted for degradation in a GSK3 $\beta$ -independent manner by binding to FBXL14 [81, 94, 95] or, in the case of Snail2, through Mdm2 ubiquitination [222]. In addition, the intracellular localization of Snail factors is also controlled through import and export signals [96, 97]. In addition to GSK3 $\beta$ , phosphorylation of Snail1 by PKD1 also triggers its nuclear export [98]. On the other hand, phosphorylation by PAK1 promotes its nuclear localization, which is also dependent on the breast cancer-associated zinc transporter protein LIV1 [99, 100].

As with other EMT-ATFs, signals regulating Snail proteins in cancer parallel those operating during development. Snail factors are induced by TGF $\beta$ , Notch, TNF $\alpha$ , EGF, FGF, Wnt, Shh, SCF/c-kit, hypoxia, and estrogens [3].

Transcriptional activation of Snail1 by TGF $\beta$  requires interaction of R-Smads with HMGA2 [101]. In some cancer cell types, induction of Snail1 by TGF $\beta$  requires simultaneous oncogenic Ras signaling [102]. On its part, TGF $\beta$ -mediated induction of Snail2 depends on the downregulation of KLF4 and FOXA1 that, interestingly, form a double repressor loop with Snail2 [103]. Notch signaling induces Snail1 through direct and indirect mechanisms. In addition to direct activation of the Snail1 promoter, Notch stabilizes Snail1 through HIF-1 $\alpha$ -mediated activation of LOX [104]. NF $\kappa$ B signaling by AKT, hypoxia or inflammatory cytokines transcriptionally activates Snail1. Induction of NF $\kappa$ B by inflammatory cytokines also stabilizes Snail1 by inhibiting GSK-3 $\beta$ -mediated ubiquitination [105]. Engagement of receptor tyrosine kinases signaling and COX2/PEG2 also induces expression of Snail1 by inhibition of GSK3 $\beta$  [70]. Snail1 is also able to repress its own transcription [106]. Finally, Snail1 is activated at the translational level by YB-1 [80].

### Twist1 and Twist2

Twist1 and Twist2 share a basic/helix-loop-helix (bHLH) domain that mediates their binding to DNA and homo/hetero-dimerization (Fig. 1). At the C-terminal end, there is a “Twist box” that has been implicated in both transcriptional activation and repression (reviewed in [107] and [312]). Regulation of gene expression by Twist factors depends on their binding to other transcriptional regulators, post-translational modifications, and choice of partner for dimerization. Binding of Twist proteins to E-boxes in the promoters of target genes could either activate (e.g., N-cadherin, AKT2 or Gli1) or repress (e.g., E-cadherin) transcription [108, 109]. In some cases, Twist-mediated repression involves binding to other transcription factors and cofactors to inhibit their activity (e.g., Runx2, myogenic bHLH, NF $\kappa$ B, and p300/pCAF). Direct repression of E-cadherin by Twist1 entails recruitment of multiple chromatin remodeling complexes, whose composition and dynamics is currently being uncovered. Twist1 activates transcription of the PRC1 component Bmi1 and binds to polycomb repressor complexes PRC1 and PRC2 at the E-cadherin promoter [310]. Twist1 interaction with components of the NuRD complex is also required for E-cadherin repression [110], a result in line with the finding that recruitment of PRC2 is specified by NuRD histone deacetylation [111]. In addition, it was recently reported that binding of Twist1 to the H4K20 methyltransferase SET8 could simultaneously contribute to repression of E-cadherin and activation of N-cadherin [112].

Gene regulation by Twist factors is also modulated by control of their intracellular localization and the identity of



their dimerization partner. Nuclear/cytoplasm shuttling of Twist factors is modulated by integrin-mediated adhesion to the extracellular matrix, post-translational modifications, and partner dimerization [108]. In *Drosophila*, Twist homodimers activate transcription whereas heterodimers with Daughterless function as repressors. However, in mammalian systems, Twist/E12 heterodimers can both activate and repress transcription [107, 312]. Homo- or heterodimerization of Twist proteins is determined by availability of E12. Likewise, Twist can titrate away E12 from other bHLH proteins thus inhibiting their function. Phosphorylation of the bHLH domain of Twist alters not only dimerization partner choice but also binding affinity for DNA [107].

Understanding the signaling pathways upstream of Twist1 and Twist2 is not as complete as for ZEB and Snail factors. Still, Twist factors are upregulated by classical EMT-inducing pathways during development, inflammation, and cancer, such as TGF $\beta$ , Wnt, hypoxia, and ligand-binding activation of receptor tyrosine kinases and inflammatory cytokines receptors [107, 312]. EGF and IL6 induce Twist1 via activation of JAK/STAT signaling and direct binding of STAT3 to the Twist1 promoter [113, 114]. Twist1 and Twist2 form a negative loop with inflammatory cytokines, as Twist factors are transcriptionally induced by NF $\kappa$ B and in turn bind to the TNF $\alpha$  and IL1 $\beta$  promoters blocking NF $\kappa$ B transcriptional activity [115]. As discussed below, upregulation of Twist1 by HIF-1 $\alpha$  during hypoxic conditions has significant implications in tumor invasion and angiogenesis [116].

Twist factors are also regulated at the mRNA and protein level as well by their intracellular localization. As with other EMT-ATFs, YB-1 fosters cap-independent translation of Twist1 [80]. Twist1 is also controlled post-translationally by cytoplasmic polyadenylation sites in its 3'UTR [117]. Heterodimerization of Twist1 with E12 and Hand2, fostered by phosphorylation by PKA, stabilizes Twist1 protein that is, on the other hand, targeted for degradation upon binding to FXBL14 [81, 118].

#### Regulatory networks between EMT-ATFs and microRNAs

In recent years, a plethora of publications have identified miRs regulating each and every step during cancer progression, from cell proliferation, cancer cell stemness, and apoptosis to angiogenesis or tumor invasiveness (reviewed in [13]). miRs regulate invasiveness and metastasis by targeting the transcripts of a large number of genes involved in EMT/MET regulation, including those of EMT-ATFs (Fig. 2).

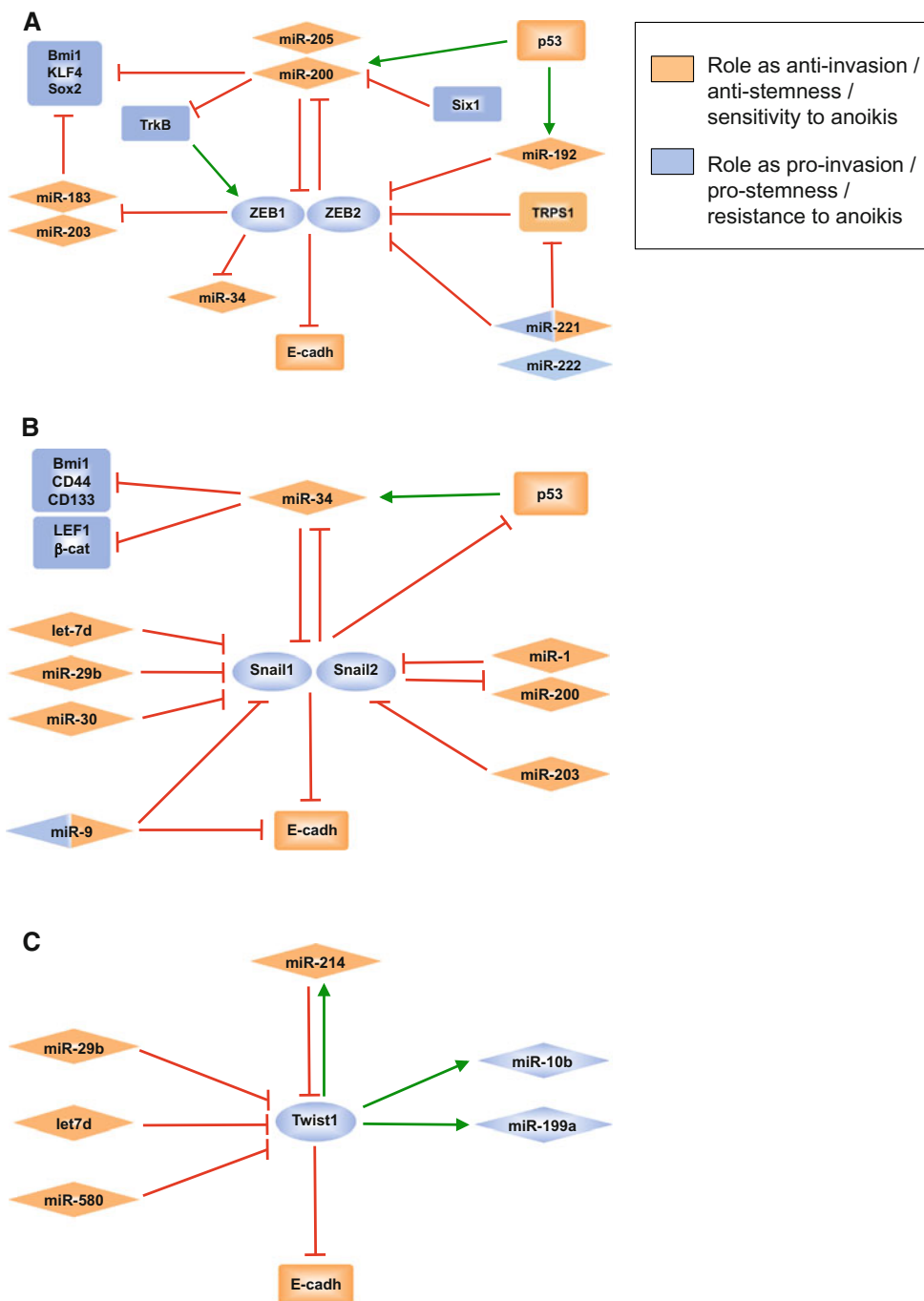
Members of the miR-200 family (miR-200a/b/c, miR-141, and miR-429) maintain an epithelial status and

