THE SNAIL SUPERFAMILY OF ZINC-FINGER TRANSCRIPTION FACTORS

M. Angela Nieto

The Snail superfamily of zinc-finger transcription factors is involved in processes that imply pronounced cell movements, both during embryonic development and in the acquisition of invasive and migratory properties during tumour progression. Different family members have also been implicated in the signalling cascade that confers left–right identity, as well as in the formation of appendages, neural differentiation, cell division and cell survival.

GASTRULATION
The morphogenetic movements of the early embryo that lead to the generation of the third embryonic layer — the mesoderm.

MESODERM
The third embryonic layer generated during gastrulation, which occupies an intermediate position between the ectoderm and the endoderm. It will give rise to the skeleton, muscles and connective tissue.

TRIPOBLAST
An animal that is composed of three embryonic cell layers: ectoderm, endoderm and mesoderm.

NEURAL CREST
A cell population that originates in the dorsal part of the neural tube and gives rise to many derivatives, including most of the peripheral nervous system, the cranio-facial skeleton and pigmented cells of the body.

Instituto Cajal, Doctor Arce, 37, 28002 Madrid, Spain.
e-mail: anieto@cajal.csic.es
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Assuming the veracity of Lewis Wolpert's popular statement[1] that it is not birth, marriage or death, but GASTRULATION, that is the most important event in the lifespan of an individual, it seems almost trivial to mention that the study of MESODERM formation is a must for developmental biologists. To put it in more conventional terms, the formation of the third embryonic layer in TRIPOBLASTIC animals is, indeed, the time at which the embryonic axes are coordinated and when the important morphogenetic movements that shape the embryo commence.

In this regard, the Snail family of zinc-finger transcription factors occupies a central role in morphogenesis, as its members are essential for mesoderm formation in several organisms from flies to mammals[2–10]. The analysis of different vertebrate Snail homologues has highlighted their role not only in the development of the mesoderm, but also in other processes that require large-scale cell movements, such as the formation of the NEURAL CREST[6,11–14].

More recently, this role in promoting cell movement has been extended and includes more generalized phenomena such as the EPITHELIAL–MESENCHYMAL TRANSITION (EMT)[15,16]. EMT is the mechanism by which epithelial cells that are generated in a particular region can dissociate from the epithelium and migrate to reach different locations[17]. As such, EMT is fundamental to both normal development and the progression of malignant epithelial tumours[17]. In addition to triggering EMT, Snail superfamily members have been implicated in various important developmental processes, including neural differentiation, cell fate and survival decisions, and left–right identity[18].

From an evolutionary point of view, the Snail family provides a good model to study ancestry and the acquisition of functions that are related to changes in the BODY PLAN. In this respect, this family is associated with the appearance of the neural crest, which is essential for the formation of the vertebrate head[19]. The recent identification of new family members and the association of these members with new functions has attracted researchers in many fields, from embryonic pattern formation to cancer research. In this review, I describe the diversity and organization of the Snail superfamily, and then address the roles that have been assigned to the different family members.

The Snail superfamily of repressors

The first member of the Snail family, *snail*, was described in *Drosophila melanogaster*[20,21], where it was shown to be essential for the formation of the mesoderm[2]. Subsequently, Snail homologues have been found in many species including humans, other vertebrates, non-vertebrate CHORDATES (protochordates), insects, NEMATODES, ANNELIDS and molluscs (TABLE 1).

Snail family members encode transcription factors of the zinc-finger type. They all share a similar organization, being composed of a highly conserved carboxy-terminal region, which contains from four to six zinc fingers, and a much more divergent amino-terminal region. The fingers correspond to the C_2H_2 type[22] and function as sequence-specific DNA-binding motifs. The fingers are structurally composed of two β-strands followed by an α-helix, the amino-terminal part of which binds to the major groove of the DNA.

Table 1 | **Snail superfamily members**

Species	Common name	Gene	Synonyms	Accession no.	Map	References
Caenorhabditis elegans	Nematode	*ces1**		AAF01678	I:2.9	37
		snail-like	K02D7.2	T32983	IV:-26.1	36
		*scratch-like**	C55C2.1	T15225	I:-9.3	36
Helobdella robusta	Leech	*snail1*	*Hro-sna1*	AF410864		43
		snail2	*Hro-sna2*	AF410865		43
Patella vulgata	Limpet	*snail1*	Pv-sna1	AY049727		32
		snail2	Pv-sna2	AY049791		32
Drosophila melanogaster	Fruitfly	*snail*		S06222	35D2–3	20,100
		escargot		AAF12733	35D1	111
		worniu		S33639	35D2–3	95
		*scratch**		AAA91035	64A2–3	33
		*scratch-like1**	CG12605	AAF47818	64A1	36
		*scratch-like2**	CG17181	AAF47394	61C7	36
Lytechinus variegatus	Sea urchin	*Snail*		AAB67715		unpublished
Halocynthia roretzi	Ascidia	*Snail*		BAA75811		8
Ciona intestinalis	Ascidia	*Snail*		AAB61226		42
Branchiostoma floridae	amphioxus	*Snail*		AAC35351		7
Takifugu rubripes	Pufferfish	*Snail1*		CAB54535		112
		Snail2		CAB54536		112
Danio rerio	Zebrafish	*snail1*		CAA52795		5,49
		snail2		AAA87196		11
		slug		AI722148		36
		*scratch**		AI883776		36
Xenopus laevis	African clawed toad	*Snail*	*Xsna*	P19382		113
		Slugα	*Xslu*	AF368041		78,114
		Slugβ	*Xsluβ*	AF368043		78
Silurana tropicalis	Western clawed frog	*Slug*	*Xslug*	AF368038		78
Gallus gallus	Chicken	*Snail*	*SnR*	CAA71033		50,84
		Slug		CAA54679		6
Mus musculus	Mouse	*Snail*		Q02085	Chr.2-97.0	3,4
		Slug	*Slugh*	AAB38365	Chr.16-9.4	50,73,85
		*Scratch**		AY014997		35
		Smuc	Zfp293	NP038942		26
Homo sapiens	Human	*SNAIL*	*SNAIL1, SNAILH*	AF155233	20q13.1	115,116
		SNAILP	*SNAI1P*	AF153502	2q34	115,116
		SLUG	*SLUGH, SNAIL2*	AAC34288	8q11	117
		*SCRATCH1**		AY014996	8q24.3	35
		*SCRATCH2**		AL121758	20p12.3–13	35

