Characterization of Snail nuclear import pathways as representatives of C_2H_2 zinc finger transcription factors

José-Manuel Mingot[1,*], Sonia Vega[1], Beatriz Maestro[2], Jesús M. Sanz[2] and M. Angela Nieto[1,*]

[1]Instituto de Neurociencias, CSIC-UMH, Avda. Ramón y Cajal s/n, San Juan de Alicante, Spain
[2]Instituto de Biología Molecular y Celular, UMH, Elche, Spain
*Authors for correspondence (e-mails: j.mingot@umh.es; anieto@umh.es)

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Summary

Snail proteins are C_2H_2 class zinc finger transcription factors involved in different processes during embryonic development, as well as in several adult pathologies including cancer and organ fibrosis. The expression of Snail transcription factors is tightly regulated at the transcriptional level and their activity is modulated by their subcellular localization. Given the importance of this gene family in physiology and pathology, it is essential to understand the mechanisms by which Snail proteins are imported into or exported out of the nucleus. Here we show that several importins mediate the nuclear import of the human Snail proteins and we identify a unique nuclear localization signal (NLS), recognized by all the importins, that has been conserved during the evolution of the Snail family. This NLS is characterized by the presence of basic residues at defined positions in at least three consecutive zinc fingers. Interestingly, the consensus residues for importin-binding are also involved in DNA binding, suggesting that importins could prevent non-specific binding of these transcription factors to cytoplasmic polyanions. Importantly, the identified basic residues are also conserved in other families of C_2H_2 transcription factors whose nuclear localization requires the zinc finger region.

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Introduction

Transcription factors of the Snail family are transcriptional repressors best known for being direct repressors of *E-cadherin* transcription and inducers of the epithelial to mesenchymal transition (EMT) (Barrallo-Gimeno and Nieto, 2005). During embryogenesis Snail factors regulate the cell movements necessary for the formation of the mesoderm in species as distant as flies and mammals, as well as those involved in the development of the neural crest and other vertebrate tissues. Snail confers migratory and invasive properties on cells and whereas these cellular functions are necessary for embryonic development, they become deleterious when aberrantly activated in the adult. Therefore, *Snail* gene expression in primary tumours favours the acquisition of metastatic properties (Olmeda et al., 2007a; Olmeda et al., 2007b), whereas in non-transformed cells Snail1 induces organ fibrosis (Boutet et al., 2006).

The Snail transcription factors belong to the Snail superfamily, which also contains the Scratch proteins (Manzanares et al., 2001). More than 150 *Snail* genes have been identified to date from all metazoan groups (A. Barrallo-Gimeno and M.A.N., unpublished), with five family members in vertebrates: *Snail1*-*Snail3* and *Scratch1* and *Scratch2*. The organization of all Snail and Scratch transcription factors is conserved, with a divergent N-terminal half of the protein and a highly conserved C-terminal half, the DNA binding domain (DBD), containing from four to six zinc fingers (ZFs) of the C_2H_2 type (Manzanares et al., 2001).

As transcription factors, Snail proteins must translocate to the nucleus in order to be functional. Although small molecules (smaller than 20-40 kDa) can pass through nuclear pore complexes by passive diffusion, the translocation of the majority of proteins requires energy and in most of the cases, it is mediated by nuclear transport receptors (NTRs) of the importin-β (Impβ; also known as KPNB1) family (Gorlich and Kutay, 1999; Pemberton and Paschal, 2005). These 90-130 kDa soluble proteins interact with three different elements: the cargo they are going to transport, the nucleoporins and the small GTPase Ran. The importins mediate transport between the cytoplasm and the nucleus, interacting with their substrates through specific motifs called nuclear localization signals (NLSs). These interactions may be direct or they may be mediated by adaptor proteins such as importin α1-α7 (Impα1-Impα7; also known as KPNA1-KPNA7) that recognize and bind to the NLS present in many of the proteins imported by Impβ. The directionality of the nuclear transport is imposed by a RanGTP gradient across the nuclear envelope, with high concentration of RanGTP in the nucleus and a very low concentration in the cytoplasm. Importins only interact with their substrates in the cytoplasm, translocating them to the nucleus where they interact with RanGTP. Once bound to RanGTP, the affinity of the importins for their substrates is extremely low and they are released. The RanGTP-bound importins translocate back to the cytoplasm where, thanks to the activity of RanGAP and RanBP1, the GTP bound to Ran is hydrolyzed and Ran released from the importin. The importin can then start a new cycle of nuclear import (Gorlich and Kutay, 1999; Pemberton and Paschal, 2005).

The expression of *Snail* genes is regulated at the transcriptional level by many signalling molecules, including FGF, Wnt, TGFβ,

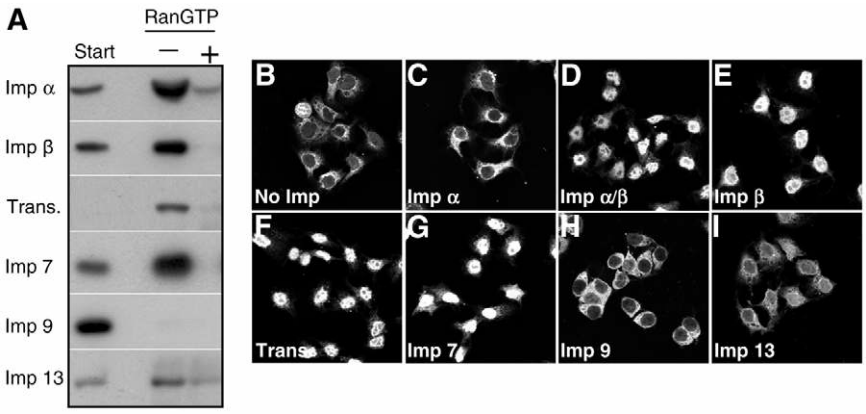

Fig. 1. Several importins mediate Snail1 nuclear import. (A) Identification of importins that bind to immobilized Snail1 in pull-down assays using a cytosolic HeLa extract as the source of importins. The assay was performed in the presence or absence of RanGTP (5 μM) as indicated. Bound importins were identified by western blotting. (B-I) Nuclear import assays with digitonin-permabilised HeLa cells. (C-G) GFP-Snail1 was efficiently imported by the importins that strongly bound Snail1: Impβ, alone or in combination with Impα, transportin and Imp7, but not by the inactive adapter Impα alone. (H,I). Imp9 and Imp13 could not efficiently import Snail1 in to the nucleus.

