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Snail Nuclear Transport: the Gateways Regulating Epithelial-to-Mesenchymal Transition?

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Abstract

Epithelial-to-mesenchymal transition (EMT) and the reverse process (MET) plays central role in organ developmental biology. It is a fine tuned process that when disturbed leads to pathological conditions especially cancers with aggressive and metastatic behavior. Snail is an oncogene that has been well established to be a promoter of EMT through direct repression of epithelial morphology promoter E-cadherin. It can function in the nucleus, in the cytosol and as discovered recently, extracellularly through secretory vesicular structures. The intracellular transport of snail has for long been shown to be regulated by the nuclear pore complex. One of the Karyopherins, importin alpha, mediates snail import, while importin beta/exportin 1 (Xpo1) or chromosome maintenance region 1 (CRM1) is its major nuclear exporter. A number of additional biological regulators are emerging that directly modulate Snail stability by altering its subcellular localization. These observations indicate that targeting the nuclear transport machinery could be an important and as of yet, unexplored avenue for therapeutic intervention against the EMT processes in cancer. In parallel, a number of novel agents that disrupt nuclear transport have recently been discovered and are being explored for their anti-cancer effects in the early clinical settings. Through this review we provide insights on the mechanisms regulating snail subcellular localization and how this impacts EMT. We discuss strategies on how the nuclear transport function can be harnessed to rein in EMT through modulation of snail signaling.

Keywords

Epithelial-to-mesenchymal transition; EMT; Nuclear Transport; Karyopherin; Importin; Exportin; Xpo1; CRM1; SNAIL; SNAIL2; HMLE-SNAIL

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1. Introduction

Epithelial-to-Mesenchymal transition (EMT) is a complex phenomenon in which cancer cells lose their polarity undergo changes from epithelial to mesenchymal morphology thereby achieving plasticity that confers an invasive and metastatic behavior [1]. It is a fine tuned process regulated by a number of proteins that are strategically distributed in the nuclear and cytosolic compartments of cancer cells. A number of different parallel signaling pathways interact in the development of EMT [2]. Over the years, these pathways have been well studied leading to deeper characterization of different EMT promoting proteins and transcription factors (TFs), as well as their localization within and outside the cellular compartments [3]. Major EMT regulating proteins and TFs, such as wnt/β-catenin, notch, TGF-β, Twist and Snail are recognized to undergo nuclear-cytosolic shuttling using specialized transporters: Karyopherins [4]. These observations highlight that the nuclear protein transport process may be playing an integral part in the EMT signaling. The karyopherin importin alpha shuttles in proteins with nuclear localization signal sequence (NLS) [5]. The export of most of the EMT regulating TFs is mediated exclusively by Exportin1/XPO1 [also known as chromosome maintenance region 1 (CRM1)] through nuclear exclusion sequence (NES) recognition [6]. For long it has been fairly well recognized that in addition to transcriptional regulation, the activity of the TFs can be controlled by changing their cellular location which permits rapid response to signals, resulting in a powerful modulation of the biological system [7]. While, disease induced changes in expression of nuclear importer proteins have not been very well characterized, it is fairly unequivocally well recognized that nuclear exporters particularly CRM1, are often aberrantly expressed in cancer [8]. Nevertheless, till date, not many studies have looked into how disturbed nuclear export may interfere with EMT signaling. As discussed below, we propose that the role of aberrant nuclear export is critical to understanding the regulation of EMT through major TFs that they directly target (here a special focus on the Snail protein). As a number of different specific inhibitors of nuclear export are being developed and tested clinically, this may become an attractive therapeutic strategy to interfere in the EMT pathways.

2. The Snail family transcription factors and EMT

The transcription factors (TFs) of the Snail family are best recognized for being the direct repressors of epithelial morphology promoter *E-cadherin* transcription that drives EMT [9]. Snail family TFs play central role during the embryogenesis processes of both invertebrates and higher order animals where they regulate the cell movements necessary for the formation of the mesoderm [10]. Their involvement in the formation of vertebrate neural crest cells has been appreciated for more than two decades [11]. While Snail induced migratory and invasive behavior in developmental cells is vital for embryonic development, the same becomes problematic when aberrantly activated in later stages especially in pathological states such as cancer [12]. It is well established that enhancement in *Snail* gene expression in primary tumors promotes cellular motility and the consequent acquisition of metastatic properties [13,14]. On the other hand, in non-transformed cells, the enhancement in snail protein expression induces fibrosis like features [15]. Studies have clearly demonstrated that targeted down-regulation of snail can reverse EMT [16]. Human snail is a 264-amino

