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REVIEW

Molecular regulation of Snai2 in development and disease

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ABSTRACT

The transcription factor Snai2, encoded by the *SNAI2* gene, is an evolutionarily conserved C2H2 zinc finger protein that orchestrates biological processes critical to tissue development and tumorigenesis. Initially characterized as a prototypical epithelial-to-mesenchymal transition (EMT) transcription factor, Snai2 has been shown more recently to participate in a wider variety of biological processes, including tumor metastasis, stem and/or progenitor cell biology, cellular differentiation, vascular remodeling and DNA damage repair. The main role of Snai2 in controlling such processes involves facilitating the epigenetic regulation of transcriptional programs, and, as such, its dysregulation manifests in developmental defects, disruption of tissue homeostasis, and other disease conditions. Here, we discuss our current understanding of the molecular mechanisms regulating Snai2 expression, abundance and activity. In addition, we outline how these mechanisms contribute to disease phenotypes or how they may impact rational therapeutic targeting of Snai2 dysregulation in human disease.

KEY WORDS: Slug, Cancer, Development

Introduction

Snai2, encoded by the *SNAI2* gene (formerly known as Slug), is one of three members of the Snail family of zinc-finger transcription factors (TFs). It is highly conserved among vertebrate species and widely regarded as a prototypical epithelial-to-mesenchymal transition transcriptional factor (EMT-TF) (Barrallo-Gimeno, 2005; Nieto, 2002; Thiery, 2002). As an EMT-TF, Snai2 promotes loss of cell adhesion and polarity while conferring migratory and invasive capabilities (Bolós et al., 2003; Hajra et al., 2002). These processes are fundamental aspects of many developmental stages conserved in vertebrate and non-vertebrate organisms. As such, Snai2 is known to play critical roles in primitive streak formation, neural crest migration, left-right asymmetry and morphogenesis of various tissues (Barrallo-Gimeno, 2005; Cobaleda et al., 2007; Nieto, 2002; Nieto et al., 1994). However, over the past decade, the known functional repertoire of Snai2 has expanded considerably beyond its classical role in developmental biology (Box 1). Most notably, the discovery of how EMT contributes to cancer progression and metastasis led to an appreciable body of evidence supporting a critical role for Snai2 and other EMT-TFs in promoting malignant cancer cell behavior (De Craene and Berx, 2013; Lamouille et al., 2014; Peinado et al., 2007). Indeed, Snai2 overexpression is a widespread phenomenon in human cancers and notably predicts poor prognosis in cancer patients (Cobaleda et al., 2007; Coll-Bonfill et al., 2016; De Craene and Berx, 2013; de Herreros et al., 2010; Lamouille et al., 2014; Shih and Yang, 2011).

A defining feature underpinning the functional versatility of Snai2 is its ability to act as a transcriptional repressor. As a member of the Snail TF family, Snai2 contains five consecutive C-terminal zinc fingers (Fig. 1), which facilitate its binding to E-box consensus CAGGTG motifs of target genes. It also contains an evolutionarily conserved N-terminal SNAG domain that mediates the recruitment of various chromatin regulators to epigenetically silence the expression of its target genes (Fig. 1). The most notable of its target genes is *CDH1*, which encodes the epithelial cell adhesion molecule E-cadherin; however, a plethora of other Snai2 target genes have been identified more recently (Bai et al., 2017; Bolós et al., 2003; Cobaleda et al., 2007; Hajra et al., 2002; Phillips et al., 2014; Tien et al., 2015; Wu et al., 2005). In contrast to the above structural features, the central region of Snai2 differs substantially from that of other Snail family members. Specifically, Snai2 lacks several regulatory elements present in the paralogous protein Snai1 (encoded by *SNAI1*), such as the destruction box and nuclear export signal (Cobaleda et al., 2007; Nieto, 2002). Instead, Snai2 contains a unique, 28-amino acid sequence called the SLUG domain, which facilitates interaction with several co-factors that subsequently impact Snai2 function and abundance (Hemavathy et al., 2000; Molina-Ortiz et al., 2012; Sefton et al., 1998; Zhou et al., 2016).

While EMT-TFs perform an overlapping set of biological functions, they can also regulate distinct morphogenic and tissue-specific processes (Phillips and Kuperwasser, 2014; Shirley et al., 2010; Stemmler et al., 2019). For example, Snai1 and Snai2 are capable of inducing unique gene expression programs and appear to contribute to adult tissue homeostasis and/or tumorigenesis in distinct ways (Gross et al., 2019; Phillips and Kuperwasser, 2014; Phillips et al., 2014; Shirley et al., 2010; Stemmler et al., 2019; Ye et al., 2015; Zhou et al., 2016). More specifically, Snai2 has recently been implicated in controlling a variety of critical biological processes beyond classical EMT, including stem and/or progenitor cell activity, cellular differentiation, DNA damage response and vascular remodeling (Fig. 2) (Cobaleda et al., 2007; Coll-Bonfill et al., 2016; Gross et al., 2019; Nassour et al., 2012; Phillips et al., 2014; Sánchez-Duffhues et al., 2015; Storci et al., 2010; Welch-Reardon et al., 2014). Given this broad array of biological functions that Snai2 controls, it is not unexpected that Snai2 requires strict regulation of its expression and activity in normal tissues (De Craene and Berx, 2013; Díaz et al., 2014; Vernon and LaBonne, 2006; Zhou et al., 2016). Such attributes raise fundamental questions regarding the molecular mechanisms responsible for Snai2 regulation and whether these are altered during disease progression. Therefore, in this Review, we aim to provide a comprehensive discussion of the molecular mechanisms controlling Snai2 abundance and activity at the transcriptional, post-transcriptional, translational and post-translational levels (Fig. 3). In addition, we highlight how its dysregulation impacts cancer development and provides potential therapeutic strategies (Box 2).

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Box 1. Snai2 is a regulator of stem cell fitness and differentiation

While Snai2 is a well-established regulator of early development in many model organisms, it is also expressed in normal adult tissues. Accordingly, many recent studies have identified roles for Snai2 in regulating stem cell fitness and differentiation in several different tissue systems (see Fig. 2 for an overview).

Snai2 has been established as a major regulator of adult stem and/or progenitor cell function and differentiation in hematopoietic, breast, epidermal and mesenchymal tissues. Loss of Snai2 in hematopoietic stem cells (HSCs) leads to defective lineage specification and also suppresses HSC repopulation under non-homeostatic conditions (Pérez-Losada et al., 2002; Sun et al., 2010). In mammary epithelium, Snai2 promotes stem cell function and directs lineage specification through direct transcriptional repression of luminal differentiation genes (Guo et al., 2012; Nassour et al., 2012; Phillips et al., 2014; Ye et al., 2015). Snai2 also controls differentiation of epidermal progenitor cells, as its loss promoted keratinization and increased adhesion junctions in the epidermis (Mistry et al., 2014). Likewise, differentiation of adult mesenchymal tissue into muscle, cartilage or bone is regulated by Snai2 (Kim et al., 2010; Lolli et al., 2014; Seki et al., 2003; Soleimani et al., 2012; Tang et al., 2016b).