BMPs, etc. (Barrallo-Gimeno and Nieto, 2005). The activity of Snail1 is also modulated by post-translational modifications that regulate its subcellular localization (Dominguez et al., 2003; Yang et al., 2005; Zhou et al., 2004). The phosphorylation of Snail1 by p21-activated kinase (PAK) seems to increase Snail1 nuclear import in breast cancer cells (Yang et al., 2005). In addition, Impβ appears to be involved in the translocation of Snail1 to the nucleus, a process that requires the zinc fingers of this protein (Yamasaki et al., 2005). In order to gain further insight into how the subcellular localization of Snail is regulated, we have characterized several pathways capable of driving the nuclear translocation of human Snail1, Snail2 and of the product of a primate-specific Snail retrogene, called Snail-like. We show that the pathways are conserved and that in addition to Impβ, importin 7 (Imp7; also known as IPO7) and transportin can also mediate the nuclear translocation of Snail. All these importins recognize a unique NLS that consists of basic residues situated in three consecutive zinc fingers. Interestingly, this motif is not only conserved in all metazoan members of the Snail family described so far but also, in all C_2H_2 zinc-finger proteins whose nuclear localization requires the zinc finger region.

Results

Identification of Snail1 nuclear import pathways

To characterize the pathways responsible for the nuclear import of Snail1, we first identified the importins that can interact with this protein. Human Snail1 was recombinantly expressed as a ZZ-tagged fusion protein, immobilized on IgG Sepharose and incubated, either in the absence or the presence of RanGTP, with a complete HeLa extract that we used as a source of human importins. In addition to Impβ in this assay Snail1 strongly interacted with Impα Imp7 and transportin (Fig. 1A). The association of these importins was clearly displaced by RanGTP suggesting that the interaction takes place in the cytoplasm and that upon nuclear translocation, the binding of RanGTP releases Snail1. Interaction with Imp9 was not detected and the binding of Imp13 was poorly regulated by RanGTP, suggesting that this importin does not play any significant role in Snail1 nuclear import (Fig. 1A).

After identifying Snail1-interacting importins in vitro, we checked whether these importins could translocate Snail1 to the nucleus using classical nuclear import assays in permeabilized HeLa cells. We found that in the absence of importins or in the presence of Impα alone (an Impβ adaptor with no nuclear import activity), GFP-Snail1 was mainly excluded from the nucleus of permeabilized HeLa cells (Fig. 1B,C). Similarly, Imp9 and Imp13 were unable to induce Snail1 nuclear translocation, consistent with the undetectable binding of Imp9 and with the poor regulation of Imp13 binding by Ran-GTP (Fig. 1H,I). By contrast, GFP-Snail1 was efficiently transported to the nucleus in the presence of Impβ, either alone or in combination with Impα, Imp7 and transportin (Fig. 1D-G). Together, these results show that the nuclear import of Snail1 is mediated by several pathways.

Characterization of the Snail1 NLS

Having identified different importins capable of translocating Snail1 to the nucleus, we examined the protein residues that might act as the NLS. The carboxyl half of Snail1 interacts with Impβ and is required for its nuclear translocation (Yamasaki et al., 2005). Indeed, when we split Snail1 into two and analyzed their respective binding to importins, we found that not only Impβ but also, Impα, Imp7 and transportin bound exclusively to the C-terminal half of the protein (Fig. 2A). NLSs are usually small clusters of solvent-exposed basic residues, such as the classical monopartite and bipartite NLSs (Lange et al., 2007). Alternatively, they may involve basic domains occupying a significant portion of the protein as in the case of histones and ribosomal proteins (Jakel and Gorlich, 1998; Moreland et al., 1985; Mosammaparast et al., 2002; Mosammaparast et al., 2001; Schaap et al., 1991). Since the structure of Snail1 had not been resolved, we could not anticipate the solvent-exposed basic residues in the carboxyl half of the protein. However, given that zinc fingers are structurally well conserved, we generated a three-dimensional model of this part of the protein using the synthetic six-finger zinc finger Aart polypeptide bound to DNA as a template (Segal et al., 2006) (Fig. 2B). Accordingly, we mutated residues predicted to be exposed in zinc fingers 1-3 of the human Snail1 protein (Fig. 2B,C). These fingers correspond to numbers 2-4 in the Snail protein consensus since human *Snail1* has lost the first finger (Manzanares et al., 2001). Among the mutant proteins generated, importin binding was impaired in four of these (M2, M3, M5 and M6; Fig. 2C,D). These results indicate that some of the residues mutated in fingers 1-3 are important for the interaction with the importins, and that each (Impα, Impβ, Imp7 and transportin) interacts with the same residues in Snail1.

To analyze the contribution of each residue to importin binding, we independently mutated the two residues in M2 (K161, K170), those in M3 (K187, R191) and the three residues mutated in M6 (R220, N222, R224). In contrast to the effect produced by the double mutations in M2 and M3, substitution of only one of the residues had no significant effect on importin binding (Fig. 2E). Similarly, single mutations in R220, N222 or R224 in M6 did not significantly alter importin binding (Fig. 2E). However, when both R220 and