prevent EMT through inhibition of ZEB1 and ZEB2 (reviewed in [54], see also [119–121], and other references in [54]). In turn, miR-200 members are transcriptionally repressed by ZEB factors—as well as Snail1—thus forming a double-negative loop that maintain cells in either an epithelial or mesenchymal state [54, 122–124]. Expression of miR-200 and ZEB factors presents an inverse pattern in a number of human cancers. Invading mesenchymal-like cancer cells that extravasate and metastasize eventually need to revert to an epithelial phenotype for the metastatic colony to grow into a secondary tumor. Cancer cells that form macroscopic metastasis have higher levels of miR-200 compared to those that invade but are not able to colonize and, paradoxically to their roles as EMT repressors: overexpression of miR-200 in mouse breast cancer isogenic cell lines fosters lung and liver metastasis by inhibition of Sec23a, a component of the tumor secretome that blocks metastatic colonization [125–313]. The 5'CpG islands of miR-200 loci are also subjected to dynamic epigenetic regulation [126]. In epithelial cell lines, miR-200 expression is silenced by hypermethylation during TGF $\beta$ -induced EMT but reverted by demethylation in MET. Likewise, 5'CpG islands of miR-200 loci are hypermethylated in cancerous cells of primary colorectal carcinomas but remain unmethylated in epithelial cells of the normal colonic mucosa.

miR-200 members mediate the anti-EMT activities of other factors and signaling pathways. For instance, Six1 induces ZEB1 and EMT through transcriptional repression of miR-200 [127]. By contrast, the tumor suppressor p53 inhibits a mesenchymal and stem cell phenotype and/or the invasive capacity of mammary and pancreatic epithelial cells and hepatocarcinoma cell lines by upregulating miR-200—as well as miR-192—to repress ZEB1 and ZEB2 [128–130]. Conversely, oncogenic mutant forms of p53 decrease miR-200 and increase ZEB1 expression [128].

ZEB1 and ZEB2 participate in other miR regulatory networks. ZEB factors repress miR-183 and miR203, which together with miR-200, inhibit the expression of stemness factors Bmi1, Sox2 and KLF4 [131, 132]. ZEB1 and ZEB2 are also targets of miR-205 [13]. In aggressive basal-like breast carcinomas, but not the luminal type, Fra-1 activates miR-221 and miR-222 [133], miRs that have been associated with EMT and tumor invasiveness and are downregulated by EGFR inhibitors. miR-221 and miR-222 downregulate TRPS1, a GATA-like repressor, that in turn inhibits ZEB2 [133]. In contrast, in endothelial cells, ZEB2 is a direct target of miR-221 [134], confirming evidence elsewhere that miR may have different, even opposing, functions depending on the cell type.

Contrary to its inhibitory effect on E-cadherin in breast epithelial cells [135], miR-9 downregulates Snail1 expression in melanoma cells by targeting NF $\kappa$ B, thus



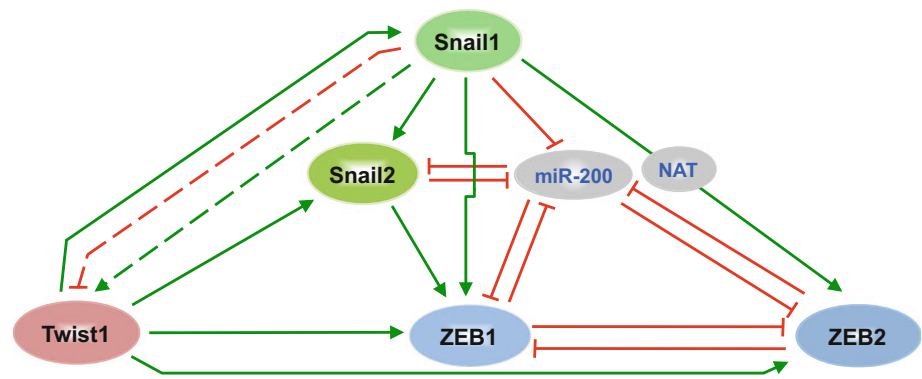
**Fig. 2** Regulatory networks between microRNAs and EMT-ATFs of the ZEB (a), Snail (b) and Twist (c) families. For simplification, some relationships have been consolidated or are not shown. *Double color*

identification refers to miRs that have been described as having both pro- and anti-invasion/metastatic roles depending on the context and/or cell system analyzed. See text for discussion and references

inhibiting proliferation and invasiveness [136]. p53 also downregulates Snail1 and Snail2 via induction of miR-34a/b/c. Conversely, Snail1 and Snail2 (and ZEB1) transcriptionally repress miR-34a/b/c, thus forming a double-negative loop similar to the one between ZEB factors and miR-200 [137, 138]. As with miR-200, miR34a inhibits stem cell-related factors Bmi1, CD44, and CD133 [137].

Members of the miR-30 family also target Snail1 [139, 140] and, as with miR-200, overexpression of either miR-30 and miR-34 prevents TGF $\beta$ -induced EMT. It is worth noting here that miR-34 also targets several Wnt signaling genes like Wnt1, Wnt3, LEF1 and  $\beta$ -catenin, suggesting that loss of p53 tumor suppressor activity could be accompanied by activation of  $\beta$ -catenin/TCF signaling,

**Fig. 3** Cross-regulation among EMT-ATFs. *Dashed lines* indicate that different relationships have been reported depending on the cell system analyzed. See text for discussion and references



which may reinforce EMT and stemness programs [141]. Snail1 is also repressed by let-7d and miR-29b, microRNAs that block EMT and invasiveness in HNSCC and prostate carcinomas, respectively [142, 143].

Snail2 participates in yet another double-negative loop with miRs: miR-1 and miR-200 inhibit Snail2 expression and are repressed by binding of Snail2 to their promoters [144]. Snail2 transcript is also inhibited by miR-203, which is upregulated in primary tumors and hypermethylated and silenced in metastatic cell lines [145].

To date, fewer miRs have been linked to Twist factors. Overexpression of let-7d inhibits Twist1 expression in oral squamous cell carcinomas [142]. Twist1 is also inhibited by miR-29b, miR-580, and miR-214, but it is not clear whether downregulation of Twist1 by miR-29b is mediated by inhibition of Snail1 [117, 143, 146]. In turn, Twist1 activates the expression of miR-10b, miR-199a, and miR-214 genes through direct binding to E-boxes in their promoters [147, 148]. miR-10b is expressed in metastatic breast cancer cells and induces cell motility and migration by indirectly inducing RhoC.

The emergence of all these new regulatory loops between miRs and EMT-ATFs provides evidence of dynamic epigenetic silencing of miR-200 (and likely other miRs) during cancer progression, which endows cancer cells with enhanced plasticity to reversibly switch between EMT and MET during tumor invasiveness and metastasis.

#### Cross-regulation among EMT-ATFs

Previous sections have highlighted the existence of significant overlap among EMT-ATFs in their regulatory signals, target genes, and mechanisms of action, raising the question of what is the specific contribution of each factor in the regulation of EMT and cancer. Recent evidence indicates that ZEB factors are downstream of the Snail and Twist families in the EMT interactome [149] (Fig. 3). The hierarchical Snail–Twist/ZEB relationship supports earlier evidence of the strongest correlation of ZEB factors, especially ZEB1, with E-cadherin loss and EMT across cancer cell types [150].