In addition to promoting regenerative function and regulating differentiation, Snai2 also controls other aspects of stem cell fitness. Snai2 promotes survival of hematopoietic progenitor cells following irradiation through transcriptional repression of the pro-apoptotic gene *PUMA* (also known as *Bbc3*) (Inoue et al., 2002; Wu et al., 2005). Relatedly, Snai2 regulates apoptosis during post-lactational involution of the mammary epithelium, as loss of Snai2 *in vivo* impaired STAT3-mediated apoptosis (Castillo-Lluva et al., 2015). More recently, Snai2 was also found to facilitate efficient DNA damage repair (DDR) in mammary epithelial cells (Gross et al., 2019).

Taken together, these findings suggest that Snai2 regulates many aspects of stem and/or progenitor cell biology, ranging from regulating canonical stemness features, such as self-renewal and differentiation, to other aspects of stem cell fitness, such as survival and DDR. The discovery that Snai2 regulates a broader repertoire of stem cell checkpoint decisions, such as DDR, apoptosis and differentiation, than previously assumed warrants further investigation, particularly within physiological contexts that preferentially invoke its mediation of checkpoint decisions.

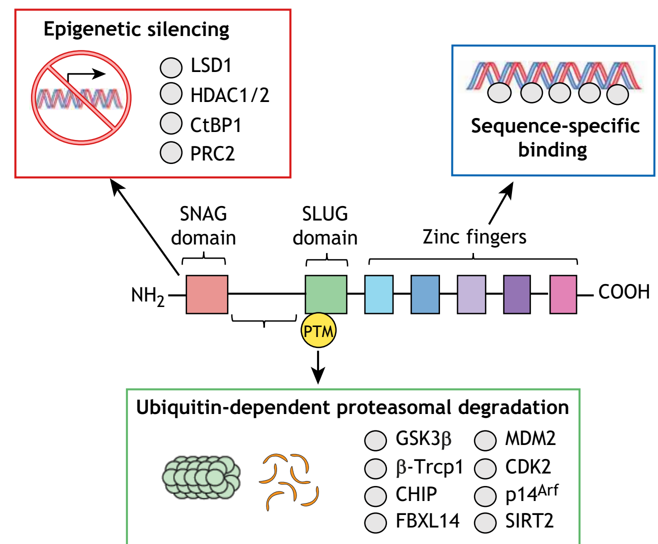

Fig. 1. The molecular anatomy of the transcription factor Snai2. The transcription factor (TF) Snai2 is a well-conserved member of the Snail family. It contains several protein motifs characteristic of this TF family, such as a SNAG domain at the N-terminus and consecutive zinc finger domains at the C-terminus. These domains are particularly critical for Snai2's role as a transcriptional regulator. The five C-terminal zinc finger domains of Snai2 bind to E-box consensus motifs in gene regulatory regions, while chromatin regulators, such as CtBP1, HDAC1/2, LSD1 and PRC2, are recruited via the SNAG domain (Bai et al., 2017; Phillips et al., 2014; Tien et al., 2015). Through this recruitment of epigenetic silencers, Snai2 facilitates transcriptional repression of its target genes. The SLUG domain also impacts Snai2 function. A variety of proteins, such as GSK3β, β-Trcp1, CHIP, FBXL14, MDM2, CDK2, p14^{Arf} and SIRT2, can facilitate the deposition of post-translational modifications (PTM), including phosphorylation, ubiquitylation and acetylation, on SLUG domain residues that can dictate its proteolytic turnover or cellular localization (Hemavathy et al., 2000; Molina-Ortiz et al., 2012; Sefton et al., 1998; Zhou et al., 2016).

Transcriptional regulation

A diverse repertoire of molecular mechanisms controlling Snai2 regulation at the transcriptional level has been characterized in a variety of model organisms. Generally, activation of receptors by extracellular cues triggers an intracellular signaling cascade that leads to the binding of a transcription factor to the *SNAI2* promoter and regulation of its expression. Importantly, several key developmental pathways have been found to regulate *SNAI2* gene expression in this manner, and their dysregulation in cancer cells promotes malignant characteristics. It should be noted that many of the growth factors and signaling pathways that transcriptionally regulate Snai2 also control expression of other EMT-TFs, including that of the Snail family member Snai1. While it is important to consider such similarities, extensive evaluation of this topic is beyond the scope of this article and has been covered in other recent reviews (Stemmler et al., 2019).

Transforming growth factor β signaling

The transforming growth factor β (TGFβ) signaling pathway is a well-characterized transcriptional regulator of *Snai2* expression for induction of EMT during development. In response to the activation of TGFβ family receptors, the SMAD signaling complex forms and translocates into the nucleus, where it readily recruits other transcription factors and thus indirectly upregulates *SNAI2* gene expression. More specifically, SMAD3 recruits myocardin-related transcription factors (MRTFs) and bind the cis-element in the *SNAI2* promoter region to activate its transcription in kidney, liver, and mammary epithelial cells (Morita et al., 2007). Moreover, SMAD2 and SMAD3 signaling recruits hairy/enhancer-of-split protein 1 (Hey1) and high mobility group A2 (HMGA2) to upregulate *SNAI2* gene expression in mammary epithelial cells (Morita et al., 2007; Thuault et al., 2006). Following its transcriptional upregulation, Snai2 cooperates with other EMT-TFs to promote the EMT transcriptional program, thereby enabling proper function of TGFβ signaling during key developmental events, such as neural crest formation, endocardial cushion formation and palate fusion. Importantly, Snai2 overexpression induced by constitutive stimulation of TGFβ signaling and/or overexpression of its transcriptional regulators (i.e. HMGA2 or MRTFs) endowed migratory and invasive capabilities in cancer cells (Li et al., 2014).

Growth factor signaling

A substantial number of growth factor signaling pathways also regulate *SNAI2* transcription. Notably, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and insulin growth factor (IGF) readily increase both *SNAI2* mRNA and Snai2 protein abundance to promote mesenchymal phenotypes in epithelial cells (Billottet et al., 2008; Grotegut et al., 2006; Kusewitt et al., 2009; Savagner et al., 1997; Vallés et al.,