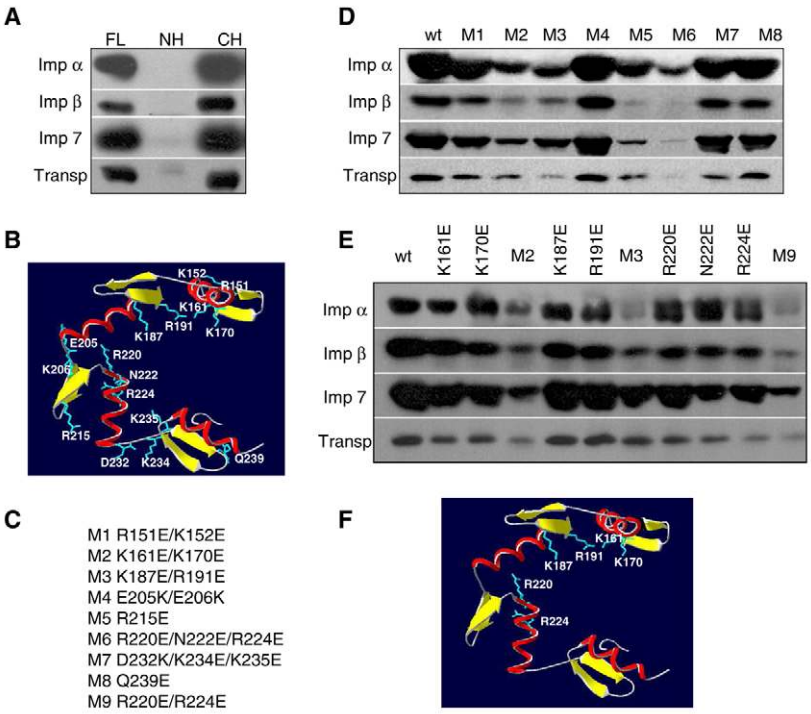

Fig. 2. Identification of the Snail1 NLS. (A,D,E) Pull-down assays were performed with the indicated Snail1 protein fragments in the absence of RanGTP as described in Fig. 1. Bound importins (Impα, Impβ, Imp7 and transportin) were identified by western blotting. (A) Assays carried out with either the full length (FL) or the N-terminal (NH; 1-151) or C-terminal (CH; 152-264) halves. (B) Three-dimensional model of the four zinc fingers of Snail1 showing the position of all the mutated residues. (C) List of the mutated residues in all the Snail1 mutants (M1-M9) used in the pull-down assays shown in D and E. (F) Three-dimensional model showing the position of the residues that when mutated, impair Snail1 nuclear import.

R224 were simultaneously mutated (M9) the effect was similar to that obtained with the triple mutation (Fig. 2E).

We examined whether mutations in the residues necessary for importin binding affected the subcellular localization of a GFP-Snail1 fusion protein transfected in MCF7 epithelial cells. Whereas GFP could be detected in the nucleus and cytoplasm (Fig. 3A), GFP-Snail1 was only localised in the nucleus (Fig. 3B). Versions M2 (K187E, R191E), M3 (K161E, K170E) and M6 (R220E, N222E and R224E) of GFP-Snail1 were not exclusively localized in the nucleus and they behaved like GFP alone (Fig. 3C,D,F). Snail1 subcellular localization was not affected after mutating R215 (M5; Fig. 3E) indicating that this residue is not necessary in vivo for the nuclear localization of Snail1. This nuclear localization was not changed by single substitutions in any of the residues mutated in M2, M3 or M6 (Fig. 3G-K and not shown). However, the behaviour of M9 (R220E/R224E) was indistinguishable from that of M6 (compare Fig. 3F with 3L), suggesting that N222 is not necessary for Snail1 nuclear import.

Although binding of the importins and therefore the nuclear localization of the Snail1 mutants M2, M3 and M9 was clearly impaired (Fig. 2D,E), the GFP fusion proteins always showed some nuclear localization when expressed in MCF7 cells. The small size of the fusion proteins (around 55 kDa) and the fact that the subcellular localization of the combined M3-M9 mutant was indistinguishable from that of the individual mutants (not shown) indicates that the partial nuclear localization of the mutants is probably due to diffusion through the NPCs. To address this possibility, we increased the size of these proteins by fusing them to GST. Indeed, when transfected in MCF7 cells, the GST-GFP-Snail1 protein was localized in the nucleus whereas the corresponding M2, M3 and M9 mutated versions were fully excluded from it (Fig. 3M-P). Although the loss of nuclear localization correlates with the loss of importins binding in mutants M2, M3 and M9, increased nuclear export might also contribute to this effect. This does not seem to be the case since binding of CRM1, the only known exportin involved in Snail1 nuclear export, is not affected in the mutated versions (not shown).

As expected from our importin binding data, full nuclear localization of Snail1 is not affected by single mutations in any of the identified basic residues (K161/K170, K187/R191 and R220/R224) indicating that any five of these basic residues are sufficient for Snail1 nuclear localization. In order to characterise in more detail the minimal requirements for this NLS, we simultaneously mutated two or three residues, which were each located in different fingers. All the four mutant versions generated were unable to bind to the importins (Fig. 3Q) and GFP-GST fused to any of these mutant proteins, showed a clear cytoplasmic localization when expressed in MCF7 cells (Fig. 3R-U). These results clearly indicate that five out of six of the identified basic exposed residues are the minimal requirement for Snail1 nuclear localization.

In summary, we have identified a NLS that serves for all Snail1 binding importins and that consists of basic residues localised in the three first fingers of Snail1 (Fig. 2F).

NLS residues also participate in DNA binding

Once the basic residues needed for Snail1 nuclear import were identified and considering that they are located in the zinc finger region, we wondered whether these residues were involved in the known DNA binding activity of this domain or if they were only necessary to make direct contact with the importins. In an in vitro assay we found that, unlike wild-type Snail1, the M2, M3 and M9 mutant versions were unable to bind to a DNA probe containing the E-box1 of the *E-cadherin* promoter (Fig. 4A). Consistent with this, the mutated versions were unable to repress the activity of the *E-cadherin* promoter (Fig. 4B). Thus, these residues could be important to maintain the structural organization of the zinc finger region and their mutation would therefore affect all the functions associated to it. We carried out a circular dicroism (CD) spectroscopic analysis on purified wild-type and mutant Snail1