Snail1 increases ZEB1 protein levels through both transcriptional and post-transcriptional mechanisms [57, 151, 152] (Fig. 3). Snail1 also enhances the stability of Twist1 and triggers nuclear translocation of Ets1, both directly activating the ZEB1 promoter. Additionally, Snail1 not only represses miR-200, thus derepressing ZEB1 mRNA, but also stabilizes ZEB1 protein through still undefined mechanisms. Snail2 also activates ZEB1 by direct binding to its promoter [308].

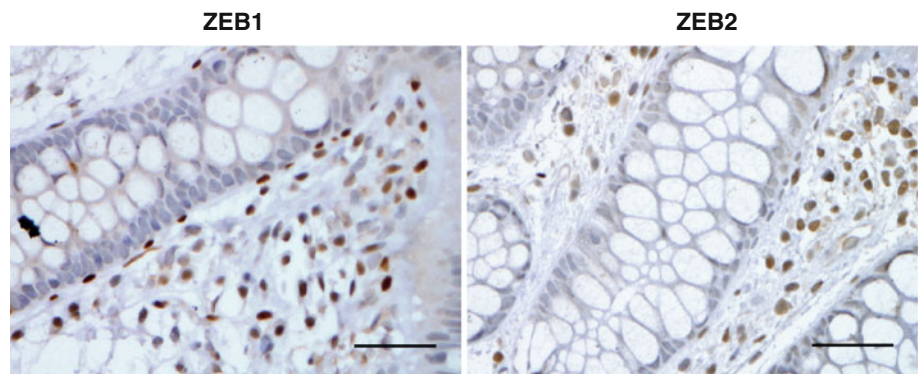
ZEB2 expression is also regulated by Snail1 through several mechanisms. Repression of miR-200 by Snail1 upregulates ZEB2 mRNA (Fig. 3). In addition, Snail1 induces a natural antisense transcript (NAT) that overlaps with an internal ribosomal entry site in the 5' UTR of ZEB2, preventing its splicing and increasing ZEB2 [153].

Twist1 mediates some of its EMT-activating effects through Snail2 by binding to its promoter and activating its transcription [154]. Expression of Twist1 and Snail1 are also mutually dependent although in different directions, depending on the cell system. Knock-down of Snail1 downregulates Twist1 protein and mRNA, while knock-down of Twist1 interferes with TGF $\beta$ -mediated induction of Snail1 [152]. Alternatively, it has also been reported that Snail1 transcriptionally represses Twist1, which only increases as Snail1 declines in later stages of EMT [155].

Microarray gene expression signatures of human mammary epithelial cells (HMEC) driven towards EMT have confirmed this hierarchical/network relationship among EMT-ATFs. HMEC that have been either treated with TGF $\beta$  or subjected to overexpression of Snail1, Twist1, or Goosecoid, or knock-down for E-cadherin, display an overlapping gene signature characterized by the induction of ZEB1, ZEB2, and FOXC2 [149]. In any case, several of the signaling pathways (e.g., TGF $\beta$ , Wnt, Notch, TNF $\alpha$ /IL1 $\beta$ , receptor tyrosine kinase, etc.) inducing Snail and Twist factors also activate ZEB1 and ZEB2 expression directly. There is also considerable cross-talk among many of the upstream signals regulating all three EMT-ATF families. For instance, besides regulation of ZEB factors via TGF $\beta$ /Snail1-mediated inhibition of miR-200 [152],



**Fig. 4** ZEB1 and ZEB2 are expressed in stromal cells of normal colonic mucosa. Immunohistochemistry of colon samples from normal individuals stained by the 3,3'-diaminobenzidine (DAB) method with ZEB1 (H-102; Santa Cruz Biotechnology) and ZEB2 (H-260; Santa Cruz Biotechnology) as described in [69]. Scale bars 25  $\mu$ m



ZEB1 induction by Notch involves NF $\kappa$ B [71] and growth hormone induces ZEB2 via NAT [75]. TrkB induces EMT through a ZEB1-dependent mechanism that requires the cooperation of Snail1 and Twist1 [63].

Expression and functional redundancy among EMT-ATFs suggests that their specificity could occur at the spatial and temporal level, with Snail1 being required to initiate the EMT and, subsequently, Twist and ZEB factors to consolidate it [57, 152, 155].

#### EMT-ATFs in EMT and tumor invasiveness

##### ZEB1 and ZEB2

Expression of ZEB factors drives an EMT by repressing and activating epithelial and mesenchymal specification genes, respectively (reviewed in [33, 53, 54]). ZEB1 and ZEB2 both bind to E-box sequences in the E-cadherin promoter but recruit different set of corepressors: CtBP and SWI/SNF in the case of ZEB1, and CtBP and NuRD by ZEB2. ZEB proteins bind and repress the promoters of other epithelial markers such as P- and R-cadherins, cell polarity markers (Crumb3, Pals1-associated tight junction protein, lethal giant larvae homologue 2), components of tight junctions (occludin, claudin 7, junctional adhesion molecule 1, zonula occludens protein 3), gap junctions (connexins 26 and 31), and desmosomes (desmoplakin, plakophilin 3) (reviewed in [53] and see also [14–16, 20]). Conversely, ZEB proteins activate mesenchymal markers such as vimentin and N-cadherin. Although the mechanism of ZEB1- and ZEB2-mediated induction of mesenchymal genes has not been fully elucidated, in some cases (e.g., vimentin) it involves direct binding of ZEB proteins to the promoter regions of mesenchymal genes and transcriptional activation [156, 157]. ZEB1 and ZEB2 also repress epithelial splicing regulatory proteins-1 and -2 (ESRP-1 and -2) that coordinate the epithelial pattern of alternative splicing of FGFR2 and whose overexpression inhibits EMT [158].

Breakdown of the basement membrane, that separates the epithelial compartment from the surrounding stroma, is considered a key step in the progression from in situ to invasive carcinoma. Interestingly, ZEB1 regulates the expression of several components of the epithelial basement membrane (e.g.,  $\alpha$ 3 chain of laminin 5,  $\alpha$ 2 chain of collagen IV,  $\gamma$ 2 chain of laminin 5), although in a tissue-specific manner [69, 159, 160]. In colorectal carcinomas, the  $\gamma$ 2 chain of laminin 5 coexpresses with MT1-MMP in cancer cells at the tumor invasive edge and cleavage of the former by the latter promotes cancer cell migration [69, 161].

As an inhibitor of the epithelial phenotype, ZEB1 is not expressed in normal epithelium, although it is found in isolated fibroblasts and immune cells in the interstitial stroma (Fig. 4). ZEB1 expression is also absent at the center of relatively well-differentiated carcinomas expressing E-cadherin [29, 159]. However, ZEB1 is highly expressed in invading dedifferentiated cancer cells of many tumors including colorectal, breast, liver, endometrial, lung, prostate, and pancreatic carcinomas (Table 1). Like ZEB1, ZEB2 is expressed by stroma cells in epithelial tissues (Fig. 4), but, interestingly, is also detected in normal E-cadherin-positive epithelial cells in several organs [162]. Nevertheless, upregulated expression of ZEB2 at the invasive front has been reported in most carcinomas expressing ZEB1, such as colorectal, breast, gastric, bladder, liver, and pancreatic (Table 1). In most of these tumors, there is also an increase in the number of ZEB1- and ZEB2-positive cells (fibroblasts, macrophages, and endothelial cells) in the peritumoral stroma—often at higher levels than in invading cancer cells—representing both tumoral cells that have undergone an EMT and activated stromal cells (Table 1; e.g., [16, 31, 159, 163–166]). It has been postulated that ZEB-dependent paracrine signaling from the stroma could cooperate in E-cadherin repression in other parts of the tumor [163].