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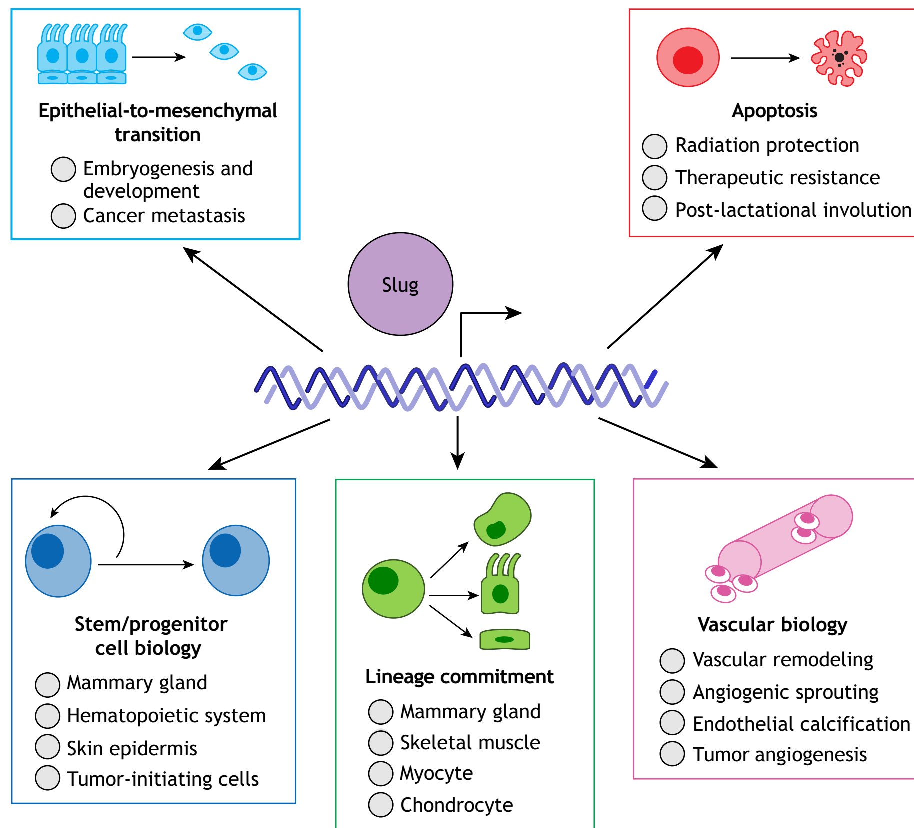

Fig. 2. The diversity of Snai2 function in development and tumorigenesis. As an EMT-TF, Snai2 represents a central convergence point of signaling processes to facilitate key morphogenic events during embryonic development. Such functions of Snai2, in addition to its expression in adult tissues, suggest that it is capable of regulating stem and progenitor cell biology beyond early development. Indeed, Snai2 function has been shown to be integral to maintaining adult stem cell compartments in the hematopoietic system, mammary gland, epidermis and mesenchymal tissues (Guo et al., 2012; Kim et al., 2010; Mistry et al., 2014; Nassour et al., 2012; Pérez-Losada et al., 2002; Phillips et al., 2014; Seki et al., 2003; Soleimani et al., 2012; Sun et al., 2010; Ye et al., 2015). Aside from conferring self-renewal and regenerative functions, Snai2 also regulates other aspects of stem and progenitor cell biology, such as lineage commitment and differentiation decisions, proliferative potential and cell survival, within many of these tissue compartments through additional mechanisms (Castillo-Lluva et al., 2015; Gross et al., 2019; Guo et al., 2012; Inoue et al., 2002; Kim et al., 2010; Lolli et al., 2014; Mistry et al., 2014; Nassour et al., 2012; Pérez-Losada et al., 2002; Phillips et al., 2014; Seki et al., 2003; Soleimani et al., 2012; Sun et al., 2010; Tang et al., 2016b; Wu et al., 2005). Importantly, Snai2 performs these functions not only in normal adult tissues but also in contexts of cancer. For example, while Snai2 is an important suppressor of apoptosis in normal hematopoietic stem cells, this function promotes therapeutic resistance in cancer cells (Arienti et al., 2013; Chang et al., 2011; Dong et al., 2016; Haslehurst et al., 2012; Jiang et al., 2016; Kurrey et al., 2009; Vitali et al., 2008; Wu et al., 2005). Emerging evidence has additionally highlighted a role for Snai2 in vascular biology. Snai2 facilitates vascular remodeling during pulmonary hypertension by altering expression of genes involved vascular smooth muscle differentiation, proliferation and migration (Coll-Bonfill et al., 2016). Snai2 also controls endothelial cell (EC) behavior during angiogenesis and osteogenic differentiation of ECs during vascular calcification, suggesting that Snai2 may be a key regulator involved in vascular disease.

1996; Yao et al., 2016). In contrast to TGFβ, these growth factors utilize the receptor tyrosine kinases (RTKs) and downstream phosphorylation cascades to upregulate *SNAI2* transcription. For example, following RTK-mediated RAS and RAF activation, extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK) signaling induces the transcription of *SNAI1* and *SNAI2* to drive EMT programs involved in embryonic development (Chen et al., 2009; Hardy et al., 2011; Lamouille et al., 2014; Stevens et al., 2008). Importantly, migratory and invasive behavior in colon cancer cells is driven by oncogenic activation of KRAS and BRAF, and thus it is possible that this phenomenon occurs via the above mechanism (Lamouille et al., 2014; Makrodouli et al., 2011). Despite these findings, there is limited knowledge of the transcriptional regulators responsible for *SNAI2* induction upon growth factor receptor and kinase signaling. However, a recent study illustrated that in colon cancer cells, IGF-II (also known as IGF2) activates the alternative Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway, resulting in nuclear translocation of STAT3 and its association with the Nanog transcription factor (Yao et al., 2016). Consequently, Nanog binds to the *SNAI2* promoter and directly upregulates its expression. As IGF receptor expression is often elevated in colon cancers, the link between IGF, STAT3, Nanog and Snai2 constitutes an integral signaling pathway mediating EMT and stemness that may serve as a prominent driver of aggressive malignant phenotypes in colon cancer (Yao et al., 2016).

Notch signaling

Another important developmental pathway that regulates *SNAI2* transcription is Notch. In response to delta-like or jagged protein ligand stimulation, the Notch receptor undergoes proteolytic processing to release the Notch intracellular domain (NICD); subsequently, NICD translocates into the nucleus and interacts with transcriptional regulators that affect *SNAI2* expression (Niessen et al., 2008; Shao et al., 2015). During heart development, for example, delta-like 4 ligand (DLL4)-mediated activation of Notch

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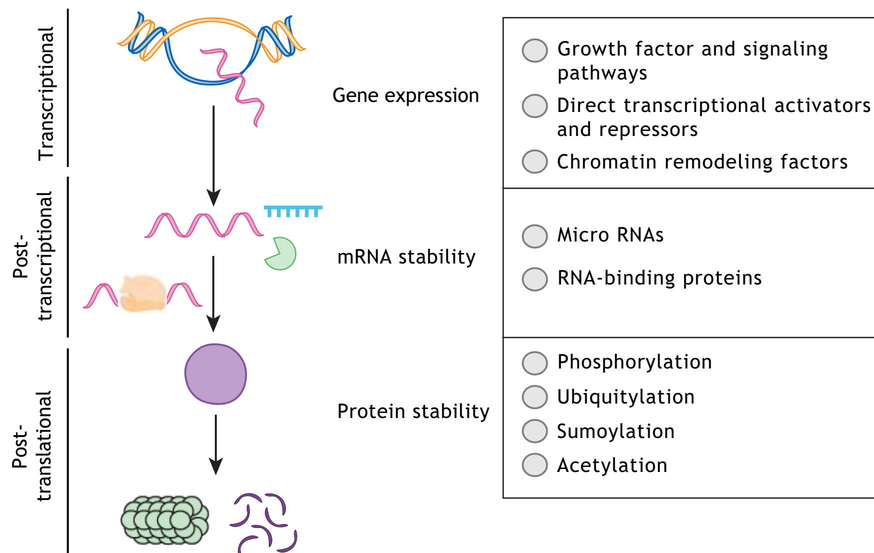