The Snail superfamily is subdivided into two families: Snail and Scratch (marked by an asterix). Accession numbers are from Entrez (http://www.ncbi.nlm.nih.gov/Entrez).

EPITHELIAL–MESENCHYMAL TRANSITION
The transformation of an epithelial cell into a mesenchymal cell with migratory and invasive properties.

BODY PLAN
The organization of the embryonic tissues to generate an individual with specific characters.

CHORDATE
An animal with a notochord. These include ascidians, amphioxus and all vertebrates.

NEMATODE
An unsegmented worm.

ANNELID
A segmented worm.

BASIC HELIX–LOOP–HELIX PROTEIN
A transcription factor with a basic domain that binds to a hexanucleotide called the E box, and a hydrophobic domain (the helix–loop–helix) that allows the formation of homo- and heterodimers. They can also have leucine repeats called a leucine zipper.

The two conserved cysteines and histidines (C_2H_2) coordinate the zinc ion. Both random selection and transfection experiments with different promoters have shown that the consensus binding site for Snail-related genes contains a core of six bases, CAGGTG[15,16,23–26]. This motif is identical to the so-called E box, the consensus of the core binding site of BASIC HELIX–LOOP–HELIX (bHLH) transcription factors, which indicates that Snail proteins might compete with them for the same binding sequences[26–28].

On binding to the E box, Snail family members are thought to act as transcriptional repressors[9,14–16,24,26,27,29,30]. The repressor activity depends not only on the finger region, but also on at least two different motifs that are found in the amino-terminal region. One of these is the so-called SNAG (Snail/Gfi) domain, which was initially described as a repressor domain in the zinc-finger protein Gfi1 (REF. 31). This motif is important for repression in mammalian cells[27]. The SNAG domain is conserved in all vertebrate *Snail* genes, and is also found in echinoderms, cephalochordates[7], in one of the limpet genes[32] and in *Drosophila scratch*[33]. Its wide distribution might reflect an early ancestry. This, in turn, would imply that it has been lost in other *Drosophila* family members, *Caenorhabditis elegans* and urochordates. Alternatively, the SNAG domain might have been added independently in each of the different species. The availability of complete coding sequences from other groups will help to distinguish between these two possibilities.

Despite the absence of a SNAG domain, *Drosophila snail* also acts as a transcriptional repressor. This activity is mediated through an interaction with a co-repressor, CtBP (carboxy-terminal binding protein)[34]. Consensus motifs for the binding of CtBP are present in other *Drosophila* Snail family members (but not *scratch*) and a partial consensus is found in several vertebrate family