Expression of ZEB proteins by both cancer and stromal cells at the invasive front of carcinomas translates into increased tumor invasiveness: ZEB proteins promote

**Table 1** Upregulated expression of EMT-ATFs in selected human cancers

EMT-ATF	Human cancer	Reference	
ZEB1	Bladder	[164, 284–286]	
	Breast	[165, 287]	
	Digestive tract (colorectal)	[16, 69, 159]	
	Digestive tract (esophagus squamous cell carcinoma)	[288]	
	Digestive tract (gastric)	[171] <sup>a</sup>	
	HNSCC	[289]	
	Hepatocarcinoma	[174] <sup>a</sup>	
	NSCLC	[70, 163]	
	Pancreas	[131], [176] <sup>a</sup> , [273]	
	Prostate	[65]	
	Uterus (leiomyosarcoma, endometrial adenocarcinoma)	[168, 170]	
	ZEB2	Bladder	[164] <sup>a</sup> , [284, 286]
		Breast	[169]
Digestive tract (colorectal)		[173] <sup>a</sup>	
Digestive tract (gastric)		[175] <sup>a</sup> , [290]	
HNSCC		[291] <sup>a</sup> , [292]	
Hepatocarcinoma		[166] <sup>a</sup> , [293]	
Ovarian		[169, 172] <sup>a</sup>	
Snail1	Pancreas	[176] <sup>a</sup> , [294]	
	Breast	[165], [169] <sup>a</sup> , [182, 192, 295, 296]	
	Digestive tract (colorectal)	[181] <sup>a</sup> , [184, 297]	
	Digestive tract (gastric)	[290]	
	HNSCC	[292, 298]	
	Hepatocarcinoma	[185], [187, 191] <sup>a</sup>	
	NSCLC	[254] <sup>a</sup>	
Snail2	Ovarian	[169, 189] <sup>a</sup>	
	Pancreas	[299]	
	Breast	[182, 192]	
	Digestive tract (colorectal)	[189]	
	Digestive tract (esophagus)	[188] <sup>a</sup>	
	Digestive tract (gastric)	[300]	
	HNSCC	[292]	
Snail3	NSCLC	[253] <sup>a</sup>	
	Ovarian	[169]	
	Pancreas	[299]	
	Lung	[301]	
	Melanoma	[301]	
	Twist1	Bladder	[201]
		Breast	[19], [165] <sup>a</sup> , [192, 248, 255]
Digestive tract (colorectal)		[248, 302] <sup>a</sup>	
Digestive tract (esophagus squamous cell carcinoma)		[200, 207] <sup>a</sup> , [248]	
Digestive tract (gastric)		[206, 290]	
HNSCC		[199, 292]	
Hepatocarcinoma		[191, 205] <sup>a</sup> , [303]	
Ovarian		[203, 204] <sup>a</sup>	
Pancreas		[304]	
Prostate	[282, 309]		

**Table 1** continued

EMT-ATF	Human cancer	Reference
Twist2	Breast	[202]
	Digestive tract (esophagus)	[248]
	HNSCC	[305] <sup>a</sup>
	Uterus (cervical intraepithelial neoplasia, cervical squamous cell carcinoma)	[306]

Only some representative publications are included

<sup>a</sup> Studies demonstrating prognostic value of EMT-ATFs expression (correlation with metastasis, recurrence and/or survival)

metastasis in xenograft models [131, 167] and associate with increased aggressiveness and higher metastatic capacity in a wide range of primary human carcinomas (e.g., [65, 70, 131, 164, 168–171]). ZEB factors have been shown to have an independent prognostic value for nodal dissemination, metastasis, response to treatment, and/or survival in multiple carcinomas, inter alia, of the ovary, bladder, colorectal, gastric, pancreas, and hepatocarcinoma [164, 166, 169, 171–176] (see representative entries with superscript letter in Table 1).

### Snail1, Snail2 and Snail3

Independently of their inductive effects on ZEB1 and ZEB2, Snail factors also directly regulate epithelial and mesenchymal markers ([21, 177, 178] and references therein). In fact, the emerging consensus in the literature points to Snail1 as the factor responsible for the initiation of EMT in response to inducing signals [152, 155]. In addition to direct binding to the E-cadherin promoter and inhibition of its transcription, Snail factors repress other epithelial markers independently of their effect on E-cadherin—e.g., desmoplakin, adherens junction (claudin-1, -3, -4, -7), tight junctions (occludins), cytokeratins, and Mucin-1. On the other hand, Snail factors activate the expression of mesenchymal-like and pro-invasive genes (e.g., vimentin, fibronectin MMP1, MMP2, MMP7, MT1-MMP) that promote cell migration. While the epithelial and mesenchymal genes controlled by Snail1 and Snail2 overlap to a great extent, there is also evidence of some level of differential regulation in the degree of induction or repression and even in its direction [21]. For some genes, regulation requires cooperation between Snail1 and Snail2 or between Snail1 and Twist1: for example, Snail1 and Snail2 cooperate to transcriptionally repress the vitamin D receptor by which engagement by vitamin D3 promotes cell differentiation which is associated with a good prognosis in colorectal carcinomas [179, 180].

Snail factors are not present in normal epithelial cells but their expression is evidenced, usually higher for Snail1,

in cancer cells at the invasive front of carcinomas of the breast, digestive tract, liver, pancreas, ovary, and lung among others (Table 1). Characterization of Snail3 lags behind, but ESTs have been identified in melanomas and lung carcinomas. In addition to the tumor front, Snail1 is found at areas of inflammation in colorectal carcinomas [181]. Tumor progression correlates with increasing nuclear expression of Snail1, but Snail1 can also be aberrantly detected in the cytoplasm of cancer cells of several carcinomas. Additionally, strong Snail1 staining is found—often at a higher percentage than in tumor cells, when not even exclusively—among fibroblast-like cells, macrophages, and endothelial cells at the peritumoral stroma [181–184].

Expression of Snail factors correlates with malignancy and less differentiated tumors, lymph node invasion, and metastasis, and Snail1, but not always Snail2, is considered an independent prognostic factor of worst evolution and poorer survival in a large number of carcinomas [182, 185–189] (Table 1). Snail1 promotes the recurrence of Her2/neu-induced primary breast tumors in mice, and recurrent human carcinomas tend to display mesenchymal-like characteristics [186]. Thus, Snail1 is spontaneously induced in recurrent breast carcinomas and high levels of Snail1 are an independent predictor for decreased relapse-free survival in breast cancer patients. At least in head and neck carcinomas, only Snail1 nuclear staining, but not cytoplasmic, is associated with a worse clinical outcome [190]. Interestingly, reactivity for Snail1 in stromal but not in cancer cells correlates with distant metastasis and lower survival in early stage colorectal carcinomas [181].

Up to now, evidence for differential roles of Snail1 and Snail2 in cancer progression is scarce. In hepatocarcinomas, only Snail1 but not Snail2 correlates with E-cadherin loss [191], whereas another study reported that Snail2 but not Snail1 is associated with a poorer outcome in breast carcinoma patients [192].

### Twist1 and Twist2

Twist factors induce EMT directly and indirectly through their effect on other EMT-ATFs. Thus, Twist1 has been shown to repress E-cadherin by binding to its promoter [19], but also through induction of Snail1 [193] or Snail2 [154]. In HMEC, Twist1 overexpression yields a similar gene signature than Snail1 with repression of E-cadherin and other epithelial specification genes and induction of mesenchymal markers [149]. Several array studies found that overexpression of Twist1 also upregulates the expression of cytoskeletal and extracellular matrix genes involved in cell motility. However, the extent to which

induction of mesenchymal genes by Twist is dependent on E-cadherin repression is less clear [194]. Twist1 induces N-cadherin via directly driving its transcription [108] and/or through post-transcriptional mechanisms [195]. Nevertheless, in glioblastoma cells, Twist1 promotes expression of mesenchymal markers without eliciting an E-cadherin/N-cadherin switch [196]. Likewise, Twist1 induces fibronectin in gastric and ovarian cancer cells [195] but not in breast cancer cells [109]. In ovarian carcinoma cells, Twist1 also activates MMP2 and MT1-MMP, and its knock-down reduces cell adhesion to extracellular matrix components in parallel with a decline in several adhesion molecules (e.g.,  $\beta$ 1 integrin, CD44) [197].

A role for Twist1 in cancer was first evidenced by its ability to promote greater invasiveness and metastasis in xenograft models [19]. Like other EMT-ATFs, Twist1 and Twist2 are absent in normal epithelium but are induced in a number of human carcinomas, including those of the digestive tract, breast, liver, prostate, endometrium, and ovary (Table 1). Interestingly, the Twist1 gene has also been found to be deleted or amplified in some osteosarcomas [198]. Again, mirroring ZEB and Snail factors, Twist1 and Twist2 are not only upregulated among cancer cells at the invasive front of carcinomas but also in stromal cells. Likewise, upregulation of Twist factors is not limited to the nuclei of cancer cells but, in many cases, also in their cytoplasm (e.g., [199–202]).