Fig. 3. Overview of Snai2 regulation spanning from transcript to protein level. Snai2 levels are strictly regulated in normal tissues by multiple transcriptional, post-transcriptional and post-translational control mechanisms. *SNAI2* transcription is controlled by many signaling pathways fundamental to developmental processes, including TGFβ, growth factors, Notch and Wnt signaling. Either through these known pathways or other mechanisms, its transcription is impacted or directly facilitated by a variety of transcriptional activators and repressors, as well as chromatin remodeling factors. At the post-transcriptional level, the expression level or stability of the *SNAI2* gene product can be regulated by several known microRNAs or RNA-binding proteins. While not much is currently known about how Snai2 is regulated at the translational level, a wide array of post-translational modifier proteins have been established as critical regulators of Snai2 proteolytic turnover, cellular localization and biological function. Phosphorylation and ubiquitylation are the most abundant modifications made by these regulators, but recent findings have shown that Snai2 can also undergo sumolyation and acetylation, suggesting that post-translational modifications of Snai2 capable of regulating its stability and activity may be more diverse than previously thought.

stimulates the binding of the TF RBPJ [also known as CSL (CBF1, Suppressor of hairless, Lag-1)] to the *SNAI2* promoter and induces its expression (Niessen et al., 2008). Such upregulation of *SNAI2* suppresses the endothelial phenotype within the developing tissue while facilitating migratory capabilities necessary for cardiac cushion morphogenesis (Niessen et al., 2008). Notch signaling also critically regulates *Snai2* transcription during neural crest formation in several species (Endo et al., 2002; Glavic, 2003). While delta-like protein (DLL1)-mediated Notch activation was found to induce a transient, stage-specific repression of *SNAI2* expression in chick embryos, *SNAI2* expression was shown to be indirectly induced downstream of Notch-mediated activation of the transcriptional regulators Iroquois homeobox protein (Xiro1) and Hairy/enhancer-of-split related with YRPW motif protein 2 (Hey2) in *Xenopus* (Endo et al., 2002; Glavic, 2003). Finally, Notch hyperactivity caused by aberrant expression of jagged-1 ligand (JAG1) and NOTCH1 in human cancer cells results in increased NICD binding to the *SNAI2* promoter and upregulation of Snai2; this leads to increased metastasis through EMT initiation (Leong et al., 2007).

Wnt signaling

The canonical Wnt/β-catenin pathway also seems to regulate *SNAI2* transcription. Upon Wnt ligand binding to Frizzled receptors, β-catenin is stabilized, undergoes nuclear translocation, and binds to the lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) transcription factors to form an active transcriptional complex. LEF/TCF binding sites have been identified within the *SNAI2* promoter in several vertebrate species, and a functional Lef1 binding site in the *Xenopus SNAI2* promoter is necessary and sufficient to induce Snai2 expression during neural crest cell determination (Lambertini et al., 2010; Sakai et al., 2005; Vallin et al., 2001). *SNAI2* gene expression is positively regulated by Wnt/β-catenin signaling in human osteoblasts, specifically through direct recruitment of Lef1 to the *SNAI2* promoter (Lambertini et al., 2010). Furthermore, the secreted Wnt signaling antagonist secreted frizzled-related protein 3 (sFRP3, also known as FRZB) suppresses *SNAI2* expression and its related EMT program in prostate cancer cells (Zi et al., 2005). As sFRP3 is frequently deleted in human tumors, it is plausible that the promotion of malignant behavior in cancer cells may occur through exploitation of this regulatory mechanism (Zi et al., 2005). Indeed, aberrant regulation of Wnt signaling is a common feature in cancer cells, and dysregulation beyond the transcriptional level can alter expression of Snai2 and other EMT-TFs (DiMeo et al., 2009; Guo et al., 2007; Lamouille et al., 2014; Peinado et al., 2007; Wu et al., 2012).

Other signaling pathways

Bone morphogenetic protein 4 (BMP4) is a member of the TGFβ superfamily and shares similar SMAD signaling complexes to mediate is downstream functions. As discussed above, nuclear SMAD can directly bind to the *SNAI2* promoter and upregulate its expression (Gordon et al., 2009; Morita et al., 2007; Thuault et al., 2006). Through this regulation, BMPs cooperate with Wnt and FGF signaling to coordinate the EMT program and neural crest formation during embryogenesis. In the context of cancer progression, several BMP family members are upregulated in pancreatic cancer, thereby promoting Snai2 overexpression to facilitate EMT-associated cancer invasion (Gordon et al., 2009).

Sonic hedgehog (Shh) signaling has also been implicated in regulating *SNAI2* expression, as its inhibition downregulates *SNAI2* transcription (Wen et al., 2015). However, it remains undetermined whether the Shh downstream transcription factor Gli1 directly mediates this effect.

Hypoxia represents a unique environmental factor that has been established as a positive regulator of *SNAI2* expression (Xu et al.,

Box 2. Snai2 in tumor biology: beyond EMT

Aberrant Snai2 expression is frequently observed in many cancer types and can predict cancer progression and patient prognosis (Barrallo-Gimeno, 2005; Come et al., 2006; Kajita et al., 2004; Nieto, 2002; Villarejo et al., 2014). Although Snai2 was initially thought to contribute to cancer progression strictly through its EMT functions, recent studies have revealed that Snai2 also contributes to cancer biology through additional mechanisms.

The role of Snai2 in promoting stem cell function in normal tissues is thought to contribute to cancer stem cell (CSC) behavior. Snai2 is highly expressed in $CD44^+/CD24^-$ CSCs from breast tumors, and Snai2 overexpression was sufficient to generate a highly regenerative, tumorsphere-initiating population from breast cancer cells (Bhat-Nakshatri et al., 2010; Guo et al., 2012). The role of Snai2 in promoting CSC-like behavior has also been observed in glioblastoma, pancreatic and liver cancer cells (Huan et al., 2017; Lin et al., 2018; Ma et al., 2017; Lin et al., 2018; Ma et al., 2017).

Snai2 also represents a molecular determinant of breast tumor subtype. Snai2 is upregulated in BRCA-mutated normal human mammary epithelial cells (HMECs), which generate basal-like tumors following oncogenic transformation (Proia et al., 2011). Snai2 transcriptionally silences luminal differentiation, biasing progenitor cells towards basal differentiation. Accordingly, Snai2 depletion reversed basal characteristics of $BRCA1^{mut/+}$ HMECs, revealing that Snai2 is a critical regulator of basal-like breast cancer (Proia et al., 2011). As Snai2 regulates differentiation in many other normal tissues, it will be useful to investigate whether disrupted lineage commitment driven by Snai2 dysregulation similarly biases tumor subtype development in other cancers.