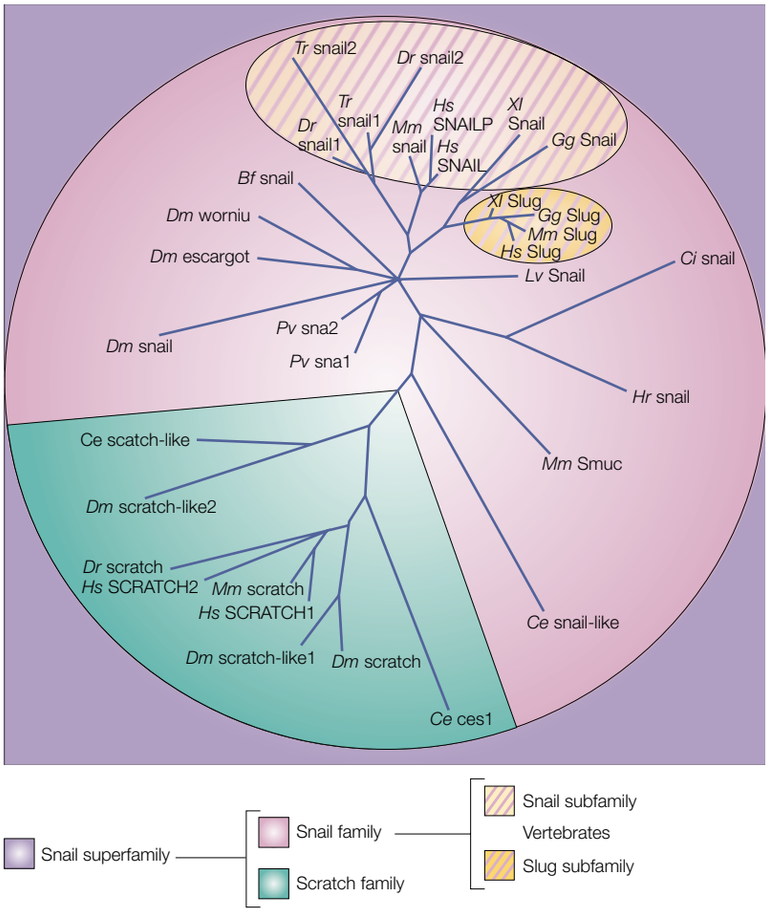


Figure 1 | **Phylogenetic tree of the Snail superfamily.** The dark purple square engulfs all the superfamily members. A light purple background groups the members of the Snail family and a green background highlights the Scratch family members. The vertebrate Snail and Slug subfamilies are shown with a light or heavy yellow hatching, respectively. The species shown represent members of the lophotrochozoans: *Pv*, *Patella vulgata* (limpet); ecdysozoans: *Ce*, *Caenorhabditis elegans* (nematode); *Dm*, *Drosophila melanogaster* (fruitfly); and deuterostomes: *Bf*, *Brachiostoma floridae* (amphioxus); *Ci*, *Ciona intestinalis* (ascidion) and *Hr*, *Holocynthia roretzi* (ascidians); *Dr*, *Danio rerio* (zebrafish); *Gg*, *Gallus gallus* (chicken), *Hs*, *Homo sapiens* (human); *Lv*, *Lytechinus variegatus* (green sea urchin); *Mm*, *Mus musculus* (mouse); *Tr*, *Takifugu rubripes* (pufferfish); and *Xl, Xenopus laevis* (African clawed toad). This is an updated version of the tree published in REF. 36.

members. Interestingly, urochordate *snail* genes, which lack a SNAG motif, have CtBP consensus sites. So, it is tempting to speculate that the repressor activity of Snail proteins has been evolutionarily conserved, but could use different mechanisms: CtBP co-repression or a SNAG domain acting alone, or both in conjunction.

A new classification for the Snail family

Recently, new family members have been found in different organisms. In particular, several new genes that have been described in *C. elegans*, *Drosophila*, fish, mouse and human[35,36] are much more similar to *Drosophila scratch*[33] and the *C. elegans* cell death gene *ces-1* (REF. 37) than to any other Snail family member (TABLE 1). This has led to the proposal that Snail is a superfamily that can be subdivided into two related but independent groups: the Snail and the Scratch families[36].

This proposal is supported by the phylogenetic relationships that are established when the sequences of the zinc-finger regions of all Snail superfamily members are compared[36]. An updated version of such a phylogenetic tree is shown in FIG. 1, in which the *Scratch* genes are closely grouped and the *Snail* genes are less tightly associated, with several branches that emanate from the base of the tree. The vertebrate *Snail* genes seem to be subdivided into two subfamilies that have already been described: Snail and Slug. The recently isolated mouse gene *Smuc*[26] occupies a very unusual position in the tree, which cannot be easily explained at present. It is either a gene that originated very early, or it is only present in the mouse and has undergone many changes.

Sequence comparisons have allowed the identification of consensus sequences for the individual fingers, both for the Snail and Scratch families as well as a combined consensus for the zinc-finger region of the whole superfamily[36] (FIG. 2). Signature domains have been identified in the non-finger region that permit members to be ascribed to the Scratch family and the Slug subfamily (FIG. 2). On the basis of this phylogenetic analysis, a model for the evolution of this superfamily that incorporates the gene duplication events that might have led to the generation of the family from ancestral genes is shown in BOX 1.

Snail in mesoderm and neural-crest formation

In *Drosophila* embryos, *snail* is initially expressed in the prospective mesoderm[38] (FIG. 3), where it acts as a repressor to inhibit the expression of neuroectodermal genes such as *rhomboid*[39] and *single-minded*[40]. So, in *Drosophila*, mesoderm specification is partly carried out by the exclusion of alternative cell fates, and *snail* is central to this process. The isolation of Snail homologues in different species has confirmed a conserved role for Snail in mesoderm specification in other insects[41], ascidians[8,42] and amphioxus[7], and mesoderm development in vertebrates (see below). However, the expression pattern in the limpet[32] and leech[43] embryos does not correlate with a role in mesoderm formation, which indicates that this function cannot be extended to LOPHOTROCHOZOANS at present.

In addition to their function in the mesoderm, vertebrate family members have also been linked with the development of the neural crest. From an evolutionary point of view, the appearance of this cell population is extremely attractive, as, together with the EPIDERMAL PLACODES, the neural crest has been crucial in the formation of the 'new head' of vertebrates[19]. These two tissues differentiate vertebrates from the rest of the chordates, and their origin correlates with the shift to active predation and the appearance of paired sense organs. Indeed, non-vertebrate chordates (ascidians[8,42] and amphioxus[7]) do not have a neural crest. However, these chordates do express *Snail* in dorsal neural cells, just at the position in which the neural crest forms in vertebrates (FIG. 3). So, non-vertebrate chordates could have the beginnings of a genetic programme for neural-crest formation, and the *Snail*-expressing cells could represent a neural-crest

LOPHOTROCHOZOAN
This group includes two important animal groups, the Lophophorata (brachiopods, flat worms and nemerteans) and the Trochozoa (molluscs and annelids).