acid nuclear protein with an amino-terminal basic amino acid-rich domain (SNAG domain) and a carboxyl-terminal DNA-binding domain (zinc finger domain) (Figure 1). The Snail superfamily also include the Scratch proteins [17]. More than 100 targets of *Snail* have been identified to date from all metazoan groups, with five family members in vertebrates: *Snail1, Snail 2, Snail3* and *Scratch1* and *Scratch2*. The domain structures of all Snail and Scratch TFs is conserved, i.e. having a divergent N-terminal half of the protein and a highly conserved C-terminal half as the DNA binding domain (DBD) which contains four to six zinc fingers (ZFs) of the C2H2 type [17] (Figure 1). The expression of *Snail* family genes is regulated at the transcriptional level by many signaling molecules, including FGF [18], Wnt [19], TGFβ [20] which collectively form the building blocks of the microenvironment that serves as a niche for EMT [21].

3. Cellular localization of snail family proteins

As transcription factors that require sequence specific alignment on DNA for gene regulation, snail proteins must translocate to the cell nucleus in order to be functional. Like all the proteins, snail family members cannot passively diffuse through the nuclear membrane and require a carrier for their nuclear import or export. Their translocation requires energy and in most of the cases, it is mediated importin-β (Impβ/KPNB1) belonging to the karyopherin family proteins [22]. These receptors are 90–130 kDa soluble proteins interacting with the cargo they are going to transport, the transport proteins and the GTPase Ran [23] (Figure 2). The importins mediate transport between the cytoplasm and the nucleus, interacting with their cargoes that carry specific amino acid sequences called nuclear localization signals (NLSs) [24]. These interactions can be direct or they may be mediated by karyopherin family that recognize and bind to the NLS present in many of the proteins imported by Importin β [25]. The directionality of the nuclear transport is imposed by a gradient formed by RanGTP across the nuclear envelope (higher concentration of RanGTP in the nucleus and a lower concentration in the cytoplasm) [26]. Importins exclusively interact with their cargoes in the cytoplasm and relocate them to the nucleus where they interact with RanGTP. The RanGTP binding in the nucleus causes lowering of the affinity of the importins for their substrates leading to their release. The RanGTP-bound importins relocate in the cytosol where, through the involvement of RanGAP and RanBP1, the GTP bound to Ran is hydrolyzed and Ran is released from the importin [27]. The importin can then start a new cycle of nuclear import. Taken together, these findings show that nuclear transporters (both importin and exportins) play central role in the biology of Snail (or other EMT promoting TFs with NES and NLS). Therefore, modulation of nuclear transport proteins should in principle result in alterations in Snail cellular localization and consequently impact snail mediated EMT signaling.

3.1 Nuclear import mechanisms of snail family proteins

The regulatory mechanisms that promote Snail nuclear import and enhance its stability have been well investigated. Among the earliest studies, Yamasaki and colleagues utilized a number of different fusion proteins containing a green fluorescent protein (GFP) to generate a series of the Snail fragments to analyze their subcellular localization [28]. In their studies, the fusion of the four zinc fingers to GFP led to the targeting of GFP to the nucleus,

indicating that the zinc finger domain is sufficient for nuclear localization. More convincing evidence came from experiments where an *in vitro* transport system was used and the nuclear import of Snail was reconstituted by importin in the presence of Ran and NTF2. This approach further demonstrated that Snail binds directly to importin in a zinc finger domain-dependent manner. These results indicated that zinc finger domain of Snail functions as a nuclear localization signal and Snail can be transported into the nucleus in an importin-mediated manner. Interestingly, the above studies also highlighted that all four zinc fingers are necessary for efficient nuclear localization, because removing of any one zinc finger alone or in combination resulted in a decreased nuclear accumulation. It is likely that all four fingers are required for the coordination of the structure of the carboxyl terminal domain to interact efficiently with the nuclear import machinery and actually function as the NLS of Snail. It should be noted that there remains a possibility that deletion of any zinc finger domains or disruption of the ternary structure of zinc fingers may cause a loss of DNA binding ability, resulting in a loss of nuclear retention. Therefore, strategies disrupting the ternary complex or blocking access of the importins to the 4 zinc finger domains can certainly be postulated to influence the nuclear retention and the importin binding activity that may provide opportunity for targeted inhibition of nuclear localization of snail.