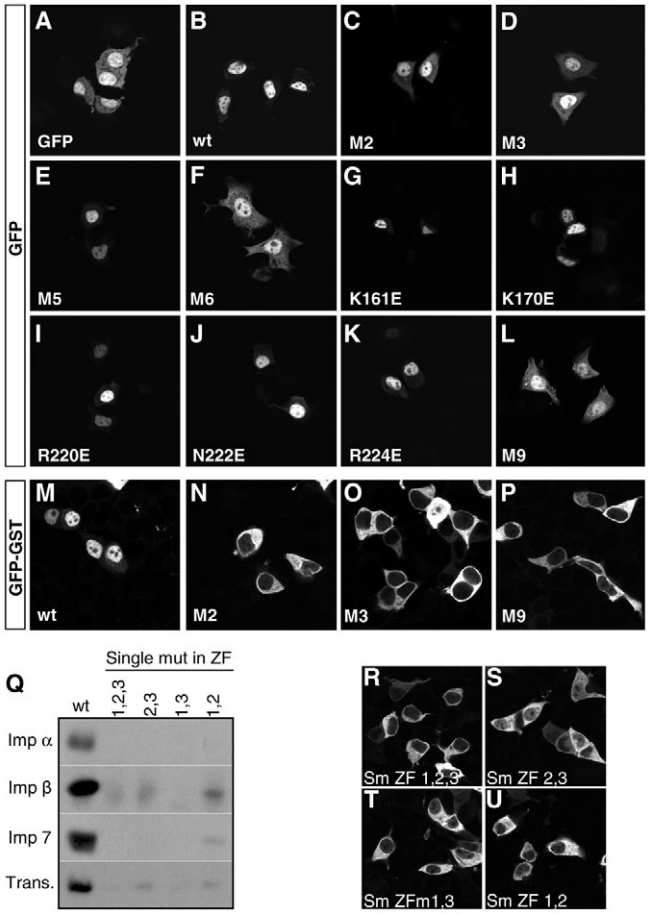

Fig. 3. Snail1 NLS is functional in cell lines. Constructs driving the expression of GFP (A) or the indicated GFP- (B-L) or GFP-GST- (M-P) Snail1 fusion proteins were transiently transfected into MCF7 cells. The cells were fixed 24 hours after transfection and the subcellular localization of the proteins was analysed by confocal microscopy. (Q) Pull-down assays with the indicated Snail proteins were performed as described in Fig. 2. (R-U) Subcellular localization of the indicated GFP-GST-Snail1 mutants in transiently transfected MCF7 cells. Single mutations in zinc fingers (Sm ZF) were as follows: ZF1: K161E; ZF2: K187E and ZF3: R220E.

proteins to examine this possibility, and we also measured their thermal denaturation in order to check whether the stability of the proteins were affected upon mutation. The CD spectrum of the mutants was virtually indistinguishable from that of the wild-type protein and their thermal unfolding patterns were almost identical (Fig. 4C,D) indicating that the mutated basic residues are not necessary for a proper Snail1 conformation. However, since CD spectroscopy mainly provides information on secondary structure, these data do not rule out an effect of the mutations on tertiary structure. To address this issue, we generated four Snail1 mutant versions, each with one of the four zinc fingers unfolded by mutating one of the two zinc chelating cysteines to alanine. Individual unfolding of any of the four Snail1 zinc fingers caused, to different extend, a clear loss in importins binding (Fig. 5A) and a decrease in nuclear localization in MCF7 cells (Fig. 5B-G). When the second finger was unfolded (C182A) at least three of the importins could still weakly bind to the Snail1 mutant protein (Fig. 5A) and this binding was sufficient for a partial nuclear localization (Fig. 5E). Thus, a proper conformation of the ZF region seems to be necessary to generate a pocket with the distant basic residues accessible for the interaction with the importins.

Although only the crystal structure of Snail1 when bound to its target can provide definitive proof for the DNA-protein interactions, we modelled the interaction of Snail1 with its best known target DNA, the E1 box of the human *E-cadherin* promoter and found that those residues in Snail1 identified as necessary for the interaction with the importins might be also necessary for its interaction with DNA (supplementary material Fig. S1) consistent with the loss of DNA-binding activity of the mutated proteins. In our model, one of the identified residues, R191, interacts directly with one of the nitrogen bases of the E-box (G-76) whereas K161, K170, K187, R220 and R224 (the latter probably indirectly via R220) probably interact with the phosphate backbone (supplementary material Fig. S1). Thus, the model predicts that seven of the nine versions of the Snail1 protein we have generated contain mutations in residues necessary for the interaction with

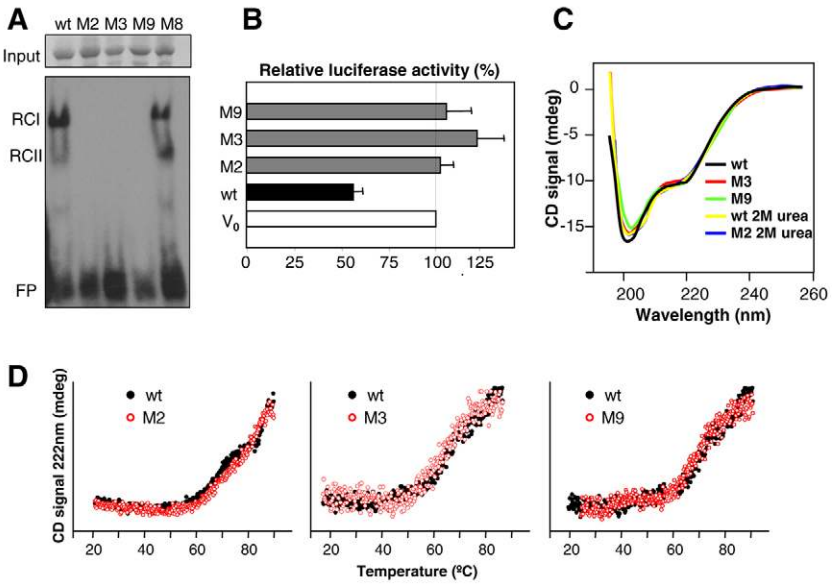

Fig. 4. The Snail1 NLS residues are necessary for DNA binding but not for the conformation of the binding domain. (A) EMSA showing that Snail1 mutants M2 (K161E, K170E), M3 (K187E, R191E) and M9 (R220E, R224E) have lost their ability to form retardation complexes (RCI and RCII) with a Snail1-specific DNA probe, unlike the M8 mutant (Q239E) that forms complexes comparable with those of the wild type (wt). Upper panel: Snail proteins input used in the assay. FP: free probe. (B) Luciferase reporter assay showing that the Snail1 mutant versions M2, M3 and M9 are unable to repress the activity of the *E-cadherin* promoter (–1000 bp). Relative luciferase activity is related to the activity detected in cells cotransfected with the luciferase reporter construct and an empty vector (V_0). (C) The CD spectra of the indicated wild-type and mutant Snail1 proteins do not display significant differences. (D) Thermal denaturation of the indicated Snail1 proteins monitored by far-UV CD reveal two main transitions at around 63 and 83°C in all cases.