Additionally, in multiple cancer types, Snai2 depletion sensitizes many cancers to chemotherapy drugs, radiation therapy and/or targeted therapies (Arienti et al., 2013; Chang et al., 2011; Dong et al., 2016; Haslehurst et al., 2012; Jiang et al., 2016; Kurrey et al., 2009; Vitali et al., 2008; Wu et al., 2005). Thus, Snai2 represents a rational target to combat therapeutic resistance; this raises the possibility of using Snai2 inhibition as an adjuvant treatment to boost efficacy of existing cancer therapeutics. Consequently, identifying the molecular mechanisms that drive Snai2-dependent therapeutic resistance will be important in order to determine the most promising approaches for targeting Snai2 clinically.

2015). Specifically, the transcription factor HIF-1α was found to mediate *SNAI1* and *SNAI2* induction (Xu et al., 2015). A hypoxic tumor microenvironment is associated with more malignant phenotypes such as invasion, and *SNAI2* upregulation in this context promotes hypoxia-mediated metastasis (Huang et al., 2009).

Other transcriptional regulatory mechanisms

The transcriptional regulation of *SNAI2* expression is not limited to the growth factor and developmental pathways discussed above. Additional transcriptional regulators regulate *SNAI2* expression in postnatal tissue development, tissue homeostasis and cancer progression.

The chromatin remodeling factor jumonji domain-containing protein 3 (Jmjd3, also known as KDM6B) decreases trimethylation of H3K27 on the *SNAI2* promoter to promote its transcription (Tang et al., 2016a). Functionally, Jmjd3 overexpression leads to upregulation of Snai2 expression and promotes EMT, stem-like traits, and invasive behavior in liver cancer cells, whereas silencing Jmjd3 reduces Snai2 expression and its associated self-renewal and tumor-initiating capacities *in vivo* (Tang et al., 2016a). Clinically, elevated Jmjd3 expression in hepatocellular carcinoma inversely correlates with patient survival (Tang et al., 2016a). In endothelial cells, epigenetic regulation of *SNAI2* transcription is mediated by the chromatin remodeling factor high mobility group protein A1 (HMGA-1) (Sánchez-Duffhues et al., 2015). In pulmonary hypertension patients, HMGA-1 is highly expressed in endothelial cells (ECs) and causes transcriptional upregulation of *SNAI2*, leading to expression of smooth muscle differentiation genes (Sánchez-Duffhues et al., 2015).

In the mammary gland, *SNAI2* promoter activity and transcription can be directly repressed by the transcription factor E74-like factor 5 (Elf5), resulting in inhibition of the EMT program during normal postnatal development and breast cancer metastasis (Chakrabarti et al., 2012). Accordingly, Elf5 deletion *in vivo* results in an increased presence of mammary stem cells (MaSCs), induction of EMT features during pregnancy and lactation, and increased lung metastases in a mouse tumor model. Reduced levels of Elf5 have been observed in early disease-stage breast hyperplasias, and this loss may represent an instigating step for breast cancer cells to acquire malignant features through upregulation of *Snai2* and its associated EMT program (Chakrabarti et al., 2012).

Similarly, the basic helix-loop-helix (bHLH) transcription factor single-minded 2 short splice variant (Sim2s) also directly binds the *SNAI2* promoter and represses its expression in the mammary epithelium (Laffin et al., 2008). Loss of Sim2s causes aberrant mammary ductal development characterized by increased proliferation, loss of polarity, downregulation of E-cadherin, and epithelial invasion into the surrounding stroma (Laffin et al., 2008). Importantly, Sim2s deletion increases tumor burdens in mice, and Sim2s is frequently lost or its expression reduced in human breast tumors; this loss likely leads to elevation of *SNAI2* expression and activation of its associated EMT programs during breast cancer progression (Kwak et al., 2006).

Another negative regulator of *SNAI2* expression is CCAAT-enhancer-binding protein δ (C/EBPδ, also known as CEBPD). In normal mammary tissue or less aggressive breast tumors, the estrogen receptor stabilizes C/EBPδ, which binds the *SNAI2* promoter and silences its gene expression (Mendoza-Villanueva et al., 2016). This C/EBPδ-mediated *SNAI2* silencing limits the motility and invasion of breast cancer cells, consistent with the observation of better prognoses for patients with tumors expressing high levels of C/EBPδ (Mendoza-Villanueva et al., 2016).

Furthermore, the transcription factors Krueppel-like factor 4 (Klf4) and forkhead box protein A1 (Foxa1) cooperate to directly repress *SNAI2* expression in prostate cancer (Liu et al., 2012). Klf4 depletion in prostate cancer cells leads to elevation of *SNAI2* expression and EMT program initiation, which is in accordance with the finding that advanced stage, malignant prostate tumors demonstrate low Klf4 and high Snai2 expression (Liu et al., 2012).

Several transcriptional activators of *SNAI2* have also been described. The transcription factor forkhead box protein M1 (FoxM1) directly binds to the *SNAI2* promoter to upregulate its expression and promote EMT induction in breast cancer cells (Yang et al., 2013a). EMT-TFs also positively regulate each other's expression to activate EMT in a coordinated fashion. In the case of Snai2, both Snai1 and Twist proteins are capable of binding to the *SNAI2* promoter to induce its gene expression (Casas et al., 2011; Chen and Gridley, 2013). This regulation allows the simultaneous activation of all pathways of the EMT program, consistent with the co-expression of multiple EMT-TFs observed in developmental processes.

Finally, Snai2 can bind to its own promoter and activate its own expression. Auto-activation of Snai2 has been documented during

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neural crest development, wherein the transcription factor Sox9 or the protein kinase A (PKA) family of signal transducer proteins can further enhance Snai2 binding to its own promoter (Chen and Gridley, 2013; Sakai, 2006). This finding strongly suggests that, in addition to its canonical repressive activity, Snai2 may also possess transcriptional activator activity. As the paralogous protein Snai1 was recently shown to activate cytokine expression by coupling with CREB-binding protein, it is plausible that Snai2 can act as a transcriptional activator for its own set of gene targets (Hsu et al., 2014).

Post-transcriptional regulation

Post-transcriptional control represents a fundamental mechanism to regulate gene expression. Once transcribed, *SNAI2* transcripts are susceptible to multiple gene regulatory mechanisms. The majority of the existing literature addressing post-transcriptional control of Snai2 centers on microRNAs (miRNAs), but a handful of alternative mechanisms for the regulation of Snai2 transcript stability have also been observed, as discussed below.

microRNAs

miRNAs are small, non-coding RNAs that selectively target gene transcripts to regulate their stability and/or translation. Through this post-transcriptional control of gene expression, miRNAs are capable of regulating EMT-TFs, including Snai2 (Diaz-Lopez et al., 2014). Multiple miRNAs have been found to directly bind to the 3′ untranslated region (UTR) of *SNAI2* transcripts and silence its expression, providing an additional layer of regulation to reinforce tight control of *SNAI2* expression during tissue development and homeostasis. Importantly, genetic alteration of these miRNAs causes dysregulation of *SNAI2* expression in tumors and correlates with poor prognosis in cancer patients (Ambs et al., 2008; Burk et al., 2008; Davalos et al., 2012; Liang et al., 2013; Liu et al., 2013).