3.2 Nuclear exclusion mechanisms of snail

Several mechanisms can explain the effect of the exportin recognizable domains in Snail on its own subcellular localization. These domains may act as an anchor linking Snail to specific cytosolic proteins; they may inhibit its nuclear import, or mediate its nuclear export. It is well known that the most common mechanism of nuclear export of proteins in eukaryotic cells is through Xpo1/CRM1-dependent systems. Most exportable proteins have a hydrophobic Leu-rich sequence and snail is no exception. By searching in this direction, the nuclear exclusion sequence (NES) of mouse Snail protein (aa residues 132-143) was discovered more than a decade ago [29]. It was shown that the specific Snail Leu-rich amino acid sequence is similar to those described for in other CRM1-targeted export proteins. These studies identified the sequence LGQLPKQLARLS, between the aa residues 132-143 of murine Snail, that was shown to perfectly match the consensus sequence previously defined for established NESs [$LX_{(1-3)}LX_{(2-3)}LXL$]. Interestingly, replacement of Leucine with Valine, as commonly observed to occur in human Snail (Leu135 to Val), has been observed in some other NESs, as well as longer spaces among leucines [30]. This putative NES is not present in Slug, a Snail homolog, whose localization is found to be exclusively nuclear. Deletion of part of this sequence (residues 138-151, i.e removal of last two leucines) from a GFP-N-terminal domain fusion protein was sufficient to impair Snail's exclusion from the nucleus. Further confirmation came from Xpo1/CRM1 inhibition studies using a natural product derived specific inhibitor Leptomycin B (LMB) that block the formation of the CRM1/Ran-GTP/nuclear protein complex necessary for export. In their experiments, treatment of transfected RWP-1 cells with LMB caused rapid translocation of Snail from the cytosol to the nucleus within few minutes. Similar results were obtained when the effect of LMB on the location of other exclusively cytosolic Snail constructs was studied. In the presence of this drug, both the fragment containing the complete regulatory domain (amino acids 1 to 151) and the one containing the NES (82 to 151) were detected mostly in the nucleus.

Aside from CRM1, studies have shown that exportin 5 or Xpo5, which is a major dsRNA exporter (especially pre-miRNA exporter), can also mediate Snail nuclear export [31]. However, nuclear export by Xpo5 does not occur through the typical CRM1 recognizable NES in the zinc finger domains (discussed above). Rather, Xpo5 recognizes a snag domain in Snail protein that serves as a NES mediating the nuclear export. Interestingly, Xpo5 mediated snail export essentially requires binding of eukaryotic elongation factor 1 A (eEF1A). When Xpo5 binds aminoacyl-tRNAs (aa-tRNAs), this complex can recruit and co-transport eEF1A that can interact with the snag domain for E-cadherin interacting (bound snail).

4. Snail nuclear transport modulators and their impact on EMT

There are a number of mechanisms that either enhance or retard both snail nuclear import and export processes (Figure 3). The importin α is recognized to act as a negative regulator of snail import. Importin α is known to act as an adaptor for importin β1 during different protein nuclear import processes [32]. In addition to its adaptor function, several different studies have convincingly demonstrated that importin α in many instances inhibits the nuclear import of different proteins. Thus, the inhibition of Snail nuclear import may be one of the characteristic features of importin α. It is speculated that negative biological regulators or chemical agents that enhance the expression of importin α may suppress snail nuclear localization leading to down-regulation of the protein activity.

Phosphorylation has dual effect on snail localization and function [29]. Phosphorylation of a Ser-rich sequence adjacent to the NES of Snail facilitates its export and the expression levels of snail phosphorylation regulators could serve as important regulators of the nuclear localization status of snail. Snail phosphorylation by various kinases such as protein kinase D1 (PKD1) and glycogen synthase kinase-3β (GSK-3β) primed by CK1 can trigger its nuclear export and cytosolic degradation [33]. In this direction, Du and colleagues elucidated the consequence of Snail phosphorylation on EMT signaling [34]. Their studies demonstrated that PKD1 phosphorylates Ser^{11} (S11) on Snail, triggering its nuclear export via 14-3-3σ binding. On the other hand, Snail S11 mutation causes acquisition of mesenchymal traits and expression of stem cell like markers. Conversely, Snail can be stabilized by small C-terminal domain phosphatase-meditated dephosphorylation or through CK2 and PKA phosphorylation, or O-GlcNAc modification and the nuclear localization of snail can be increased in cancer cells, upon PAK1 phosphorylation (snail phosphorylation mechanisms comprehensively reviewed in [35]). In another important study, using a bioluminescence cell-based assay to evaluate Snail1 protein stability, Zhang and colleagues investigated positive and negative post-translational regulators of total cellular Snail1 protein level [36]. A human kinome RNAi screen identified the protein kinase Lats2 stabilizing cellular Snail1 protein levels post-translationally. In this study Lats2 was shown to modulate snail phosphorylation by interacting with Snail1 in cells and directly phosphorylating Snail1 at T203 in response to multiple signals that activate Lats2, such as TGFβ-induced EMT. Lats2 mediated phosphorylation of Snail1 at T203 occurs in the nucleus and serves to retain Snail1 in the nucleus thereby enhancing protein stability and function. Interestingly a number of studies have shown that de-regulation of Lats2 results cancer invasiveness and poor prognosis [37,38]. Lats2 down-regulation and the consequent enhancement in tumor