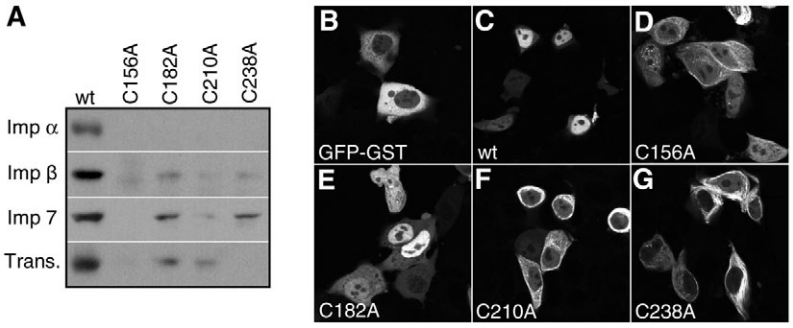

Fig. 5. The proper folding of the four zinc fingers is necessary for Snail1 binding to the importins and subsequent nuclear localization (A) Pull-down assays with the indicated Snail1 proteins were performed in the absence of RanGTP as described in Fig. 1. (B-G) Subcellular localization of GFP-GST or the indicated GFP-GST-Snail1 fusion proteins in transiently transfected MCF7 cells.

the DNA. In support of this prediction, these seven mutant proteins have lost the ability to bind the Snail1 DNA target (Fig. 4A, and data not shown). These mutant forms not only include those containing the residues necessary for importin binding (M2, M3 and M6) but also M4 and M7 that contain mutations in E206 and K234, probably close to the DNA according to our model. The only mutant form that still binds to the DNA probe as efficiently as wild-type Snail1 is M8 (Q239E). The model predicts that the Q239 residue is too far from the DNA to have any role in DNA binding (supplementary material Fig. S1). Thus, our data are consistent with the idea that the residues that interact with importins are a subset of those necessary for Snail1 binding to its target DNA, although they are not essential to maintain the conformation of the protein.

Conservation of nuclear import pathways in the Snail family

We then assessed whether the crucial residues we identified for Snail1 nuclear import might be conserved in other family members. The four Snail proteins described in humans (Snail1, 2 and 3, and Snail-like) are strongly conserved at the amino acid level, ranging from 78% (Snail1 vs Snail-like) to 42% (Snail1 vs Snail3). The residues identified as participating in the nuclear localization of Snail1 are conserved in all Snail family members in human, suggesting that the nuclear import pathways may also be conserved. Indeed, the importins that bind to Snail1 (Impα, Impβ, Imp7 and transportin), also bind to Snail-like and Snail2 in a Ran-dependent manner (Fig. 6A). Import assays in HeLa cells with GFP-tagged versions of Snail-like and Snail2 also show that the interacting importins are functional (not shown) and deletion analyses indicate that importins bind exclusively to the C-terminal half (Fig. 6B). Site-directed mutagenesis confirmed that the same three pairs of basic residues identified in Snail1 are required for the interaction of Snail-like and Snail2 with importins (Fig. 6C). Thus, the nuclear import pathways appear to be conserved in different human Snail proteins.

A conserved motif in zinc finger proteins of the C_2H_2 type

We checked whether this conservation might extend to other species and since amphioxus, *Branchyostoma floridae*, has only one *Snail* gene (Holland et al., 2008; Putnam et al., 2008), we examined whether the same NLS was also present in amphioxus Snail. Not only are the key basic residues conserved in this protein but also, the human importins bind perfectly to its the C-terminal half. This binding was abolished in mutated versions equivalent to M2, M3 and M9 (Fig. 6D) indicating that the NLS described is also present in amphioxus Snail. Interestingly, we have found that this NLS is not only conserved in amphioxus Snail but in all members of the Snail superfamily (Fig. 7A and not shown).

Since Snail proteins belong to the C_2H_2 class of zinc finger transcription factors, we examined whether a similar motif was present in proteins from other families included in this class. We found that in many of these proteins at least five basic residues are conserved at the same or at an adjacent position to those described in our consensus for Snail proteins in three consecutive fingers (Fig. 7B,C). According to our data, such residues could also form an exposed basic domain for importin binding and thus, they might also serve as NLSs. There are also transcription factors with three or more C_2H_2 consecutive zinc fingers in which less than five of the identified basic residues are conserved. Our prediction would be that in these cases the zinc finger region does not contain a NLS. Interestingly, in all these proteins a different type of NLS has been described, located outside of the finger region (see Discussion).

Discussion

Transcription factors of the Snail superfamily are important regulators of cell activity in response to extracellular signals (Nieto,

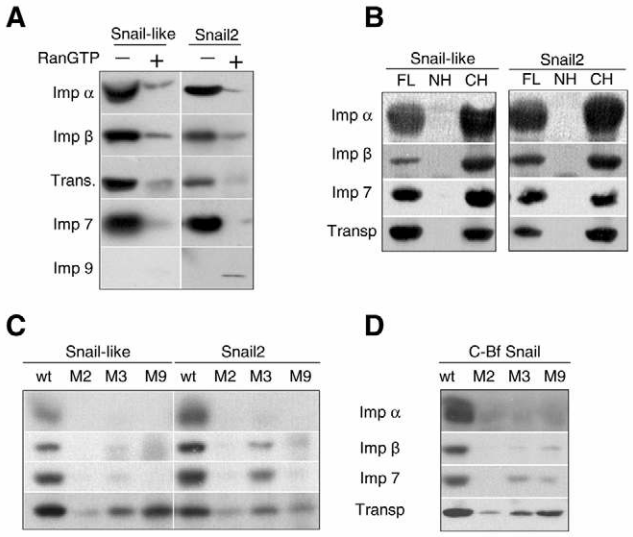

Fig. 6. Snail1 nuclear import pathways are conserved in Snail proteins. (A) Pull-down assays were performed with immobilized Snail-like or Snail2, and the bound importins were identified by western blotting. Note that the same importins that bind to Snail1 (Fig. 1A) also bind to Snail-like and Snail2. (B-D) Pull-down assays performed with the indicated immobilized proteins in the absence of RanGTP. (B) Only the C-terminal half of Snail-like and Snail2 can bind to importins. NH, N-terminal half (Snail-like: 1-145; Snail2: 1-156); CH, C-terminal half (Snail-like: 146-258; Snail2: 157-268). (C) Mutations in equivalent residues to those identified in Snail1 also impair the binding of Snail-like or Snail2 to importins. (D) The import pathways are conserved in amphioxus Snail [C-BfSnail, C-terminal half (141-253)].