EPIDERMAL PLACODE
An epidermal thickening in the embryonic head that differentiates into neurons, as well as into other cell types, at the sites at which the sense organs will form.

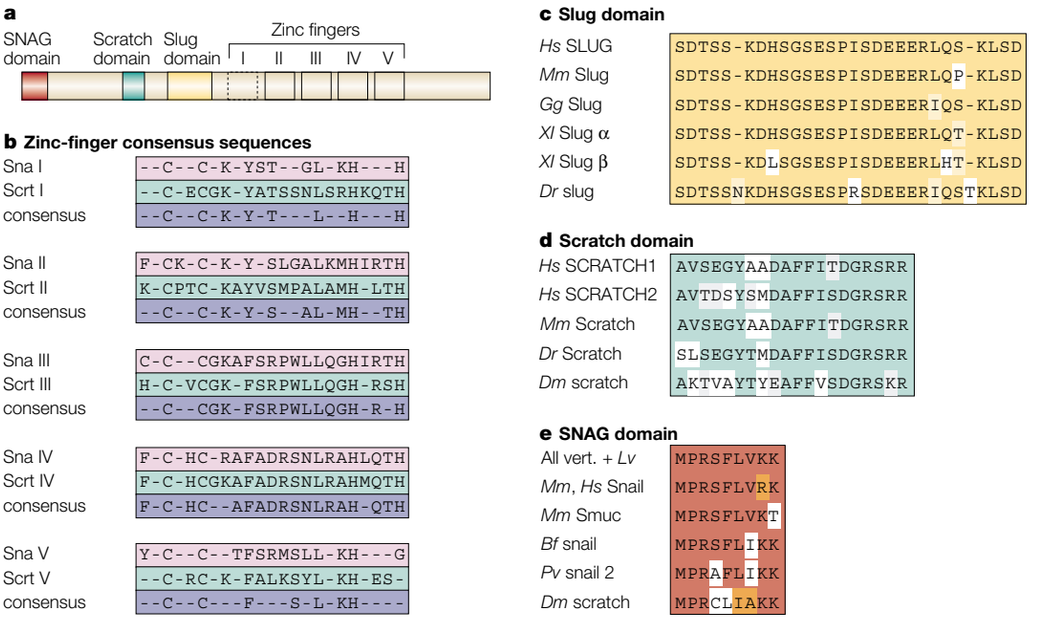


Figure 2 | **Sequence comparison of the main conserved domains and consensus sequences for the individual zinc fingers of the Snail superfamily. a** | Composite of the overall structure of Snail superfamily members, which shows the relative positions of the SNAG (Snail/Gfi) domain, the zinc fingers (I–V), and the Scratch- and Slug-specific boxes. **b** | Consensus sequences of the different zinc fingers for the whole superfamily (dark purple) and the Snail (Sna; light purple) and Scratch (Scrt; green) families. **c**, **d** | Sequence comparison of the specific domains that are present in the *Slug* or *Scratch* genes, respectively. *Dm*, *Drosophila melanogaster*; *Dr*, *Danio rerio*; *Gg*, *Gallus gallus*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus*; *Xl, Xenopus laevis*. **e** | Sequences of the SNAG domain that are present in representative members of the three big groups of bilateralians. Whereas the zinc-finger region and the SNAG domain have been shown to be fundamental for protein function, the Slug and Scratch domains represent signature domains that allow a gene to be unambiguously ascribed to the corresponding family (Scratch) or vertebrate subfamily (Slug). Abbreviations as above and *Bf*, *Brachiostoma floridae*; *Lv*, *Lytechinus variegatus*; *Pv*, *Patella vulgata*.

precursor population[44]. With respect to vertebrates and a bona fide neural crest, *Slug* (a *Snail* family member) seems to be involved in neural-crest specification in both the chick and *Xenopus* embryos[9,14,45,46].

Having been specified, both the mesoderm and the neural crest have to delaminate from the tissue in which they originate — the PRIMITIVE STREAK and the neural tube, respectively — and migrate. Their migration pathways are well defined, and this enables them to populate diverse parts of the embryo and contribute to various structures. Delamination is mediated by the triggering of EMT, and converts the epithelial cells into mesenchymal cells, which can migrate through the extracellular matrix[17,47].

The first indication that the Snail family is involved in EMT came from studies in the chick embryo. The incubation of early chick embryos with antisense oligonucleotides to *Slug* inhibited both neural crest and mesoderm delamination[6]. Defects in crest migration and the absence of specific crest derivatives have also been described in *Xenopus* embryos after *Slug* antisense treatment[13] or the expression of a dominant-negative *Slug* construct[9,14]. Moreover, *Slug* gain of function leads to an increase in neural-crest production in the chick embryo[46]. Interestingly, this increase in the migratory population was detected only in the head region. Therefore, different mechanisms operate for neural-crest delamination in the head and the trunk regions, explaining why inhibition of neural-crest delamination could occur in the spinal cord in the presence of *Slug* expression[48]. So, in the chick, *Slug* is involved in crest specification all along the anteroposterior axis of the embryo and has an additional role in crest migration in the head region. It is tempting to speculate that *Snail* genes had an ancestral role in the specification of tissues such as the mesoderm and the neural crest and that the function in emigration might have been acquired subsequently. In addition to these data on chick embryos and the studies in *Xenopus* that show the role of *Slug* in both specification and migration[9,14,45], the expression patterns of *Slug* and *Snail* in other vertebrate embryos, such as in zebrafish[5,11,49] and mouse[3,4,50], are also compatible with their role in neural-crest development.