One major miRNA repressor of EMT-TFs is the miR-200 family. Expression of miR-200ba429 and miR-200c141 helps protect epithelial cell identity, and somatic loss of several miR-200 family members is associated with aggressive cancer types and metastasis (Davalos et al., 2012). Indeed, miR-200b can bind to the 3′ UTR of *SNAI2* and directly inhibit its expression, as does another miRNA, miR-1 (Liu et al., 2013). Similarly, miR-200c can bind the 3′ UTR of *SNAI1* transcript and inhibit its expression (Perdigão-Henriques et al., 2015). Overexpression of both miR-200b and miR-1 depletes *SNAI2* transcript levels and impairs EMT activation, which ultimately decreases metastases and tumor burdens in an *in vivo* mouse model (Ambs et al., 2008; Burk et al., 2008; Liu et al., 2013).

Many other miRNAs have also been found to regulate *SNAI2* transcripts in a variety of biological contexts. During myoblast differentiation, post-transcriptional silencing of *SNAI1* and *SNAI2* by miR-30a and miR-206, respectively, facilitates a permissive feedback loop that allows transcriptional activation of the MyoD-associated differentiation program (Soleimani et al., 2012). In ovarian cancer cells, miRNA-506 was found to bind the 3′ UTR of the *SNAI2* transcript, resulting in suppression of both *SNAI2* expression and the EMT transcriptional program (Yang et al., 2013b). Importantly, overexpression of miR-506 effectively reduced tumor burden and metastases in an orthotopic mouse model of ovarian cancer (Yang et al., 2013b). This finding is consistent with the clinical observation that cancer patients with high levels of miR-506 generally have better overall survival and also suggests that this approach may be an effective strategy to target Snai2 in cancer cells (Yang et al., 2013b). Similarly, miR-630 directly suppresses *SNAI2* expression downstream of VEGF signaling (Kuo et al., 2013). The VEGF family member angiopoietin-like protein 1 (Angptl1) couples with the integrin receptor-related focal adhesion kinase (FAK)/ERK pathway to positively regulate miR-630 expression, which results in silencing of the *SNAI2* transcript. Loss of this regulation due to low levels of Angptl1 correlates with tumor invasion, positive lymph node status, and overall poor prognosis in lung cancer patients (Kuo et al., 2013). In addition, miR-124 negatively regulates *SNAI2* transcript levels (Liang et al., 2013). Decreased miR-124 expression correlates with increased *SNAI2* expression, which in turn correlates with EMT activation and poor patient prognosis. Conversely, overexpression of miR-124 in cancer cells suppresses *SNAI2* expression, reverts EMT phenotypes, and reduces metastatic burden in an *in vivo* mouse model (Liang et al., 2013).

Collectively, these findings demonstrate that microRNAs are a critical component in regulating *SNAI2* expression. The discovery of these mechanisms has provided relevant experimental and clinical data that support the significance of this regulation during cancer progression. However, it remains somewhat unclear which molecular mechanisms govern the expression of these miRNAs and how such mechanisms are altered during cancer development. Recent studies have focused on epigenetic regulation as a possible mechanism. Indeed, hypermethylation of miR-200 family gene promoters in tumor cells correlates with reduced expression of miR-200s and enhanced EMT features, as well as poor patient prognosis (Davalos et al., 2012; Hur et al., 2013; Korpal et al., 2011). In addition, the promoter region of many of these miRNAs contains a regulatory E-box element, which can be bound by Snai2 (Liu et al., 2013). This unique feature raises the possibility of cross-regulatory feedback loops to reinforce *SNAI2* expression during EMT (Liu et al., 2013). Although the complex signaling network that controls microRNAs remains to be elucidated fully, there is clear evidence that microRNAs are potent regulators of *SNAI2* expression and thus could be exploited as a promising therapeutic target to overcome overexpression of *SNAI2* or other EMT-TFs in aggressive cancers.

mRNA transcript stability

SNAI2 transcript stability is also regulated by several RNA-binding proteins. One such protein is HuR (also known as ELAVL1), which stabilizes mRNAs by binding to AU-rich elements located in their 3′ UTR (Peng et al., 1998). *SNAI2* transcripts are stabilized via this mechanism under hypoxic conditions; specifically, nuclear β-catenin binds the 3′ UTR of *SNAI2* mRNA and recruits HuR to prevent *SNAI2* mRNA decay (D′Uva et al., 2013). Interestingly, this mechanism occurs in tandem with hypoxia-dependent transcriptional upregulation of *SNAI2* by HIF-1α, indicating that regulation of Snai2 expression can be complex and multi-layered (Xu et al., 2015). The RNA-binding protein IMP3 has also been reported to regulate *SNAI2* transcript stability. Binding of IMP3 to the 5′ UTR of *SNAI2* mRNA stabilizes the transcript, leading to increased Snai2 expression in a tumor-initiating cell (TIC) population of breast cancer cells (Samanta et al., 2016). Conversely, IMP3 depletion diminished Snai2 expression and led to decreased tumorsphere-forming ability. In the aggressive triple-negative subtype of breast cancer, IMP3 is highly expressed and aberrantly stabilizes *SNAI2* transcripts, leading to Snai2 overexpression and ultimately promoting self-renewal and tumor initiating characteristics (Samanta et al., 2016).

Beyond mRNA stability, it remains to be elucidated whether mRNA modifications such as polyadenylation, alternative splicing and methylation are potential post-transcriptional regulatory mechanisms involved in controlling *SNAI2* expression and Snai2 function.

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Translational and post-translational regulation

Although translational control has emerged as a new frontier in cancer biology, knowledge of the exact mechanisms controlling Snai2 expression at this stage remain limited. While cap-independent translation of mRNA regulates *SNAI1* expression, this mechanism does not appear to regulate *SNAI2* in a similar fashion (Evdokimova et al., 2009). However, the translation of *SNAI2* mRNA can be controlled by an upstream open reading frame (uORF) regulatory element at the 5′ UTR, as overexpression of this uORF suppressed *SNAI2* mRNA translation (Yarlagadda et al., 2011). This mechanism has the potential to be exploited to differentially regulate *SNAI2* expression in cancer cells, but further investigation is needed to probe such a connection more thoroughly.