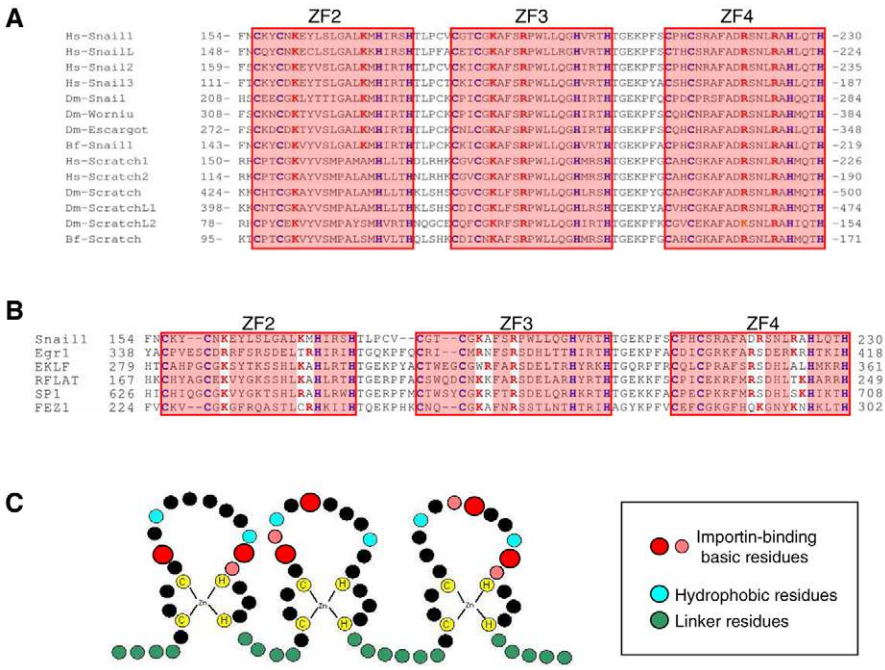

Fig. 7. Conservation of the NLS in C_2H_2 zinc finger transcription factors. (A) Alignment of zinc fingers (ZF) 2-4 of representative members of the Snail superfamily (Snail and Scratch proteins). These fingers correspond to number 1-3 in human Snail1 and Snail1L. The three pairs of basic residues necessary for nuclear import (red) are conserved in all the Snail proteins. Scratch proteins have lost the second K in finger 2. Note that only five out of the six residues are required for importin binding and nuclear localization. (B) Alignment of three consecutive zinc fingers of representative human members of five additional families of C_2H_2 transcription factors, which also contained the identified NLS. The basic residues are shown in red and the zinc coordinating cysteines and histidines are shown in blue. Zinc finger residues are shaded in red. (C) Schematic representation of the relative positions occupied by the NLS described. Exposed basic residues that conform the basic interface for importin binding are shown in red. In some cases, these basic residues can be found in adjacent positions (shown in pink) in other transcription factors. Linker regions (green);hydrophobic residues required for zinc finger conformation (Knight and Shimeld, 2001) (blue). Accession numbers of the aligned proteins: Hs-Snail1 (NP005976), Hs-SnailL (EAW70471), Hs-Snail2 (NP003059), Hs-Snail3 (NP840101), Dm-Snail (NP476732), Dm-Worniu (AAF12733), Dm-Escargot (AAA28513), Bf-Snail (AAC35351), Hs-Scratch1 (NP112599), Hs-Scratch2, (NP149120), Dm-Scratch (AAA91035), Dm-ScratchL1 (NP647845), Dm-ScratchL2 (NP612040), Bf-Scratch (Bf Scaffold 229), Hs-Egr1 (P18146), HsEKLF (NP006554), Hs-RFLAT1 (NP057079), Hs-SP1 (P08047) and Hs-FEZ1 (NP001019784). Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Bf, *Branchyostoma floridae.*

2002; Peinado et al., 2007). As transcription factors, they need to be translocated from the cytoplasm to the nucleus to be functional. Here we have performed a detailed characterization of nuclear import pathways that act on three human members of the family (Snail1, Snail2 and Snail-like) and we show that these pathways are conserved in other species. Snail proteins can interact with and be efficiently imported by Impβ, either alone as previously described for Snail1 (Yamasaki et al., 2005) or in combination with Impα. Similarly, they can be translocated by at least two other importins, Imp7 and transportin. Importins bind to exposed basic residues and in the case of Snail, they recognize a NLS constituted by pairs of basic residues in Snail1 and at equivalent positions in Snail-like and Snail2: K161/K170; K187/R191; and R220/R224. Any of these residues can be mutated individually without impairing importin binding but simultaneous mutation of more than one residue prevents both binding of the importins and nuclear localization. These residues are located in fingers 1-3 of Snail1, which correspond with fingers 2-4 in Snail2 and Snail-like, because human Snail1 has lost the first finger typical of the family (Manzanares et al., 2001). The absence of this first finger is unlikely to be determinant since it does not seem to be functional when these proteins contain more than four fingers (Pavletich and Pabo, 1993). Thus, the first finger in other Snail proteins is unlikely to be crucial for importin binding. In our study we did not initially consider the last finger because it does not comply with the consensus for a C_2H_2 zinc finger and it is not so conserved in Snail1 proteins across species (Sefton et al., 1998). However, we cannot discard the possibility that the last finger (4 or 5 depending on the species) also participates in importin binding through basic residues located at similar positions to those identified in fingers 1-3 of the human Snail1 protein.