The role of *Snail* and *Slug* in triggering EMT is not restricted to the mesoderm and neural crest. *Snail* and/or *Slug* are also observed in other cells that undergo EMT in the developing vertebrate embryo, such as during the decondensation of somites[50], formation of the parietal endoderm[51], formation of the heart cushions[52] and closure of the palate (C. Martinez and M.A.N., unpublished observations). Even in a mollusc (for example the limpet embryo), *Snail* expression in the involuting cells of the mantle tips is suggestive of a role in EMT[32]. This indicates that EMT could be one of the ancestral functions that are associated with the Snail family.

The function of *Snail* genes in mesoderm development continues after EMT. In ascidians, *snail* has been linked with the subdivision of the mesoderm in

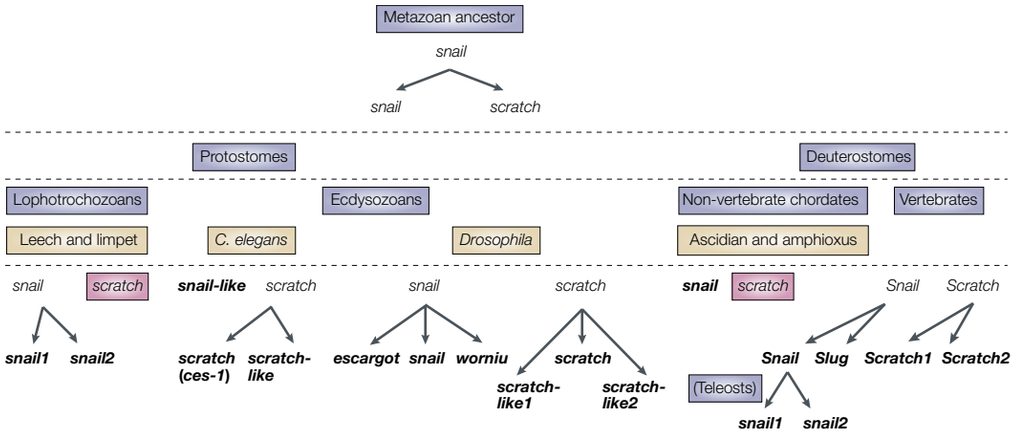
PRIMITIVE STREAK
A structure that is formed at the posterior end of amniote embryos at gastrulation stages. An area of mesoderm formation.

Box 1 | **Proposed evolutionary history of the *Snail* gene superfamily**

The duplication of a unique *snail* gene in the METAZOAN ancestor would have given rise to two genes: *snail* and *scratch*. Independent duplication events in PROTOSTOMES and DEUTEROSTOMES gave rise to a different number of family members in each group.

In *Drosophila*, intra-chromosomal duplications would give rise to three linked genes from each family. Non-vertebrate chordates seem to have retained the early metazoan situation, with only one gene from each family. This assumes the existence of a *scratch* gene that has not yet been isolated.

A whole-genome duplication event proposed to have occurred at the base of the vertebrate lineage[101], or a massive gene duplication[102], would be responsible for the presence of two genes from each family in vertebrates: *Snail* and *Slug* on the one hand, and *Scratch1* and *Scratch2* on the other hand. Again, an additional, nearly complete genome duplication[103] or massive local duplications[104] in the teleost (bony fishes) lineage would explain the existence of two very closely related snail genes (*snail1* and *snail2*) in zebrafish and pufferfish. To distinguish them from ancestral genes, present genes are shown in bold. Among the latter, the predicted genes are shown in purple.



different territories[29] — this is in agreement with a new function described for *Slug* in *Xenopus* the patterning of the dorsal mesoderm[9]. Furthermore, the expression of the fish[5,11,49], chick[50] and mouse[50] *Snail* and *Slug*, and that of the mouse *Smuc* gene[26], also indicates a role of these proteins in mesodermal patterning and differentiation.

EMT and Snail: target molecules

E-cadherin. The importance of *Snail* in triggering EMT in mammals has been confirmed using two independent approaches. First, Snail was shown to convert otherwise normal epithelial cells into mesenchymal cells through the direct repression of E-CADHERIN expression[15,16]. More importantly, *Snail* knockout animals die at gastrulation stages and show defects in EMT[10]. Mutant embryos form a mesodermal layer that expresses some mesodermal markers, but is composed of columnar cells with apical–basal polarity, microvilli and ADHERENS JUNCTIONS, which are all characteristic of epithelial cells[10]. This indicates that they have failed to undergo EMT. It is known that downregulation of E-cadherin is essential for ingression of the mesodermal cells at gastrulation in mouse embryos[53], and in the *Snail* mutant these cells retain *E-cadherin* expression. This is in agreement with *Snail* acting as a repressor of *E-cadherin* expression[15,16]. The phenotype is reminiscent of that shown by *snail* mutants in *Drosophila*, which also fail to downregulate E-cadherin during gastrulation[54].