Post-translational regulation is a versatile mechanism controlling protein stability and activity. After translation, Snai2 can be subject to extensive modifications that control its half-life, coordinate its cellular localization, and direct and/or diversify its molecular function. Phosphorylation and ubiquitylation are the two most prominent post-translational modifications (PTMs) that control proteolytic turnover and abundance of Snai2 protein. Indeed, Snai2 is a labile protein that undergoes rapid proteasomal degradation with a half-life of ∼60–80 min (Zhou et al., 2016). To date, multiple proteins have been found to regulate Snai2 degradation, and dysregulation of these proteins causes Snai2 overabundance and correlates with EMT activation in cancer cells, as discussed below. Finally, in addition to direct modifications of Snai2 by post-translational regulators, the presence or availability of protein co-factors that associate with Snai2 may impact or alter its functions both in normal and in diseased cell states (Box 3).

Box 3. Regulation of Snai2 function through association with co-factors

Generally, Snai2 regulates its transcriptional targets by recruiting chromatin remodelers to target gene promoters. Therefore, the availability of these regulators in normal or diseased states may dictate the functions and activity of Snai2.

Snai2 directly interacts with polycomb repressive complex 2 (Prc2) through its catalytic subunit enhancer of zeste homolog 2 (Ezh2) to facilitate epigenetic regulation of neural crest specification and migration genes (Tien et al., 2015). Ezh2 expression is elevated in many tumor types and is associated with aggressive or advanced cancers, but the clinical implications of association between Snai2 and Prc2 have not been investigated to date (Kim and Roberts, 2016).

In metastatic breast cancer cells, Snai2 recruits C-terminal binding protein 1 (CtBP1) and histone deacetylase 1 (HDAC1) after directly binding to an E-box motif in the promoter for *UbcH5c* (also known as *UBE2D3*), a post-translational regulator of cyclin D1 (CCND1) (Mittal et al., 2011). Their cooperative silencing of *Ubch5c* gene expression through histone deacetylation leads to increased cyclin D1 levels and increased proliferation and invasiveness of breast cancer cells (Mittal et al., 2011). Importantly, CtBP1 expression induces mesenchymal and stem cell-like features in breast cancer cells, and high CtBP1 expression predicts poor survival in breast cancer patients (Di et al., 2013). Thus, CtBP1 abundance may impact breast cancer characteristics through its functional association with Snai2, although this connection has yet to be investigated directly.

Snai2 function is also modulated through its association with the histone deacetylase lysine-specific histone demethylase 1 (Lsd1, also known as Kdm1a). Snai2 and Lsd1, along with Snai1, repress *Brca1* gene expression in breast cancer cells (Wu et al., 2012). Snai2 and Lsd1 are also both necessary for efficient repression of luminal differentiation genes, including those encoding E-cadherin in normal human breast cells, and estrogen receptor α (ERα, also known as Esr1) in breast cancer cells (Bai et al., 2017; Phillips et al., 2014). Accordingly, high Lsd1 and Snai2 expression have been observed in triple-negative or basal-like breast cancers, and both correlate with poor prognosis (Nagasawa et al., 2015; Wu et al., 2012). Interestingly, chemical or genetic inhibition of Lsd1 led to impaired Snai2-dependent repression of E-cadherin, and reduced motility and invasiveness of several other cancer cell types, though this therapeutic approach remains to be explored in the context of breast cancer (Ferrari-Amorotti et al., 2013).

GSK3β phosphorylation-dependent ubiquitylation

The E3 ubiquitin ligase β-Trcp1 (also known as BTRC) cooperates with the Skp, cullin, F-box (SCF) complex to facilitate the ubiquitylation of many protein substrates. Binding of β-Trcp1 to Snai2 promotes its ubiquitylation and subsequent proteolytic degradation, and this interaction is dependent on phosphorylation of Snai2 by GSK3β at a conserved recognition motif (Wu et al., 2012). Deletion of this motif conferred Snai2 protein stability and promoted the Snai2-associated EMT program. Interestingly, GSK3β kinase activity is inhibited by activation of Wnt signaling (Wu et al., 2012). As such, in addition to transcriptional regulation, Wnt signaling appears to contribute to the regulation of Snai2 at the post-transcriptional level, albeit indirectly. As described above, Wnt is a major developmental pathway that is frequently hyperactive in cancers. Moreover, Snai2 and its associated EMT program facilitate Wnt signaling (Lamouille et al., 2014). Taken together, these findings raise the possibility of a cross-regulatory positive feedback loop involving Wnt and Snai2. Under normal conditions, this mechanism would encourage proper progression of tissue development, but in cancer cells this mechanism may be exploited to aberrantly stabilize Snai2, leading to the promotion of malignant cell behaviors.

The carboxy terminus of Hsc70-interacting protein (CHIP, also known as STUB1) is another E3 ubiquitin ligase that regulates Snai2 in response to its GSK3β-mediated phosphorylation. Phosphorylation of Snai2 by GSK3β kinase primes it for CHIP-mediated ubiquitylation, which eventually leads to its proteasomal degradation (Kao et al., 2014). Accordingly, CHIP depletion decreased Snai2 ubiquitylation and degradation, promoting lung cancer cell migration and invasion, and dramatically increasing metastatic burden in an *in vivo* mouse model (Kao et al., 2014). Clinically, although GSK3β activity is associated with low Snai2 protein levels in lung cancer patients, the reverse trend is not strongly correlative, suggesting that additional, GSK3β-independent, regulatory mechanisms may contribute to the control of Snai2 degradation in lung cancer cells (Kao et al., 2014).

Ubiquitylation of Snai2 independent of GSK3β

The E3 ligase F-box and leucine-rich repeat protein [FBXL14, also known as partner of paired, isoform A (Ppa)] was one of the first post-translational regulators of Snai2 to be identified (Vernon and LaBonne, 2006). Upon binding of FBXL14 to the N-terminus of Snai2, Snai2 rapidly undergoes ubiquitin-mediated proteasomal degradation. Here, the hydrophobic amino acids (^{33}L, ^{44}Y, ^{58}V and ^{59}W) of Snai2 are essential for its interaction with FBXL14 (Lander et al., 2011). Interestingly, FBXL14 can also target other EMT-TFs, including Snai1, by binding to conserved hydrophobic residues, representing a common regulatory mechanism to control the EMT program (Lander et al., 2011). Physiologically, FBXL14 is temporally expressed at different stages of neural crest fold development, where it dynamically regulates Snai2 protein abundance, and reduction of FBXL14 expression by Sox9 results

in stabilization of the Snai2 protein (Vernon and LaBonne, 2006). Counteraction of FBXL14 ubiquitylation activity, mediated by the deubiquitinase ubiquitin-specific-processing protease 13 (USP13), has also been observed in tumorigenic glioma stem cells, demonstrating that cancer cells may activate mechanisms capable of disrupting proper regulation of Snai2 at the post-transcriptional level (Fang et al., 2017).