Twist factors are not only upregulated in human cancers but their reactivity increases during tumor progression from being mostly negative in benign neoplasias to be highly overexpressed in carcinomas of a wide range of tissue origins (Table 1). Knock-down of Twist1 in breast cancer cells inhibits their ability to metastasize in xenograft models but not the formation of primary tumors [19]. Twist factors also correlate with higher tumor grade, invasiveness, and metastasis, being independent prognostic factors for enhanced tumor aggressiveness, tumor recurrence, and poorer patient survival (e.g., [155, 191, 197, 199, 203–207]). Interestingly, in hepatocarcinomas, expression of either Twist1 or Snail1 is associated with shorter survival, and their simultaneous presence has an additive negative effect, suggesting that they somehow play distinct but collaborative roles in cancer progression [191]. In that line, another study in hepatocarcinomas found that Twist1 associates with increased tumor angiogenesis and metastasis but not with downregulation of E-cadherin [205]. Differential expression of Twist and Snail factors in some studies suggests a division of labor among EMT-ATFs. Breast carcinomas with lymph node involvement and poor clinical outcome display high levels of Twist1 and Snail2 while, contrary to other reports, expression of Snail1 declined [192].

### Other transcription factors inducing EMT and tumor invasiveness

Like the ZEB, Snail, and Twist factors, E12/E47 binds directly to E-cadherin promoter [6] and induces an EMT by regulating an overlapping—but still distinct—set of epithelial and mesenchymal markers compared to Snail1 and Snail2 [21]. E12/E47 complexes not only promote tumor invasiveness and growth but injection of Madin–Darby Canine Kidney (MDCK) cells overexpressing E47 into nude mice generates tumors with higher invasiveness, growth, and vascularization than those formed by MDCK cells overexpressing Snail1 [177]. T-box transcription factor Tbx3 also binds directly to the E-cadherin promoter and inversely correlates with E-cadherin in human melanomas [7].

The homeobox factor Goosecoid also induces a full EMT by repressing epithelial markers (including E-cadherin) and activating mesenchymal genes [8]. Goosecoid is induced by TGF $\beta$  in adult breast epithelial cells and, compared to normal breast tissue, is significantly overexpressed in atypical ductal hyperplasia and ductal breast carcinomas [8].

FOXC2 also induces a mesenchymal phenotype but, departing from the rest of EMT-inducing factors reviewed here, FOXC2 does not alter E-cadherin mRNA levels but rather delocalizes E-cadherin protein from the plasma membrane to the cytoplasm [208]. FOXC2 is induced by TGF $\beta$ , and in response to overexpression of Snail1, Twist1, and Goosecoid, and has been suggested to mediate activation of mesenchymal genes by these other EMT-ATFs. FOXC2 is not expressed in normal epithelium but is overexpressed in basal-like breast and esophageal squamous cell carcinomas where it correlates with adverse prognosis [208, 209].

### Other roles of EMT-ATFs: beyond EMT and tumor invasiveness

In addition to promoting tumor invasiveness, over the last few years a number of works have demonstrated that EMT-ATFs are also critical during earlier stages of cancer development, collaborating with oncogenes in malignant transformation, inducing tumor formation, contributing to bypass failsafe programs against cancer, and determining resistance to chemotherapy. While these additional cancer hallmarks are not necessarily dependent on EMT, they are jointly regulated with the EMT by EMT-ATFs. While many of these functions are interconnected, they will be addressed here in turn.

#### EMT, cancer stem cells, and tumorigenesis

The great level of heterogeneity displayed by most human tumors led to the formulation in the 1990s of a hierarchical

model of cancer initiation where a small subpopulation of cells in a tumor—referred to as cancer stem cells (CSCs)—retain the capacity for self-renewal and tumor initiation (tumorigenesis) (reviewed in [28]). CSCs have been identified in a number of human cancers including colon, breast, pancreas, ovary, prostate, and brain tumors.

As indicated earlier, Brabletz and coworkers [27] postulated that invading carcinoma cells that have undergone an EMT could function as “migrating CSCs”. Later studies by the Weinberg group and others confirmed that forcing an EMT in non-tumorigenic, immortalized HMECs—by treatment with TGF $\beta$  or overexpression of EMT-ATFs—concurrently confers on cells a phenotype similar to breast CSCs (CD44<sup>high</sup>/CD24<sup>low</sup>, miR-200<sup>low</sup>), along with the capacity to form mammospheres, self-renewal, and increasing tumorigenicity in xenotransplants [12, 26, 132, 202]. The ability of miR-200 to suppress colony formation in vitro and tumorigenesis in vivo depends on their repressor effect on Bmi1 and ZEB1 [132].

Signaling pathways that control stem cell homeostasis during embryogenesis and later in adults (e.g., Wnt, Shh, Notch) are active in cancer and trigger—via induction of EMT-ATFs—not only EMT but also stem-like properties [3]. Acquisition and maintenance of stemness by EMT-ATFs could explain the capacity of these factors to induce tumorigenesis and promote recurrence and metastasis.

Isolation of stem-like (CD44<sup>high</sup>/CD24<sup>low</sup>) and non-stem-like (CD44<sup>low</sup>/CD24<sup>high</sup>) populations from resected primary breast tumors identified high levels of mesenchymal markers (N-cadherin, vimentin, fibronectin) including EMT-ATFs (ZEB2, Snail1, Snail2, Twist1 and Twist2) in the first set but not the second [26]. While ZEB1 did not show up in this study, ZEB1 was found to be uniquely required to maintain the viability and stem-like phenotype of spheres—a property classically linked to stem cells—formed from mouse embryo fibroblasts (MEFs) with targeted deletions of Rb1 and Rb family members [210]. In pancreatic carcinomas, ZEB1 determines stemness in cancer cells by direct repression of several miRs (miR-200, miR-183, miR-203) targeting stemness regulators Bmi1, KLF4, and Sox2 [131]. By contrast, activation of miR-200c by p53 reduces ZEB1 expression and suppresses both EMT and stemness, while downregulation of miR-200c—upon loss of p53 or overexpression of oncogenic p53 mutants—increases ZEB factors and stem cell markers in mammary and pancreatic acinar epithelial cells [128, 130] (Fig. 2).

Similar results have been obtained in other models. Spheres formed out of primary colorectal cancer cells also express high levels of Snail1, whose overexpression upregulates stem cell markers [211]. Another study found that overexpression of Snail1 or Snail2 in ovarian cancer cells derepresses a stemness gene signature (e.g., Nanog, KLF4, Oct4, Bmi1, Nestin) and increases the number of



CSC-like CD44<sup>+</sup>/C117<sup>+</sup> cells, suggesting that CSCs originate from the dedifferentiation of non-stem cancer cells rather than proliferation of existing CSCs [212]. In that line, oncogenic transformation of mammary stem-like cells produces more aggressive tumors than transformation of differentiated mammary epithelial cells and enhances the conversion of non-CSCs into CSCs [213].

The capacity of EMT-ATFs to generate CSCs is important to explain some other EMT-ATFs functions, like enhanced survival, tumor recurrence, and metastasis. The possibility that cancer cells could transition between tumorigenic and non-tumorigenic states [213], and that EMT-ATFs expression could modulate this balance, represents an important avenue in therapy. In the same line, the association between expression of EMT-ATFs by cancer cell lines and primary tumors and resistance to DNA damage and chemotherapy may also be related to EMT-ATFs' capacity to generate CSCs (see below). It remains to be firmly established whether, in line with the migrating CSC model [27], the joint induction by EMT-ATFs of a proinvasive/metastatic phenotype and a tumorigenic/stemness capacity in cell systems and xenograft models also occurs in human tumors.

#### EMT-ATFs in resistance to apoptosis and anoikis

EMT allows cancer cells to overcome safeguard mechanisms and become more resistant to signals triggering programmed cell death. Aberrant expression of EMT-ATFs by tumors may thus promote tumorigenesis and tumor growth through increased resistance to apoptosis. EMT triggers pro-survival programs in response to multiple apoptotic stimuli through mechanisms closely linked to the cell cycle. For instance, in an hepatocyte cell line TGF $\beta$  triggers an EMT when cells are in G1/S but apoptosis during G2/M [214]. Both Twist1 and Twist2 inhibit myc- and p53-dependent apoptosis by repressing p19<sup>ARF</sup> [215, 216]. Twist1 also inhibits p53 targets by direct interaction and blocking of the DNA binding domain of p53 [217]. Overexpression of Snail1 induces an EMT at the same time as suppressing TGF $\beta$ -induced apoptosis of non-transformed hepatocytes and hepatocarcinoma cell lines [218]. Snail1 also protects cells against cell death caused by TNF $\alpha$  or growth factor withdrawal by activating MEK and PI3K signaling [219]. Direct repression by Snail factors of the promoters of p53 and pro-apoptotic target genes protects cells against apoptosis induced by DNA damage in response to genotoxic stress (e.g., BID, PIG8, caspase 6, DFF40) [212, 220]. In hematopoietic progenitors, Snail2 is induced by p53 in response to DNA damage and protects against apoptosis by repressing p53-mediated induction of Puma, an

inhibitor of Bcl2 [221]. By contrast, mutant p53 in NSCLC cells results in low Mdm2 and stabilization of Snail2 [222]. In neuroblastomas, overexpression of Snail2 induces Bcl2 [223], and cancer cells with mutant K-Ras that have undergone EMT require Snail2 expression for their survival [224]. In different cell systems, ZEB1 directly inhibits pro-apoptotic TAp73 but also anti-apoptotic  $\Delta$ Np73 and  $\Delta$ Np63 [225, 226]. The pro-survival effect of ZEB2 is, however, independent of cell cycle arrest and intercellular adhesion and is mediated through inhibition of cleavage of PARP and pro-caspase 3 and phosphorylation of ATM/ATR substrates [164].