However, the expression of *E-cadherin* in the mesoderm of the *Snail* mutants is lower than that in the ectoderm of the same embryos[10], which indicates that other cadherin repressors might act simultaneously with Snail during gastrulation. Candidates include bHLH-type transcription factors such as SIP1 (REF. 55) and E47 (REF. 28), which have recently been found to repress *E-cadherin* expression, and are also expressed in the embryonic mesoderm.

A tight regulation of cadherin expression is fundamental for the emigration of the neural-crest cells[56,57]. However, as the *Snail*-mutant mice die at gastrulation stages, it has not been possible to address the consequences of Snail loss of function in the neural crest.

Other targets. *E-cadherin* is the only direct target of *Snail* described so far. However, genetic analysis and overexpression experiments have generated a list of candidate targets for direct or indirect regulation. With regard to EMT, in addition to E-cadherin, Snail transfectants downregulate other epithelial markers, such as desmoplakin[15], the epithelial mucin Muc-1 and cytokeratin-18 (REF. 58; FIG. 4). Mesenchymal markers such as vimentin and fibronectin are upregulated and redistributed[15]. These changes cannot be secondary to the loss of E-cadherin, as transfection of E-cadherin is not enough to induce a reversion to an epithelial morphology[59]. This indicates that Snail must have additional targets that are independent of E-cadherin.

METAZOA
The animal kingdom. Includes sponges, diploblasts, protostomes and deuterostomes.

PROTOSTOME
An animal in which the mouth develops from the first opening that develops in the embryo. These include ecdysozoans and lophotrochozoans.

DEUTEROSTOME
An animal in which the anus develops from the first opening of the embryo, and the mouth is formed later. These include echinoderms and chordates.

E-CADHERIN
The main cell–cell adhesion molecule, which is central in maintaining the integrity of epithelial tissues, both in physiology and pathology.

ADHERENS JUNCTION
A cell–cell and cell–extracellular matrix adhesion complex that is composed of integrins and cadherins that are attached to cytoplasmic actin filaments.

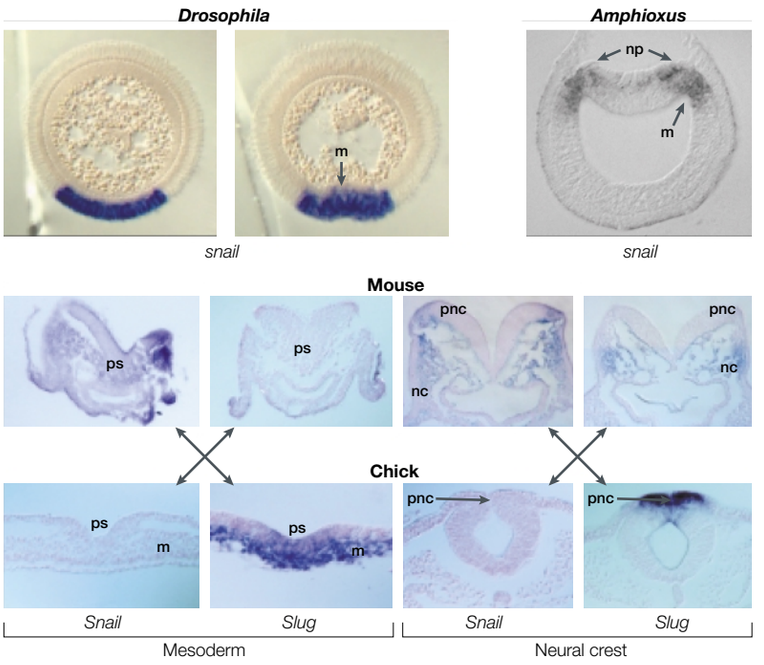


Figure 3 | **Expression of Snail family members in *Drosophila*, amphioxus, chick and mouse embryos.** In *Drosophila, snail* is expressed (blue) in the precursors of the mesoderm, and also later on, when these cells are involuting at gastrulation. In amphioxus, expression is detected in the mesoderm and at the edges of the neural plate. In vertebrates, the two family members *Snail* and *Slug* are differentially expressed in different species. Note the interchange in the patterns between chick and mouse. *Snail* in the mouse and *Slug* in the chick are expressed in the precursors of the mesoderm and the neural crest, and also in the migratory populations. m, mesoderm; nc, neural crest; np, neural plate; pnc, premigratory neural crest; ps, primitive streak. Photographs of *Drosophila* and amphioxus embryos have been kindly provided by Maria Leptin and Jim Langeland, respectively.

Also relevant to EMT is the upregulation of RhoB, which is important for neural-crest development in chick embryos[60], and is ectopically expressed in the chick neural tube after overexpression of *Slug*[46]. Regulation of this small GTPase, which is involved in actin rearrangements, links Snail and EMT with changes in cell shape and, hence, with the morphogenetic movements that occur during gastrulation and neural-crest delamination. Indeed, a Rho-mediated signalling cascade is crucial for the morphogenetic changes during *Drosophila* gastrulation, a pathway that involves the exchange factor RhoGEF2 in response to an extracellular signal called folded gastrulation (Fog)[61]. Considering that, genetically, Fog lies downstream of Snail[62], it is tempting to speculate that Rho GTPases might also be indirect targets of Snail in the gastrulating fly.