In contrast to classical NLSs, the basic residues identified are not localised in a single short stretch but rather in three consecutive zinc fingers. This suggests that a perfect folding of the DBD might be necessary to generate a basic pocket for the interaction with the importins. Indeed, the conformation of the fingers needs to be intact, consistent with previous data indicating that deletion of any of the Snail1 zinc fingers induces the loss in nuclear localization (Yamasaki et al., 2005) and that the zinc finger region is sufficient for nuclear localization (Yamasaki et al., 2005) (and data not shown). With respect to the number of fingers needed for functioning as a transcription factor, four seems to be the maximum requirement (Pavletich and Pabo, 1993) and two are insufficient (Wolfe et al., 2000). In agreement with this, here, we show that exposed basic residues in fingers 1-3 is an absolute requirement for nuclear import.

Although residues 8-16 and R151-K152 have recently been proposed as putative classical NLSs in Snail1 (Ko et al., 2007), evidence from several sources seem to be at odd with this interpretation. As such, we could not detect any importin bound to the N-terminal half of Snail1, this half of the protein did not promote

GFP nuclear translocation (Yamasaki et al., 2005) (and data not shown) and the simultaneous mutation of R151E and K152E did not decrease the binding of the importins to Snail1.

Interestingly, the residues required for importin binding are also involved in DNA binding. When these residues are mutated, Snail factors cannot bind to target DNA although the protein conformation appears to remain intact. This suggests that rather than being structurally important, these residues may participate in both functions, importin and DNA binding. The colocalization or the proximity of the NLS motifs with the DNA binding domain was first described in nucleic acid binding proteins, and this proposed proximity or overlap confers evolutionary advantage (LaCasse and Lefebvre, 1995). Evolution may have used the pre-existing DNA-binding mechanism to compartmentalize DNA-binding proteins into the nucleus (Cokol et al., 2000). However, new NLSs have clearly been generated independently, and as such, most NLSs are not used to bind DNA (Cokol et al., 2000). The finding of a putative Snail1 NLS situated outside the fingers (Ko et al., 2007) justified the analysis of the whole protein in our search for NLSs. We found that Snail1 belongs to the category that also uses the importin-binding residues for DNA binding. This is probably related to the proposed function of importins as cytoplasmic chaperones of histones and ribosomal proteins (Jakel et al., 2002). Our data extend this activity of importins as chaperones to transcription factors, therefore preventing their non-specific binding to polyanions (mRNAs, tRNAs) present in the cytoplasm.

The consensus motif that we have identified for the binding of human Snail1 to importins is conserved in all Snail proteins described so far from *Drosophila* to humans. Indeed, we show that this NLS also promotes Snail binding to importins in amphioxus, a non-vertebrate chordate that only contains one *Snail* gene.

NLSs have previously been identified in the DBD of other C_2H_2 zinc finger transcription factors and, although in the cases of Wt1 and GKLF factors one or one and a half zinc fingers seem to be enough (Bruening et al., 1996; Shields and Yang, 1997), in many cases at least three consecutive fingers are necessary for a complete nuclear localization (Matheny et al., 1994; Pandya and Townes, 2002; Quadrini and Bieker, 2002; Song et al., 2002). As expected, since importins bind to basic residues, the mutation in all of these residues in the zinc finger region of the kruppel family factor EKLF abolished nuclear localization of the protein (Pandya and Townes, 2002). Here we describe the specific residues necessary for importin binding and nuclear localization for the Snail proteins and we have found that this consensus NLS motif is conserved in representative members of all families of C_2H_2 zinc finger proteins that require the zinc finger domain for its nuclear localization. However, there are several examples of transcription factors with three or more consecutive C_2H_2 zinc fingers in which the Snail consensus NLS motif is not conserved. Interestingly, these transcription factors contain alternative NLSs. For instance, Gfi1 has a NLS located at the N-terminus of the protein (Grimes et al., 1996), whereas Gli1 contains a bipartite NLS with two basic clusters one of which is located outside the finger region (Wang and Holmgren, 1999) and MTF1 has an NLS adjacent to the zinc finger domain (Saydam et al., 2001). This suggests that different proteins have utilized different strategies to reconstitute a new NLS after the secondary loss of the canonical NLS located within the zinc finger domain. In summary, it seems that the ancestral NLS for C_2H_2 zinc finger proteins was a relaxed basic interface for importin binding compatible with the zinc finger conformation, which also allows enough flexibility in the primary sequence to adopt different DNA-binding specificities. In addition, the binding of importins to these transcription factors ensures efficient nuclear import while preventing their non-specific binding to polyanions in the cytoplasm. Once in the nucleus, the Ran system ensures the release of the transcription factor from the importin, unmasking its DNA binding site so that it can fulfil its function as transcriptional regulator.

Materials and Methods

Plasmids

Coding DNA for human Snail1, Snail2, Snail-like and *Branchyostoma floridae*, amphioxus, Snail as well as their respective truncated versions, were PCR-amplified from clones that have been described previously (Langeland et al., 1998; Locascio et al., 2002) or obtained from the RZPD, the German resource centre for genome research. These ORFs were cloned into previously described (Mingot et al., 2004) or commercial expression vectors. For *E. coli* expression, the amplified ORFs were cloned into pNzztev80 (provides an N-terminal ZZ tag, a TEV protease cleavage site and a C-terminal HexaHis tag) and pNGFP80 (provides an N-terminal GFP tag and a C-terminal HexaHis tag). For expression in mammalian cells, the coding regions for wild-type, mutated or truncated Snail1 proteins were cloned into pNGFPrevCMV or pNGFP-GSTrevCMV, generated by inserting a PCR-amplified GST coding region in frame with GFP. These vectors, derived from pNGFPrev, drive the expression of proteins with an N-terminal GFP or GFP-GST tag, respectively, under the control of the CMV promoter.