As cancer cells detach from the tumor, start migrating across the stroma, and intravasate into the circulation, they lose most (if not all) of their intercellular contacts and adhesion to the original extracellular matrix. Just like migrating progenitors during development, metastatic cancer cells are able to survive in this new environment, a condition that in normal cells triggers a caspase-dependent apoptosis program known as anoikis or anchorage-dependent cell death (recently reviewed in [227]). Acquisition of resistance to anoikis is therefore a critical cancer cell capability during tumor invasion and metastasis. Downregulation of E-cadherin expression is sufficient to determine resistance to anoikis [14, 194, 228]. ARF induces apoptosis and anoikis, and downregulation of ankyrin G—a binding partner of E-cadherin that concentrates it at sites of cell–cell contact—during EMT promotes the formation of NRAGE–Tbx2 complexes that represses ARF [229]. Several EMT-inducing pathways (e.g., receptor tyrosine kinase, oncogenic Ras, hypoxia) trigger resistance to anoikis through activation of PI3K-AKT and ERK signaling and regulation of Bcl2 family members. Likewise, in different cell systems and conditions, expression of ZEB, Snail, and Twist factors induces anchorage-independent cell growth [14, 64, 194, 230]. In a seminal contribution by Frisch's group, the capacity of ZEB1 conferring resistance to anoikis was shown to be dependent on the formation of ZEB1/CtBP E-cadherin repressor complexes, in a process reversed by E1a [14]. Snail1 expression promotes resistance to anoikis by activation of the MAPK and PI3K cascades [219]. The tyrosine kinase receptor TrkB, overexpressed in many human cancers, is a known inducer of both EMT and anoikis resistance [63]. Ligand activation of TrkB activates MAPK signaling leading to direct induction of Snail1, Twist1, and ZEB1, but with ZEB1 as the ultimate effector in TrkB-mediated resistance to anoikis. It is of note that TrkB is also a direct target of miR-200 in endometrial and breast carcinoma cells [231], forming yet another miR-regulated regulatory loop, this time for the control of anoikis resistance (Fig. 2a).



## EMT-ATFs in regulation of cell cycle, senescence and transformation

Like apoptosis, cell cycle arrest by senescence represents a crucial safeguard mechanism against cancer. In addition to telomerase shortening during replicative senescence, oncogenic transformation and DNA damage can trigger cell cycle arrest and senescence through induction of the p53 and p16<sup>INK4a</sup>/Rb pathways [232]. Oncogene-induced senescence has been identified in precancerous lesions and needs to be overcome for progression to full tumorigenic status [232, 233]. Evidence accumulated in recent years shows that EMT-ATFs allow cancer cells to bypass senescence thus contributing to the continuous proliferation of immortalized cells. In the same line, an inflammatory environment in the tumor area not only drives cancer cells into an EMT program but also overrides oncogene-induced senescence [234].

ZEB1 transcription, but not ZEB2 or Snail1, is inhibited by the p16<sup>INK4a</sup>/Rb1 tumor suppressor pathway and in turn represses p15<sup>INK4b</sup>, p19<sup>ARF</sup>, and p21<sup>CIP/WAF1</sup> ([48], [76], [235]; see [236] for a comprehensive review). Loss of Rb1 is not only involved in tumor initiation but has been reported to also induce EMT [237], and may contribute to overexpression of ZEB1 in proliferating cells and in many primary tumors. MEFs from ZEB1 (+/−) and ZEB1 (−/−) mice undergo premature replicative senescence in a dose-dependent manner compared to wild-type MEFs [235]. Likewise, progenitors in the palate, skeleton, and nervous system in the ZEB1 (−/−) mice display decreased proliferation [235]. Oxidative stress induces senescence through miR-200-mediated inhibition of ZEB1 [224]. As indicated earlier, p53 represses ZEB1 expression through induction of miR-200, [128, 129]. However, while ZEB1 represses ΔNp63 and both isoforms of p73, it does not affect TAp63 or p53 [225].

Evidence for ZEB2 points in both directions—promoting and reverting senescence—perhaps reflecting cellular background differences. ZEB2 induces a G1 arrest in epidermoid and bladder carcinoma cell lines by direct transcriptional repression of cyclin D1 [164, 238]. Overexpression of cyclin D1 uncouples ZEB2-mediated cell cycle arrest from EMT [238]. Contrary to other EMT-ATFs, ZEB2 induces senescence in hepatocarcinoma cells by inhibiting hTERT [239]. However, overexpression of ZEB2 in lung epithelial cells raises the concentration of TGFβ needed to trigger growth arrest [48], and conditional targeted deletion of ZEB2 (−/−) in the developing cerebral cortex decreases proliferation of neural precursor cells [240]. Expression of both ZEB1 and ZEB2 has been shown to abrogate EGFR-induced senescence while their knock-down induces p15<sup>INK4b</sup> and p16<sup>INK4a</sup>, thus reactivating the senescence program [241]. In this direction, miR-200

induces senescence in endothelial cells by inhibiting ZEB1 [224]. The regulatory loop between p53/miR-200 and ZEB1/ZEB2 therefore seems to also be involved in control of senescence (Fig. 2a). The ability of ZEB factors to override senescence is closely tied to their activation of EMT: triggering of senescence by knock-down of ZEB1 and ZEB2 (or p53) makes cells insensitive to the EMT-driving effects of TGFβ [241].

In the developing embryo, Snail1 inversely correlates with areas of proliferation and cyclin D2 expression, results confirmed in canine kidney epithelial cells where Snail1 transcriptionally represses cyclin D2 and associates to high levels of p21<sup>CIP/WAF1</sup> and G1 arrest [219, 242]. In this line, knock-down of Snail1 drives prostate cancer cells into senescence [243]. Snail2 is also excluded from areas of proliferation in the neural tube of the developing chick [219], and its overexpression in prostate cancer cells represses cyclin D1 [244]. However, other groups have reported opposite results, suggesting that, as in the case of ZEB2, the role of EMT-ATFs in cell cycle arrest and senescence may be cell type-dependent. Thus, in osteosarcoma cells, Snail1 inhibits E12/E47 transcriptional activation of the p21<sup>CIP/WAF1</sup> promoter, cooperating in this function with Twist1 [245]. Likewise, in breast cancer cells, Snail2 upregulates cyclin D1 and fosters proliferation by forming a complex with CtBP and transcriptionally repressing UbcH5c, an ubiquitin that targets cyclin D1 [246].

Growth arrest and senescence is also achieved by knocking down Twist1 in immortalized non-malignant prostate cells, while its overexpression inhibits p53-dependent senescence via inhibition of ARF [247]. Repression of p14<sup>ARF</sup> also results in inhibition of Chk1/2 phosphorylation in response to DNA damage [247]. Twist1 and Twist2 override Ras<sup>V12</sup>-induced senescence of cell lines, and of breast cancer cells in MMTV-Erb2/Neu transgenic mice, by inhibition of p16<sup>INK4a</sup> and p21<sup>CIP/WAF1</sup> [248]. This study also found that Twist factors cooperate with Ras<sup>V12</sup> in the transformation of MEFs, an effect that was abolished when Ras-induced senescence was inhibited. Twist1 cooperates with N-myc in the transformation of wild-type but not INK4a/ARF (−/−) MEFs, confirming that Twist activity involves inhibition of the ARF/p53 pathway [216]. As in the case of ZEB proteins, Twist1/2-mediated override of senescence is linked to their ability to induce an EMT and promote tumor invasion.

## EMT-ATFs and angiogenesis

Snail1 is required for proper vascular development during embryogenesis, and its overexpression in embryonic stem cells induces the formation of VEGFR-2-positive endothelial cells through downregulation of miR-200 [249].