EMT and Snail: inductive signals

Different signalling pathways have been linked with the induction of *Snail* family members in the EMT (FIG. 4).

Transforming growth factor (TGF)-β1 induces EMT and *Snail* expression in hepatocytes[63]. TGF-β2 has been proposed to be a signal for EMT and *Slug* induction in heart development[52]; and signalling through other members of the TGF-β superfamily — the bone morphogenetic proteins (BMPs) — participates in induction of the neural crest[64] by upregulating *Slug*[12,64,65]. In *Xenopus* and zebrafish, the neural crest is induced at a threshold concentration of BMP signalling. Higher BMP activity gives rise to non-neural ectoderm, whereas low (or null) activity generates neural plate[66,67]. Interestingly, BMP has been proposed not only as a signal to induce *Slug*, but also as a target of it, as overexpression of *Slug* induces downregulation of BMPs[9]. Nevertheless, BMP signalling alone is not sufficient for neural-crest induction, and studies in *Xenopus*, zebrafish and mouse have indicated that members of the Wnt and fibroblast growth factor (FGF) families are also needed to generate all the different premigratory precursors[45,68–70]. In the chick embryo, FGF and BMP cooperate in the generation of the neural–non-neural boundary — the territory of neural-crest specification[71]. So, the combination of BMP, Wnt and FGF signalling is needed for neural-crest development.

Given their interactions with BMPs in neural-crest development, could the FGF and/or Wnt signalling pathways induce the expression of Snail family members? FGF induces *Slug* expression in extraembryonic epithelial cells[72] and in the rat-bladder-carcinoma cell line NBT-II (REF. 73), and upregulates *Snail* and maintains *Slug* expression during limb development in the chick embryo[74–76]. In addition, mice that have a mutation for one of the FGF receptors (FGFR1) fail to undergo EMT at gastrulation, lose *Snail* expression and show ectopic expression of E-cadherin[77] in the primitive streak. This indicates that FGFR1 signalling is needed for the maintenance of *Snail* expression in the domain of the primitive streak that is fated to become embryonic mesoderm, and promotes the downregulation of *E-cadherin*[77].

With respect to Wnt signalling, the recent isolation of *Slug* promoters in *Xenopus* has led to the characterization of a functional binding site for the transcription factor Lef-1, which regulates gene expression after activation of Wnt signalling[78]. By contrast, Kwonseop *et al.*[79] did not observe *Snail* or *Slug* upregulation after overexpression of LEF in epithelial cells, nor was *Snail* regulated by LEF in human colon carcinoma cells[80]. However, an interesting relationship emerges between the FGF and Wnt signalling pathways through the role of Snail in repressing *E-cadherin* expression. Activation of the canonical Wnt signalling pathway stabilizes β-catenin in the cytoplasm, which makes it available to bind the TCF/LEF transcription factors and together translocate to the nucleus where they regulate gene expression[81]. Conversely, high levels of E-cadherin sequester β-catenin to form adhesion complexes at the cell membrane. So, FGF signalling promotes Wnt signalling by lowering the levels of E-cadherin through the maintenance of *Snail* expression. This explains why *FGFR1*-mutant mice have attenuated Wnt signalling that can be reverted by disrupting E-cadherin function[77].

Another factor that has been shown to induce Snail is the parathyroid-hormone-related peptide, PTH(rP), which is essential for triggering the EMT that leads to formation of the PARIETAL ENDODERM from the PRIMITIVE ENDODERM and the VISCERAL ENDODERM[51]. This process occurs early in mouse development, when implantation begins.

PARIETAL ENDODERM
The extraembryonic tissue that is derived from the primitive endoderm and visceral endoderm, and is composed of motile cells that secrete high amounts of extracellular matrix.

PRIMITIVE ENDODERM
The extraembryonic tissue that gives rise to the visceral and parietal endoderm.