metastatic potential has also been correlated with disturbed miRNA signaling [39]. In cellular models, it was shown that that Lats2 can affect TGFβ-induced EMT and that this effect depends upon the presence of Snail1. Lats2 has been shown to potentiate Snail1 EMT-promoting function in zebrafish and mouse embryo development models, as well as enhance Snail1's capacity to regulate tumor cell invasion/migration. While TGFβ mediated phosphorylation and degradation causes Snail turnover. Additionally it has been shown that the activation of Wnt signaling suppresses the activity of TGFβ which results in both Snail and β-catenin stabilization [40]. These findings indicate that there is some cooperation between WNT signaling and other Snail mediated pathways, such as FGF, in the triggering of the EMT. This cooperation has already been highlighted in several developmental systems, such as neural crest mesoderm [41]. For example, when Snail1 activity is maintained, E-cadherin is repressed and is therefore not available to bind β-catenin and form adherens junctions. As a result, β-catenin is available to bind to TCF/LEF and to act as a transcription factor, promoting WNT signaling. Although this situation will only occur concomitantly with an inactive β-catenin degradation system, WNT signaling can increase Snail1 function by preventing its nuclear export and degradation, allowing Snail1 promoted EMT. For example, Yadi et al, showed that the small C-terminal domain phosphatase (SCP) is a specific phosphatase for Snail [42]. SCP interacted and co-localized with Snail in the nucleus and that SCP expression induced its dephosphorylation and stabilization *in vitro* and *in vivo* leading to enhancement in EMT and consequent invasive behavior of the tested cells. Thus, it is imperative that the determination of regulation mechanisms of snail import and export will allow better understanding of its context dependent role in epithelial and/or mesenchymal tumors.

As mentioned above, Snail is a highly unstable protein that undergoes rapid turnover. In the nucleus, its turnover is decreased while in the cytosol it is rapidly degraded by proteasomes [43]. Post-translational modifications such as phosphorylation, ubiquitination, and lysine oxidation are recognized to collectively influence snail (particularly Snail1) protein stability, sub-cellular localization, and activity [44]. There are two RING finger ubiquitin ligases of the F-box subfamily containing the multimeric complex Skp1-Cullin-Rbx1-F-box (SCF) that have been shown to participate in Snail1 proteasomal degradation. $SCF^{\beta\text{-}TrCP1/FBXW1}$ is recognized to polyubiquitinate Snail1 after its phosphorylation by GSK-3β [29]. Snail1 is also targeted by $SCF^{Ppa/FBXL14}$, which is a ubiquitin ligase and acts as a master regulator of the EMT process, as it modulates not only Snail1 but also Snail2, Twist1 and Zeb2 [45]. The two ligases work in very distinct manner. Unlike $SCF^{\beta\text{-}TrCP1/FBXW1}$, FBXL14 does not require previous Snail1 phosphorylation by GSK-3β. However, both ligases are present and act exclusively in the cytosol. Additionally, the p53 negative regulator murine double minute 2 (Mdm2), a monomeric ring finger E3, can also degrade the snail family member snail2 [46]. In addition to cytosolic ubiquitination, there are certain mechanisms that negatively regulate nuclear Snail as well. Very recently, using a short hairpin RNA screening, Vinas-Castells and colleagues have identified FBXL5 as a novel nuclear snail ubiquitin ligase [47]. Their investigations pointed towards a pre-dominantly cytosolic localization of FBXL5 protein that could be reversed by LMB, indicating a CRM1 mediated export regulatory mechanism for this Snail regulator. FBXL5 was also shown to be down-regulated by numerous stresses such as γ irradiation and

transition metal ion depletion which were correlated with snail stability. Earlier work has identified the FBXL5 binding sequence in Snail1 and Snail2 that were mapped to the carboxy-terminal half of the protein (Snail-CT), mainly to ZnF2 (Figure 1). However, unlike FBXL14 which can regulate other EMT markers such as ZEB1 and Twist1, FBXL5 has more specific effects that are restricted to Snail1 and therefore have confined roles. Collectively, the above described studies clearly show that snail is under a very tight regulation in cancer cells and the over-expression or disturbances in the homeostasis of its direct and indirect regulators can certainly impact its activity and localization, which can then manifest into deregulation of its targeted genes and consequently EMT.