The growth of a tumor from an avascular hyperplasia into a larger mass requires the formation of new vessels through a process known as the angiogenic switch, which involves the production of angiogenic factors and proteases by tumor and stromal cells. Loss of E-cadherin is sufficient to trigger this angiogenic switch. Conditional knock-down of E-cadherin in mouse models of NSCLC promotes the development of tumor vasculature and growth through upregulation of vascular growth factors VEGF-A and VEGF-C and its receptor Flt-4 [250]. Angiogenesis is classically triggered by inflammation and hypoxia, conditions that are also known inducers of EMT [116]. Hypoxic conditions also increase the population of cancer cells with stem-like phenotype [251]. Stabilization of HIF-1 $\alpha$  in response to hypoxia or its overexpression promotes invasion and metastasis by directly activating Twist1 and inducing an EMT [116]. Likewise, knock-down of HIF-1 $\alpha$  or overexpression of the VHL gene—whose product targets HIF-1 $\alpha$  for ubiquitin-mediated degradation—downregulates expression of ZEB1, ZEB2, and Snail1 [60, 252]. Additionally, HIF-1 $\alpha$  feeds into upstream EMT-inducing signals (e.g., Notch, NF $\kappa$ B) [104]. Twist1-mediated induction of EMT by HIF-1 $\alpha$  is not redundant with Snail1, suggesting that both factors have distinct but complementary roles in the pro-invasive and pro-angiogenic response to hypoxia. In that line, joint expression of HIF-1 $\alpha$ , Twist1, and Snail1 in primary HNSCC associates with poorer prognosis [116].

Most EMT-ATFs promote tumor angiogenesis *in vivo*. ZEB, Snail, and Twist factors are often overexpressed by endothelial cells in the peritumoral stroma. Expression of Twist1 also associates with enhanced tumor microvessel vasculature and VEGF expression in hepatocarcinomas [205]. In mice, injection of lung adenocarcinoma cell lines overexpressing Snail1 or Snail2 generated tumors with enhanced vasculature compared to control cells [177, 253, 254], that in the case of Snail1 is accompanied by higher levels of proangiogenic factors CXCL5 and CXCL8 [254]. Likewise, compared to control cells, xenotransplant of breast cancer cell lines overexpressing Twist1 generate tumors with higher tumor angiogenesis and upregulated expression of several key vascular growth factors and receptors (e.g., VEGF, VEGFR2/KDR, Angiotensin-2, chemokine GRO- $\alpha$ , and CD31) [255, 256]. ZEB2 also promotes angiogenesis by direct transcriptional repression of the anti-angiogenic homeobox GAX factor [134]. However, and contrary to what would be expected from its induction by HIF-1 $\alpha$  and its role as promoter of tumor progression, ZEB1 can also function as a negative regulator of angiogenesis *in vivo*. Xenotransplanted melanoma cells develop larger tumors with more developed vascularization in mice with a haploinsufficient ZEB1 background [257].

EMT-ATFs in oncogenic addiction and resistance to therapy

Activation of oncogenic pathways (or loss/inactivation of tumor suppressor signals) induces pro-survival and pro-growth signals on which tumors can become dependent. This dependency of cancer cells on oncogenes—referred as “oncogenic addiction”—has been exploited in the development of new chemotherapy drugs [258]. The success of antibodies and drugs targeting specific oncogenes in the treatment of a number of solid and hematologic cancers in mice models and humans has helped to reinforce the oncogenic addiction concept.

EMT and EMT-ATFs allow cancer cells to overcome their dependency on the oncogenic signals originally involved in their transformation. Thus, the epithelial status of pancreatic and lung cancer cells with activating mutations of K-Ras determines their dependency on K-Ras for their growth and survival [259]. Cancer cells that are dependent on K-Ras exhibit epithelial characteristics, while those independent of K-Ras have a mesenchymal phenotype. Induction of EMT by TGF $\beta$  or ZEB1 overrides K-Ras addiction protecting cancer cells from apoptosis following K-Ras knock-down. Conversely, elimination of ZEB1 in K-Ras-independent cancer cells restores K-Ras dependency [261]. Snail2 is also required for the survival of colorectal cancer cells with mutant K-Ras [260]. These results support EMT as a mechanism for cancer cells to escape from oncogenic addiction and highlight the potential of EMT-ATFs as therapeutic targets.

In the same line, and in parallel with the anti-apoptotic role of EMT described earlier, a wealth of articles have shown an association between EMT and chemotherapy resistance. Cancer cell lines expressing E-cadherin are more sensitive to chemotherapy drugs compared to those displaying a mesenchymal phenotype [261–264]. Resistance to chemotherapy and hormone therapy in patients with breast carcinomas correlates with tumoral expression of stem and mesenchymal markers. Poor response of HNSCC and NSCLC to EGFR inhibitors gefitinib and erlotinib in cell lines, human tumors, and xenograft mice models is associated with expression of EMT and stem-like cell markers [265–268]. Acquisition of resistance to oxaliplatin in colorectal carcinoma cells is also accompanied by expression of mesenchymal markers [261]. Responsiveness to cetuximab (a chimeric mouse–human antibody against EGFR) in Ras wild-type colorectal cancer cells depends on high expression of epithelial markers and low levels of ZEB1, Snail1, and Snail2, and of stem-like phenotype [269]. It is worth noting that the EMT-inducing effect of some drugs is cell-cycle dependent: e.g., in breast cancer cells, doxorubicin induces a mesenchymal phenotype during G<sub>1</sub>/S and apoptosis in G<sub>2</sub>/M [270]. The

**Table 2** EMT-ATFs confer resistance to chemotherapy and radiotherapy

	Evidence	Resistance	Reference
ZEB1	Breast carcinoma cell lines	Doxorubicin	271
	Head and neck squamous carcinoma cell lines	Ertotinib	272
	Non-small lung carcinoma cell lines	Gefitinib	262
	Pancreatic carcinoma cell lines	Gemcitabine, 5-Fluorouracil, Cisplatin	71,131,273
ZEB2	Bladder and squamous carcinoma cell lines	Cisplatin, UV radiation	164
	Primary transitional cell carcinomas of the bladder	Radiotherapy	164
Snail1	Breast cancer cell line	5-Fluorouracil	276
	Lung carcinoma cell lines	Cisplatin	275
	Ovarian adenocarcinoma cell lines and primary tumors	Cisplatin	278
Snail2	Malignant mesothelioma	Doxorubicin, Paclitaxel, Vincristine	279
	Non-small cell lung carcinoma cell lines and Primary lung adenocarcinoma	Gefitinib	277
	Ovarian adenocarcinoma cell lines and primary tumors	Cisplatin	278
Twist1	Breast cancer cell lines	Doxorubicin	270
	Breast cancer cell lines	Paclitaxel	109
	Nasopharyngeal carcinoma cell lines	Paclitaxel	283
	Prostate carcinoma cell lines	Daunirubicin, cisplatin	280
	Prostate carcinoma cell lines	Paclitaxel, Cisplatin	247, 282
	Various carcinoma cell lines (bladder, nasopharyngeal, ovarian, prostate)	Paclitaxel, Vincristine	281
Twist2	Prostate adenocarcinoma cell lines	Daunirubicin, Cisplatin	280

Only some representative publications are included. See text for discussion

peritumoral stroma also plays an important role in chemoresistance to EGFR inhibitors. Interestingly, in a xenograft model of EGFR-resistant NSCLC, cancer-associated fibroblasts derived out of tumor cells that have undergone an EMT are not only EGFR-resistant but also tumorigenic [268].

The association between EMT and drug resistance is mediated, at least in part, by EMT-ATFs (Table 2). Expression of different EMT-ATFs by cancer cell lines and primary tumors confers tumor cells resistance to chemotherapy and radiotherapy. Resistance to doxorubicin in breast carcinoma cell lines correlates with higher expression of ZEB1 and SIRT1 [271]. In NSCLC cell lines, expression of ZEB1—but not of ZEB2, Snail1, or Snail2—correlates with higher resistance to gefitinib [262]. ZEB1 is also associated with resistance to erlotinib in HNSCC cell lines, and its knock-down increases drug sensitivity with the induction of E-cadherin expression [272]. Interestingly, simultaneous knock-down of ZEB1 and E-cadherin cancels out sensitization to erlotinib by ZEB1 elimination, suggesting that sensitivity to this EGFR inhibitor requires E-cadherin expression. Depletion of ZEB1 also sensitizes pancreatic cancer cell lines to gemcitabine, 5-fluorouracil, and cisplatin, and gemcitabine-resistant clones express higher levels not only of ZEB1 but also of Snail1 and Snail2 [71, 131, 273] (Table 2).