Murine double minute 2 (Mdm2), a well-known E3 ligase for p53, also directly binds to Snai2 to drive its ubiquitylation and degradation (Kim et al., 2010, 2014; Wang et al., 2009). During chondrocyte differentiation, Mdm2 levels increase and lead to proteasomal degradation of Snai2, consequently allowing the expression of chondrocyte-specific genes otherwise silenced by Snai2 (Kim et al., 2010). Mdm2 also regulates Snai2 degradation in cancer cells; this interaction occurs downstream of p53, which transcriptionally upregulates *MDM2* to promote ubiquitin-mediated degradation of Snai2 (Wang et al., 2009; Kim et al., 2014). This regulatory mechanism enables p53 to control Snai2-mediated EMT activation and cell invasion, suggesting a secondary layer of tumor-suppressive function for p53. Accordingly, lung cancer cells harboring mutant p53 that lacks transcriptional activity show increased Snai2 stabilization and cancer cell invasion (Wang et al., 2009). Clinically, the combination of p53 mutation and Snai2 overabundance represents a signature that predicts poor survival and early metastasis in lung cancer patients (Wang et al., 2009).

Snai2 is also post-translationally regulated by the cyclin E–cyclin-dependent kinase 2 (CDK2) complex during the cell cycle (Wang et al., 2015). During the G1/S phase transition, cyclin E forms a complex with CDK2, which directly binds to and phosphorylates Snai2 at specific residues. This phosphorylation leads to ubiquitylation and subsequent proteolysis of Snai2, although the exact E3 ligase mediating this step is unknown. Loss of CDK2-mediated regulation of Snai2 during the G1/S phase transition, either due to deletion of certain phosphorylated residues on Snai2 or CDK2 depletion, resulted in Snai2 stabilization, delayed S phase progression, and genomic instability (Wang et al., 2015). As such, dysregulation of the cell-cycle regulator CDK2 may drive or cooperate with aberrant Snai2 expression during tumor progression, but the clinical implications of this regulation remain to be explored.

Other post-translational regulatory mechanisms

The regulation of Snai2 in mammary gland development and tumorigenesis is indirectly regulated by TGFβ signaling (Desgrosellier et al., 2014). Cooperation of TGFβ with integrin αvβ induces MaSC expansion during pregnancy by promoting nuclear accumulation and increased stability of Snai2 (Desgrosellier et al., 2014). Loss of integrin αvβ *in vivo* diminishes Snai2 expression, which compromises MaSC expansion and ultimately leads to defective alveologenesis. Relatedly, integrin αvβ-mediated stabilization of Snai2 in breast cancer cells promotes self-renewal and tumor-initiating capabilities, while loss of integrin αvβ impairs these functions by diminishing Snai2 expression (Desgrosellier et al., 2014). Future studies to identify the precise molecular mediators involved in integrin αvβ-mediated control of Snai2 would be informative for understanding its regulation in MaSCs and breast cancer.

Beyond the well-characterized PTMs of phosphorylation and ubiquitylation, at least two other PTMs participate in the complex network regulating Snai2 protein abundance and activity. Sumoylation modifies proteins by covalent attachment of SUMO proteins, which are ubiquitin analogs. One function of SUMO modifications is to provide a regulatory signal for proteolytic processing of marked proteins. Indeed, conjugation to SUMO1 stabilizes Snai2, and p14^{Arf} (encoded by *CDKN2A*) was found to be an upstream regulator promoting this modification (Xie et al., 2014). As such, p14^{Arf} inactivation reduced Snai2 sumoylation and promoted its proteasomal degradation. This mechanism of Snai2 downregulation delayed the onset of prostate cancer progression in a mouse model, indicating that p14^{Arf}-regulated stabilization of Snai2 may contribute to driving tumorigenic behavior (Xie et al., 2014). Clinically, high p14^{Arf} expression correlates with high Snai2 protein abundance in prostate tumors, but the prognostic implication of this association has not been clearly defined (Xie et al., 2014).

Acetylation is another PTM that can modulate whether protein substrates are subject to proteasomal degradation. Several lysine residues in the zinc finger region of Snai1 were found to be acetylated, with this modification subsequently increasing its stability (Hsu et al., 2014). Similarly, recent work revealed that acetylation was found to be an essential determinant of the abundance, stability and activity of Snai2 (Zhou et al., 2016). Sirtuin 2 (SIRT2) controls this process through direct binding of Snai2 and deacetylating a lysine residue in the SLUG domain. Depletion or inhibition of SIRT2 promoted Snai2 acetylation and proteasomal degradation, while SIRT2 overexpression decreased Snai2 acetylation and thus stabilized Snai2 protein (Zhou et al., 2016). Importantly, SIRT2 is frequently amplified at the genetic level and highly expressed at the protein level in the aggressive basal-like subtype of breast cancer (Zhou et al., 2016). Thus, these findings describe a molecular pathway that may be exploited in cancer cells to drive aberrant Snai2 stabilization in this subset of breast cancers. Accordingly, genetic depletion or pharmacological inhibition of SIRT2 was sufficient to decrease Snai2 stabilization and also to suppress key EMT features and tumor growth in an *in vivo* mouse model, suggesting that targeting this pathway may be a promising therapeutic approach for treating aggressive breast cancers (Zhou et al., 2016).

Conclusions and future directions

Since the original discovery of a role for Snai2 in EMT, it has become increasing clear that this transcription factor has a multifaceted role in orchestrating key biological processes essential for tissue homeostasis and maintenance. While Snai2 levels are strictly controlled by a multitude of regulatory mechanisms in normal tissues, perturbation of this regulation alters Snai2 expression and function. Indeed, aberrant Snai2 expression is a widespread phenomenon in human cancers and notably predicts poor prognosis in cancer patients (Cobaleda et al., 2007; Coll-Bonfill et al., 2016; De Craene and Berx, 2013; de Herreros et al., 2010; Lamouille et al., 2014; Shih and Yang, 2011). Snai2 loss causes developmental defects and disturbs homeostasis across multiple tissue systems, whereas increased levels of Snai2 or its dysregulation endorse malignant characteristics during cancer development and progression (De Craene and Berx, 2013; Lamouille et al., 2014; Peinado et al., 2007). As such, Snai2 biology represents a unique platform for unraveling the connections between EMT, developmental and cancer biology, and transcriptional regulation, as well as for elucidating how these biological processes collectively shape tissue maintenance and tumor progression.

The continued exploration of the mechanistic frameworks that underpin control of Snai2 and its functions is particularly advantageous in the context of cancer, as transcription factors are not readily druggable and, consequently, an improved understanding of Snai2 regulation may provide rational

therapeutic strategies to antagonize Snai2 and its associated malignant traits. While the last few decades have resulted in enormous expansion of our understanding of the molecular regulation of Snai2, such investigations have also revealed that the biological functions of Snai2 are much more diverse than originally thought. In the future, it will be important to dissect the signaling pathways and regulatory circuitry that direct and/or prioritize the functional outputs of Snai2, as well as the coordination of the diverse biological functions of Snai2 that collectively impact tissue homeostasis and disease development. These efforts, if successful, are certain to beneficially affect our understanding of Snai2 biology and its relevance to cancer therapeutics.

Competing interests
C.K. is a shareholder of Naveris, Inc. and a member of its scientific board of advisors.

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