4.1 Modulation of snail induced EMT through exportin inhibition

Given that nuclear transport plays integral role in snail biology and EMT, targeted inhibition of nuclear export and import of the protein is speculated to become an attractive and new form of therapeutic strategy to tame this master regulator. A number of agents have been developed that can target the nuclear exporter CRM1 fairly specifically. The first agent in this series was Leptomycin B (LMB), a natural product derived drug-like compound that was originally developed as an anti-fungal agent [48]. LMB showed potent irreversible inhibitory activity against CRM1. Interestingly, some of the earlier studies utilized LMB to evaluate nuclear ubiquitination mechanisms on snail nuclear retention. These investigations clearly verified that LMB mediated CRM1 inhibition can indeed cause nuclear retention of snail. However, these studies fell short of investigating the impact of such snail retention on the EMT process, especially whether this causes reversal to MET. Additionally, LMB did not make a clinical impact and was discontinued from human application after just a single clinical trial due in part to its associated toxicity [49]. A number of newer agents (analogs of LMB) have been developed that show similar CRM1 inhibitory activity [50]. However, their clinical efficacy is yet to be evaluated. We have developed a potent exportin inhibitor Selinexor that is currently in multiple Phase I clinical trials for both solid tumors and hematological malignancies (Clinical Trial identifiers NCT01986348, NCT02093403, NCT02025985, NCT02088541, NCT02078349, NCT02091245) [51,52,53,54,55,56,57,58,59,60,61,62,63,64,65,66]. Additionally, another CRM1 inhibitor, Velidexor, is also being evaluated in canine cancer models [67]. We had earlier shown that these inhibitors can suppress growth of well recognized gemcitabine resistant pancreatic cancer cells with EMT phenotype that over-express CRM1 (unpublished work APA abstract 2013). Recently, using a cellular model system of snail transduced human mammary epithelial (HMLE) cells (HMLE-SNAIL) we also demonstrated *in vitro* activity of Selinexor, LMB and related compounds in reversing mesenchymal phenotype that was concurrent with growth inhibition and induction of apoptosis [68]. CRM1 inhibitors used at clinically relevant concentrations can reverse EMT phenotype and also restore epithelial markers in these cells. Computational analysis of KPT-185 (an analog of Selinexor) indicates that drug treatment results in global re-organization of cytosolic proteins (majority of which regulate snail and other EMT promoters) into the HMLE-snail cell nucleus. Our laboratory is currently evaluating the molecular mechanism of exportin inhibition and how it reverses EMT. Our initial results indicate that Selinexor treatment results in snail nuclear degradation that is mediated by the nuclear retention of F-Box protein FBXL5. It is recognized that FBXL5 may not be the only repressor that is causing Snail degradation and

that CRM1 inhibition by Selinexor may be inducing the relocalization of a number of different important tumor suppressors that direct or indirectly target Snail. Nevertheless, these preliminary studies do indicate that interfering with the nuclear transport can indeed impact EMT signaling and could potentially become a new approach to tame in one of the most difficult to treat, metastatic and EMT harboring tumors.

5. Conclusions and future perspectives

The foundations of metastasis, a major cause of death from cancer, are laid on EMT which has remained a major obstacle to successful anti-cancer therapies. Biologically EMT is a complex process that involves a number of different parallel signaling some of which are known, and others yet to be explored. What is clear is that subcellular localization of various EMT promoting TFs plays central role in this plasticity process and adds another tier of complexity to the already intriguingly multifaceted phenomenon. While proper subcellular localization is critical to any protein function, it is especially important for major TFs that regulate gene expression through sequence specific alignment to DNA for which nuclear retention is vital. Snail is considered as one of the primary drivers of EMT. It is under a very tight regulation by the nuclear compartmentalization mechanisms that regulate its stability and protection from ubiquitination. Aberration in nuclear transport mechanisms, which is commonly found in cancer, shifts this fine-tuned compartmentalization balance leading to mis-localization of the protein which results in many different de-regulatory signaling including EMT. A number of agents have recently been shown to target the nuclear transport machinery with great specificity. Preliminary indications in cellular models do show that such agents (for example Selinexor) can indeed cause reversal of mesenchymal phenotype (to epithelial) and consequent cell death. These findings open some distinct and less explored possibilities for targeting EMT at the nuclear pore. Such approaches may become part of future therapies against aggressive forms of cancers that sustain on EMT, hence may result in better treatment outcomes.