ZEB2 determines resistance to treatment independently of ZEB1, protecting bladder and squamous carcinoma cell

lines against DNA damage-inducing agents such as cisplatin or UV radiation [164]. Importantly, patients with ZEB2-negative bladder carcinomas also exhibit better response to radiotherapy [164].

Snail1 is upregulated in NSCLC xenotransplanted tumors resistant to EGFR inhibitors [268] and determines resistance to cisplatin in HNSCC primary tumors and HNSCC and NSCLC cell lines [274, 275] (Table 2). Snail1-related resistance to cisplatin in HNSCC cell lines is mediated by activation of the DNA excision repair protein ERCC1 [275]. Snail1 expression also confers resistance to 5-fluorouracil in breast carcinoma cell lines [276]. In contrast, other studies have found that resistance to gefitinib displayed by some NSCLC cell lines and developed by primary lung adenocarcinomas associated with overexpressed Snail2—but not Snail1, Twist1, or ZEB1—being reversed by Snail2 knock-down through a mechanism involving upregulation of Bim and activation of caspase 9 [277]. In breast carcinoma cell lines refractory to doxorubicin, knock-down of Snail1 and Snail2 have a synergistic effect inducing drug sensitiveness, suggesting that Snail1 and Snail2 determine resistance through non-overlapping mechanisms. Chemotherapy resistance induced by EMT-ATFs is not only tightly linked to the induction of mesenchymal markers but also of stemness. Knock-down of Snail1 and Snail2 in ovarian cancer cell lines increases their sensitivity to cisplatin [278]. As noted earlier, Snail1- and Snail2-induced resistance to paclitaxel and radiation in

**Table 3** EMT-ATFs regulate multiple hallmarks of cancer

Hallmarks of cancer	ZEB1	ZEB2	Snail1	Snail2	Twist1	Twist2
Activating invasion and metastasis	+	+	+	+	+	
	[167]	[15]	[177]	[253]	[19]	
Inducing angiogenesis	–	+	+	+	+	
	[257]	[134]	[177]	[253]	[255]	
Sustaining proliferative signaling	+	–			+	+
	[235]	[238]			[215, 248, 307]	[248]
Evading growth suppressors					+	+
					[215, 217, 248]	[248]
Resisting cell death	+	+	+	+	+	+
	[230]	[164]	[219]	[219]	[215]	[215]
Enabling replicative immortality	+	+	+	+	+	+
	[235, 241]	[241]	[243]	[244]	[247, 248]	[248]
		–				
		[239]				

Hallmarks of cancer refers to Ref. [1]. Only representative publication(s) were included. (+) represents activation of the cancer hallmark by the EMT-ATF. (–) represents repression of the cancer hallmark by the EMT-ATF. See text for discussion and other references

ovarian cancer cells relates to the repression of pro-apoptotic p53 targets and activation of stemness markers [212]. In malignant mesotheliomas, activation of SCF/c-kit signaling by Snail2 induces multidrug resistance [279].

Twist proteins are also involved in drug resistance. In breast carcinoma cells, Twist1 mediates resistance to paclitaxel via direct transcriptional activation of AKT2 [109]. Twist1 knock-down increases sensitivity of breast cancer cells to doxorubicin—a drug that triggers a p53-dependent DNA damage response—by disrupting p53-Mdm2 association [270]. Independently of p53 and p19<sup>ARF</sup> pathways, Twist1 and Twist2 block breast cancer cells death by daunorubicin by suppressing daunorubicin-induced phosphorylation of Bcl-2 [280]. In nasopharyngeal, bladder, ovarian, and prostate cancer cell lines, Twist1 increases resistance to paclitaxel and/or vincristine by upregulating Bcl2 and lowering Bax and Bak [281–283].

### Concluding remarks

In the span of just a few years, EMT-ATFs have evolved from simple repressors of E-cadherin to inducers of most of the traits that cancer cells need to acquire for successful tumor progression (Table 3). Cancer cells can acquire these capabilities through overexpression, gain-of-function mutations, or amplification of oncogenes and/or repression, mutation or deletion of tumor suppressors [1], and mounting evidence indicates that EMT-ATFs critically regulate these cancer hallmarks. From participating only at late stages in cancer (promoting EMT and metastasis), EMT-ATFs are now known to be also involved in the

initial phases of tumor development. First, EMT and metastasis may be a much earlier event in cancer progression than previously thought, playing an important role in tumor formation itself [31]. But, more importantly, ZEB, Snail and Twist factors regulate the acquisition of key early cancer hallmarks: EMT-ATFs contribute to overriding cancer safeguard programs (apoptosis, senescence), promoting tumor angiogenesis, cooperating with (or mediating) oncogenic signals (e.g., Ras), and antagonizing tumor suppressor pathways (e.g., p53) (Table 3). In turn, classical tumor repressors like Rb or p53 have recently been shown to regulate EMT and tumor invasiveness [128, 237].

The attention of molecular oncologists is moving from an early focus on the identification of irreversible mechanisms of cancer initiation and progression (e.g., mutations/deletions, amplifications) to the discovery of the dynamics involved in reversible programs of gene regulation in cancer cells (e.g., epigenetic/transcriptional, translational). Human tumors display a great level of heterogeneity, not only among patients but also within different areas and stages in a given tumor. Dynamic regulated expression of miRs and EMT-ATFs grants cancer cells with a great level of functional plasticity [54]. These transcriptional and translational regulatory networks allow cancer cells to reversibly transition between different states as they adapt to the environment during tumor progression, not only between epithelial and mesenchymal phenotypes but also between stemness and differentiation or between proliferation and growth arrest.

The data reviewed here attest to a significant level of overlapping among EMT-ATFs in their pattern of



expression, mechanisms of action, target genes and regulation of hallmarks of cancer. Increasing specificity and temporal and spatial hierarchies among EMT-ATFs are progressively being revealed with the identification of an expanding set of upstream regulatory miRs and translational regulators [149, 152, 155]. Similarly, the discovery of new cofactors and chromatin remodeling complexes used by EMT-ATFs to transcriptionally regulate their targets also points to some divergence; even if several EMT-ATFs are coexpressed, availability of these cofactors may dictate the functional capabilities of the EMT-ATFs.

Codification of the hallmarks of cancer by Hanahan and Weinberg [1] has helped researchers not only to systematize their findings and current understanding of cancer but has also contributed to guiding new therapy strategies. Compared to the irreversible effects of mutations and deletions, the dynamic regulation of miRs and EMT-ATFs renders them attractive targets for personalized oncology treatment. Incorporating the analysis of EMT-ATF expression in primary tumors into routine pathology diagnosis could help to prospectively identify resistance to particular chemotherapy. Since chemotherapies targeting a single oncogenic signal or cancer cell trait are not failsafe against resistance and tumor recurrence, a simultaneous approach to several signaling pathways and cancer hallmarks could be more successful. The highly modular structure and complex transcriptional activities of EMT-ATFs and their simultaneous regulation of multiple cancer hallmarks makes them attractive therapeutic targets for translational researchers.

On the other hand, reversibility of the EMT/MET balance and plasticity in its regulation represent adaptive mechanisms developed by cancer cells. Reverting the mesenchymal phenotype of invading cells by blocking the expression and/or function of EMT-ATFs may attenuate the ability of cancer cells to invade and increase their sensitivity to chemotherapy. However, as metastatic cancer cells need to regain an epithelial phenotype to grow into a macroscopic tumor at the site of distant colonization, reverting the mesenchymal status of tumor cells may actually foster metastasis. This is illustrated by the somewhat paradoxical metastatic-enhancing effect of miR-200 [125, 313]. In this regard, if reversible and dynamic regulation of miR and EMT-ATFs during cancer progression offers new avenues for therapy, it also complicates their manipulation. More information of the upstream regulatory networks and mechanisms of transcriptional regulation by EMT-ATFs is therefore needed before interference of their expression/function can be considered in cancer treatment. As far as we know, induction of EMT by EMT-ATFs occurs along with their regulation of other hallmarks (e.g., apoptosis, senescence). It remains to be ascertained how regulation of these hallmarks overlaps at the mechanistic

level. To the extent that separate mechanisms are discovered, therapeutic approaches could be more precisely targeted.

In sum, as our understanding on the expanding roles of EMT-ATFs improves, these factors are likely to become not only prognostic and predictive personalized biomarkers in cancer but also important therapeutic targets in the near future.

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