Recombinant protein expression and purification

Expression and purification of His-tagged importins (α1, β, 9, 13, transportin, *Xenopus* Imp7), as well as the components of the Ran system (Ran, RanBP1 and Rna1P-RanGAP), have been described previously (Gorlich et al., 1995; Izaurralde et al., 1997; Jakel and Gorlich, 1998; Jakel et al., 2002; Kutay et al., 1997; Mingot et al., 2001). NTF2 was purchased from Sigma. Both, ZZ- and GFP-tagged Snail1 proteins (wild-type and mutants) were expressed in *E. coli* BL21 maintained at 16°C O/N in 2YT medium and induced with 0.5 mM IPTG. Bacterial lysis was performed in 20 mM Hepes (pH 7.5) and 2 M urea for ZZ-tagged proteins or 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 2 mM $MgCl_2$ for GFP-tagged proteins. After purification on Ni Sepharose 6 Fast Flow (GE Healthcare) ZZ-tagged proteins were dialysed against 20 mM Hepes (pH 6.5), with 0.33 M (CD spectrum experiments) or 2 M urea (thermal unfolding experiments). GFP-tagged proteins were dialysed against import buffer (see below).

Pull-down assays

The ZZ versions of human Snail1, Snail2, Snail-like and amphioxus Snail were immobilized on 50 μl of IgG Sepharose (GE Healthcare) from lysates of *E. coli*, in which the recombinant proteins were expressed, were cleared by centrifugation at 100,000 ***g***. The immobilized proteins were incubated for 3 hours, in the presence or absence of 5 μM purified GTP.RanQ69L (a GTPase-deficient mutant), with 0.4 ml of a HeLa cell extract in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl and 10 mM $MgCl_2$. After four washes with 500 μl binding buffer, the bound proteins were eluted with SDS sample buffer, resolved by SDS-PAGE and analyzed by western blotting.

Antibodies

The primary antibodies against importin α, β, 7, 9, 13 and transportin have been described previously (Fassati et al., 2003) and were kindly provided by Dirk Görlich (MPI for Biophysical Chemistry, Göttingen, Germany). The secondary antibody was an anti-rabbit IgG coupled to HRP from Sigma.

Import assays

HeLa cells were grown to 50% confluence on 12 mm coverslips and permeabilized for 5 minutes with 60 μg/ml digitonin diluted in import buffer (20 mM Hepes pH 7.5, 110 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose and 0.5 mM EGTA). Once permeabilized, the cells were incubated for 5 minutes at room temperature with import buffer containing purified GFP-tagged Snail1 (1 μM), the indicated importins (1 μM), Ran mix (1.5 μM RanGDP, 0.3 μM NTF2, 0.1 μM RanBP1 and 0.05 μM Rna1p) and an energy regenerating system (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine kinase, 0.05 mM DTT and 0.05 mM $MgCl_2$).

Site-directed mutagenesis

All the mutations used in this work were introduced by the PCR-based QuickChange method (Stratagene). Mutations were verified by DNA sequencing after the purification of plasmid DNA from single colonies.

Transfections

MCF7 cells were transiently transfected with the indicated GFP-tagged proteins using FuGENE 6 transfection reagent (Roche) following the manufacturer's instructions.

Cells were fixed 24 hours after transfection with 3% paraformaldehyde for 5 minutes at 37°C, washed with PBS, mounted with vectashield (Vector) and analyzed using confocal laser scanning microscopy.

Electrophoretic gel mobility shift assays

EMSAs were performed using the indicated ZZ-tagged Snail1 proteins directly from 100,000 ***g*** cleared *E. coli* lysates and a DNA probe, with overhanging guanosine nucleotides at both ends, containing the highly conserved E-box1 from the human *E-cadherin* promoter. The probe was generated by annealing a single-stranded DNA oligonucleotide comprising nucleotides –95 to –60 of the promoter 5′-GGCTGTGGCCGGCAGGTGAACCCTCA-3′ with an appropriate complementary strand 5′-GTGAGGGTTCACCTGCCGGCCACAG-3′ and labelled by end filling using Klenow polymerase in the presence of [α-^{32}P]dCTP. The reactions were carried out on ice for 30 minutes in 25 mM Hepes-KOH (pH 7.9), 50 mM KCl, 50 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 40 μM $ZnCl_2$, 2 μg of poly(dI-dC) and 21% (vol/vol) glycerol. DNA-protein complexes were resolved in non-denaturing 8% polyacrilamide gels.

Promoter analysis

To analyze the activity of Snail1 as an *E-cadherin* repressor, a luciferase reporter construct carrying the wild-type mouse *E-cadherin* promoter (–1000 bp) was transiently transfected into MCF7 cells together with pCDNA3 (negative control) or pCDNA3 constructs driving the expression of wild-type or mutated Snail1. Firefly and renilla luciferase activity were measured 40 hours after transfection using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Results are presented as the means ± s.e. of duplicates from four independent experiments and are presented as the percentage of luciferase activity relative to the negative control (luciferase values in cells co-transfected with the empty vector).

Circular dichroism spectroscopy

Circular dichroism (CD) experiments were performed using a Jasco J-810 spectropolarimeter equipped with a Peltier PTC-423S system. Isothermal wavelength spectra were acquired at a scan speed of 50 nm/minute with a response time of 2 seconds and averaged over at least six scans at 20°C. Protein concentration was 4.4 μM and the cuvette pathlength was 1 mm. Urea (0.33 M) was necessary to ensure full solubility of the protein. CD-monitored thermal denaturation experiments were performed by monitoring the CD signal at 222 nm. The sample was layered with mineral oil to avoid evaporation, and the heating rate was 60°C/hour. In this case, 2M urea was needed in order to avoid precipitation at high temperatures.

Modelling

The three-dimensional structure of the C-terminal half of Snail1 (aa 151-264) bound to a human *E-cadherin* promoter fragment (–84 to –73) that contains the Snail1-binding E-box1 (5′-GGCCGGCAGGTG-3′ and the complementary strand) was modelled using Swiss PDB Viewer 3.7 (Guex and Peitsch, 1997). The template used was the six-finger zinc finger Aart polypeptide bound to DNA (PDB code 2I13) (Segal et al., 2006). Raw structures obtained from fitting were subjected to steepest descent energy minimization and checked for packing errors. The DNA sequence recognized by Snail1, the *E-cadherin* promoter region (–84 to –73) that contains the Snail-binding E-box1, was modelled using the *model.it* program in the DNA tools utilities hosted by the ICGEBnet server (Vlahovicek et al., 2003) and docked onto the Snail1 model using the same coordinates as the DNA from the template. Figures were rendered with PDB Viewer.

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