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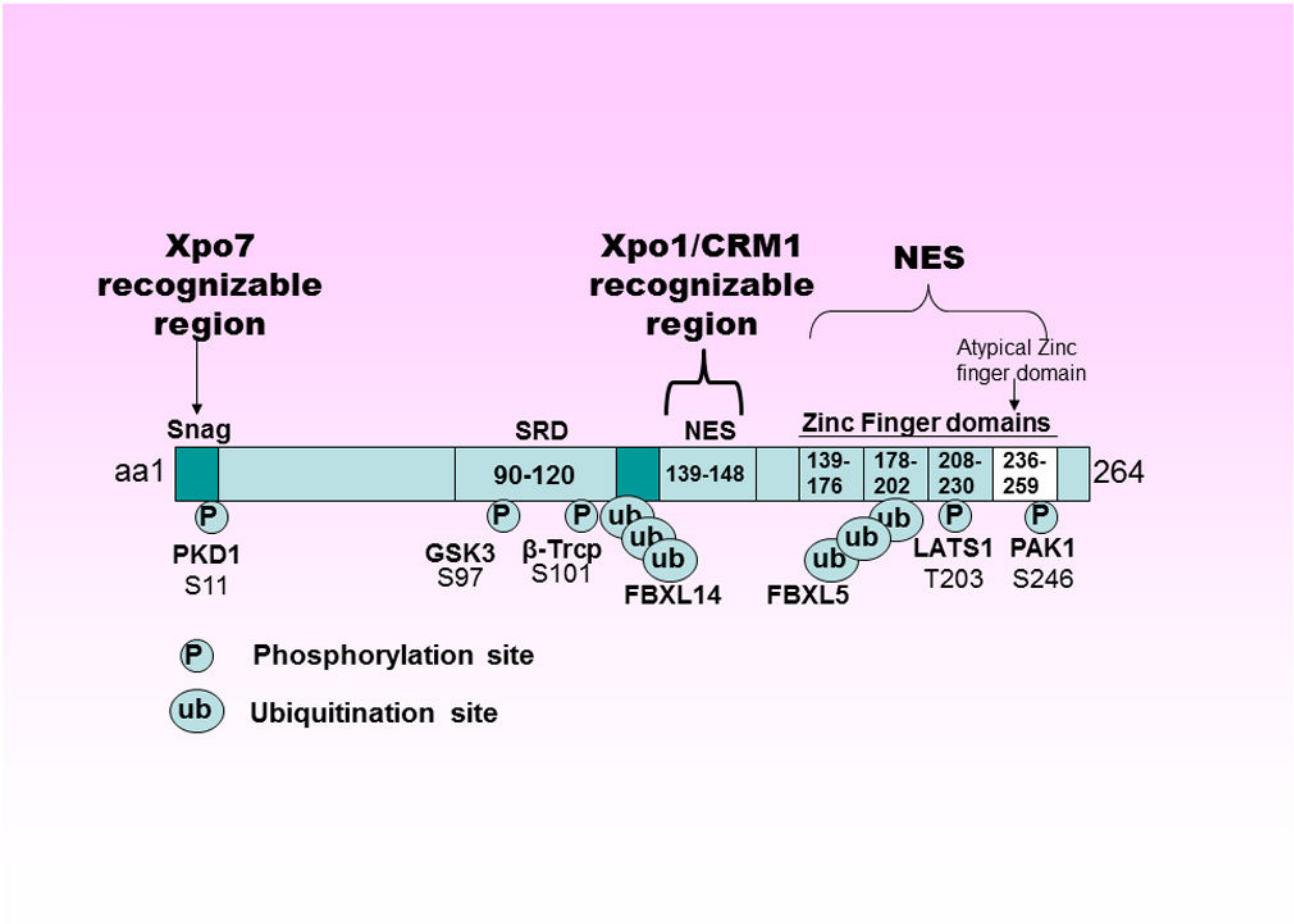

Figure 1. Snail structure

The N-terminal region [amino acid (aa) 1-150] of the Snail protein contains a SNAG (domain (aa 1-9) which includes the consensus sequence PRSFLV found in all Snail family members. This motif is highly conserved among species and also found in several other transcription factors where it is associated with repressive functions. The nuclear exporter Xpo5 recognizes SNAG domain and mediates SNAIL nuclear export. A serine-rich domain (SRD: aa 90-120) and a nuclear export sequence (NES: aa 139-148) are involved in the regulation of Snail protein stability and Xpo1/CRM1 mediated nuclear export, respectively. The C-terminal portion (aa 151-264) contains 3 typical (154-176, 178-202, 208-230) and one atypical (236-259) C2H2-type zinc finger (ZF) domains that serves as a NLS. A number of phosphorylation and ubiquitination sites exist on Snail that collectively regulates its turnover.

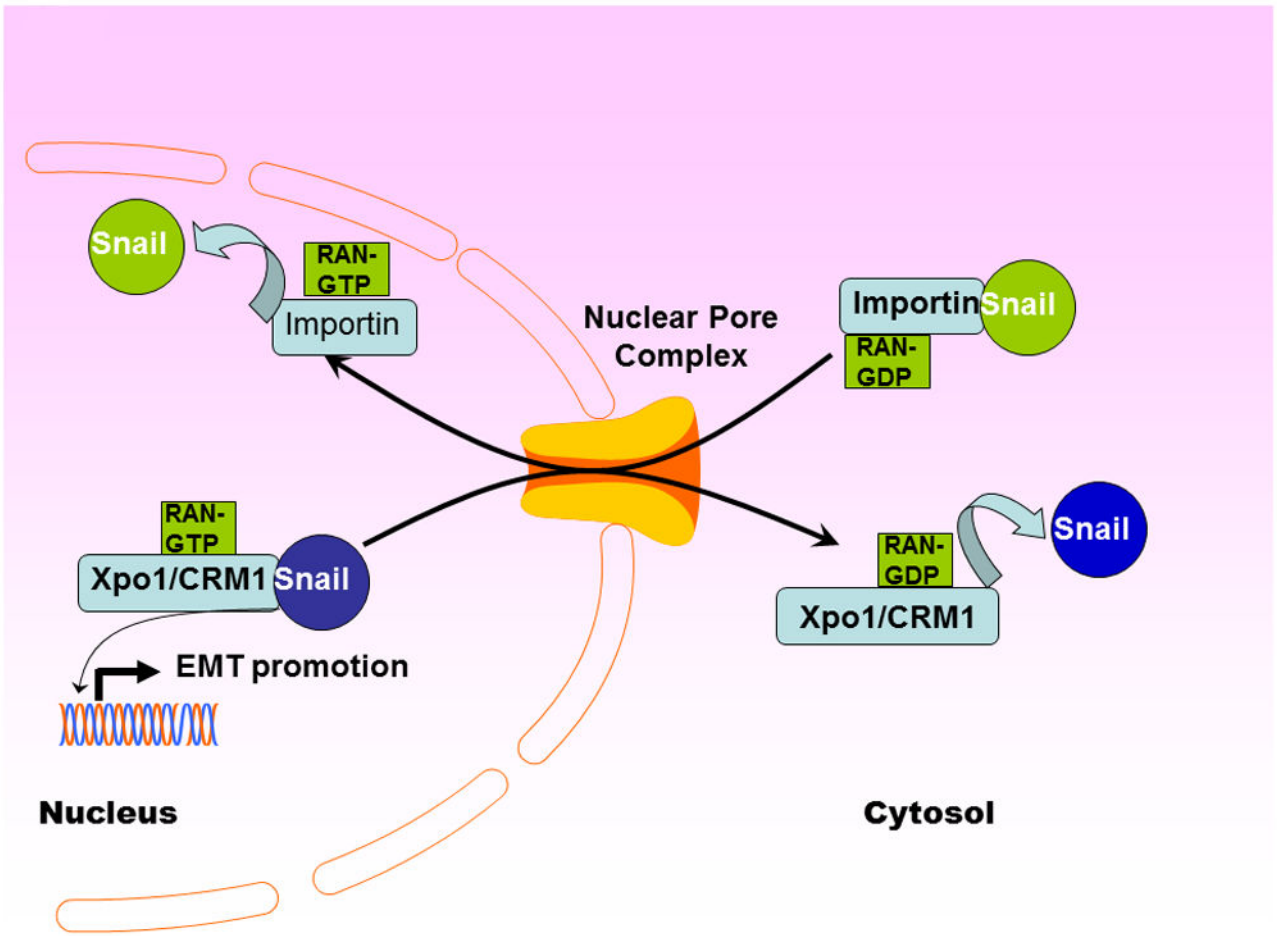

Figure 2. Snail Nuclear Import and Export a Mechanistic Summary

Snail Nuclear import and export are governed by evolutionary conserved nuclear transporters belonging to the Karyopherin family. An energy consuming process, Snail nuclear transport is mediated by nucleotide exchange factors RanGTP.

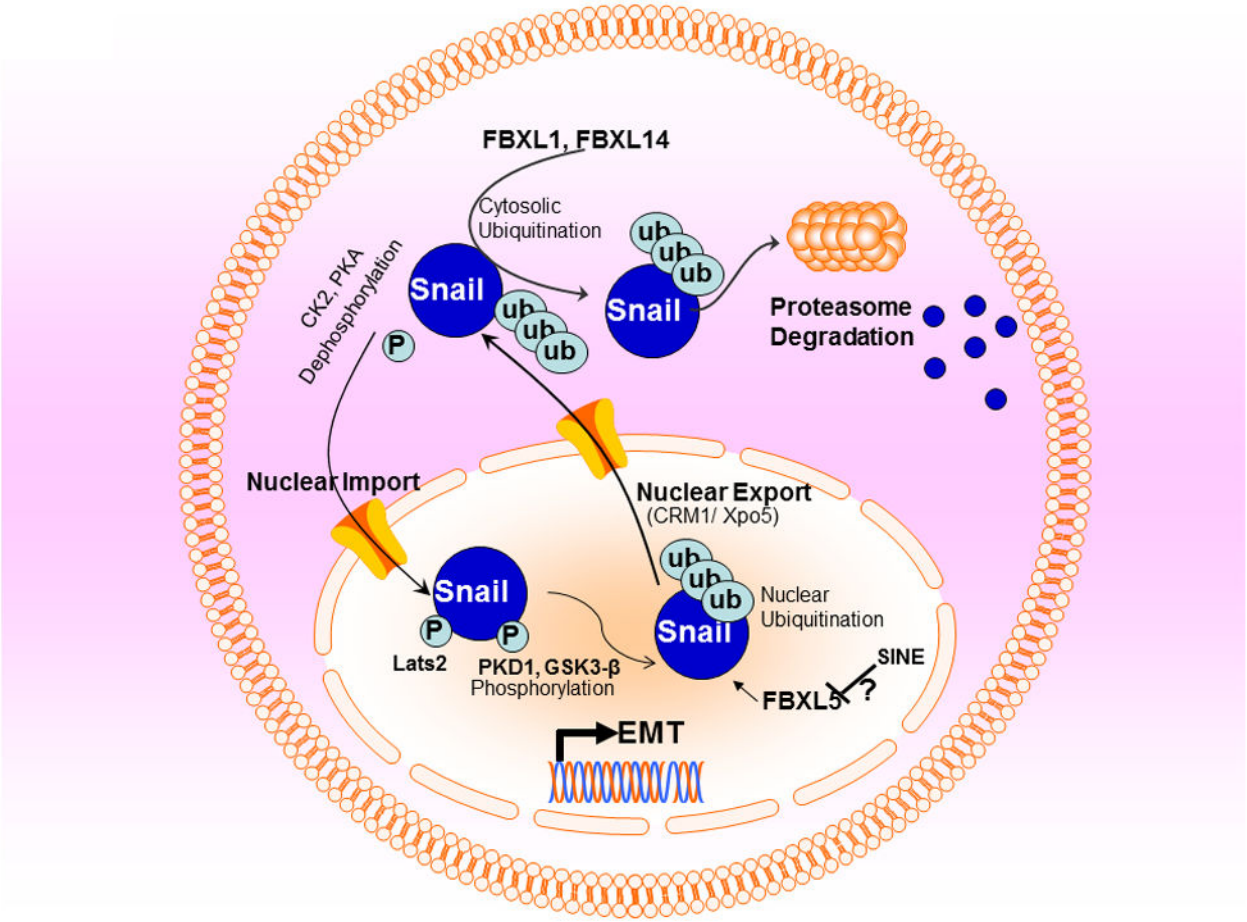

Figure 3. Snail Regulatory Mechanisms

Snail is highly labile protein that is rapidly degraded through proteasomal system. The F-box proteins (components of SCF ubiquitin-ligase complexes namely FBXL1 and FBXL14 enhance the nuclear export and induce ubiquitin-mediated proteolysis of Snail. FBXL5 induces nuclear ubiquitination of snail leading to its nuclear degradation. On the other hand phosphorylation by GSK3-β, PKA1, PDK1 and other can stabilize Snail and induce its nuclear retention.