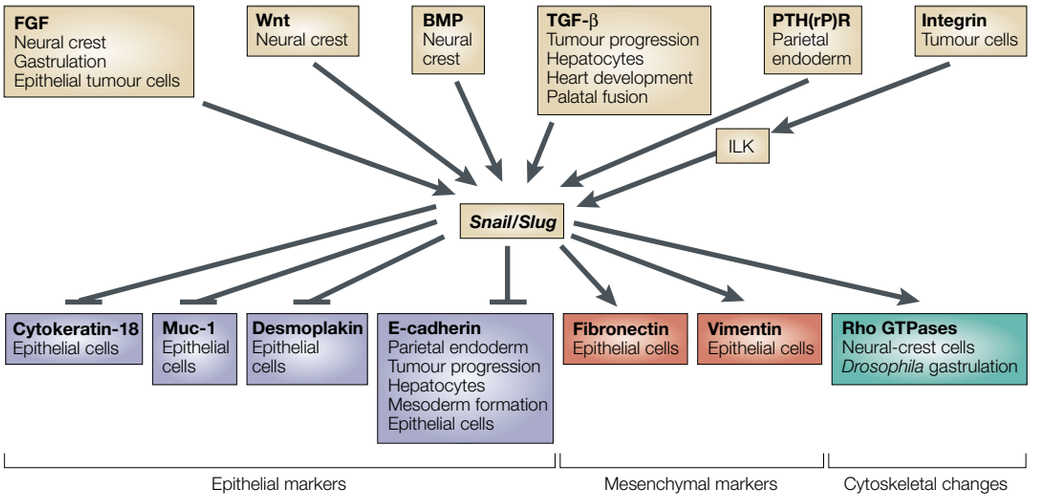


Figure 4 | **Snail genes occupy a central position in triggering EMT in physiological and pathological situations.** Different signalling molecules have been implicated in the activation of Snail genes in several processes that subsequently lead to the conversion of epithelial cells into mesenchymal cells. Although the action of Snail in the epithelial–mesenchymal transition (EMT) as a direct transcriptional regulator (repressor) has been shown only for E-cadherin, different *in vitro* and *in vivo* approaches point to a series of target genes that are directly or indirectly regulated by these transcription factors. BMP, bone morphogenetic protein; FGF, fibroblast growth factor; ILK, integrin-linked kinase; PTH(rP)R, parathyroid-hormone-related peptide receptor; TGF-β, transforming growth factor-β.

EMT processes also occur during the malignant conversion of epithelial tumours, and pathological activation of Snail participates in this process[15,16] (BOX 2). The same signalling molecules seem to operate for the induction of Snail under these pathological circumstances. Indeed, TGF-β induces EMT in epithelial cells and is necessary for acquisition of the invasive phenotype in carcinomas[82,83]. In addition, an integrin-linked kinase (ILK)-dependent pathway has also been proposed to activate *Snail* in colon carcinoma cells[80] (FIG. 4).

Different pathways converge in Snail to trigger EMT, and this places Snail in a central position in this process. Strict regulation of gene expression is therefore essential for induction of EMT and maintenance of the migratory phenotype — an indication of the cooperation that is required between different signalling cascades. An interesting model, which seems to be in keeping with the results that have been obtained in different systems, is that members of the TGF-β/BMP superfamily activate *Snail* genes, the levels of which are maintained by FGF signalling. Snail, in turn, maintains the downregulation of *E-cadherin*, and this leaves the Wnt-signalling-mediated, stabilized β-catenin available to bind TCF/LEF proteins and activate gene expression in the nucleus.

Snail and *Slug* in chick and mouse

Differences in the sites of expression of *Snail* and *Slug* between chick and mouse were the origin of some confusion. Structural homologues were thought not to be so, owing to the differences in the expression sites — indeed, this was the case for the chick Snail-related (SnR) protein[84], which is the true Snail homologue[50].

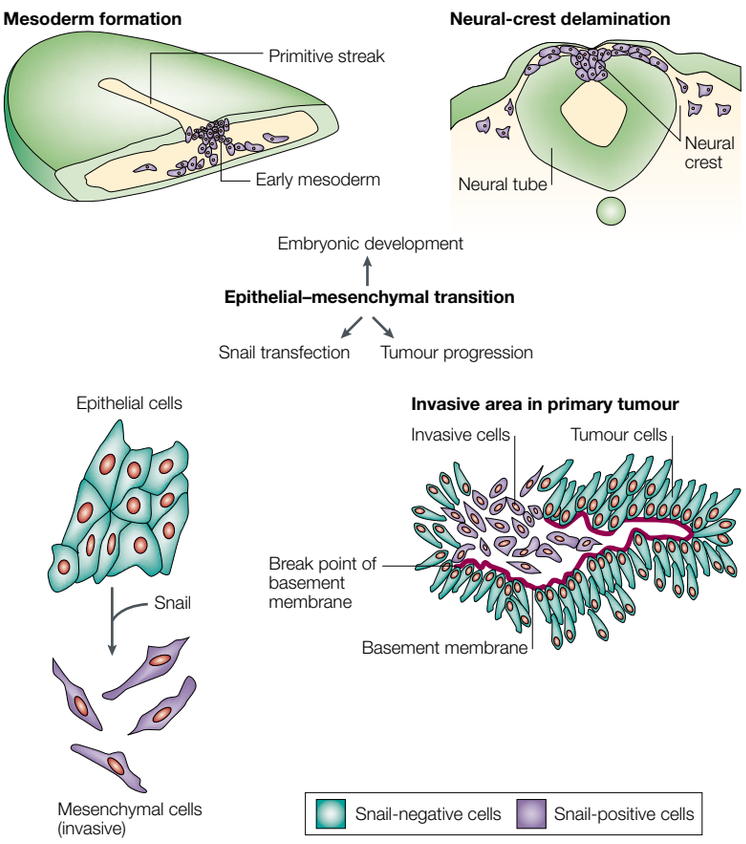
Studies of *Slug*-mutant mice showed that *Slug* is not essential for mesoderm or neural-crest formation[85]. This has been explained by the demonstration of an inversion in the expression patterns of *Slug* and *Snail* at sites of EMT[50]. In the chick embryo, *Slug* is expressed in the premigratory neural crest and the primitive streak, and *Snail* is absent from these tissues; in the mouse embryo, by contrast, *Snail* is expressed in these cells, which undergo EMT (FIG. 3). This led to the proposal that the role of *Slug* in EMT in the chick should be carried out by *Snail* in the mouse[50]. The transfection of Snail in mammalian epithelial cells[15,16], and the phenotype of the *Snail*-mutant mice[10] discussed previously, confirmed this prediction. In other vertebrates, the situation seems more similar to that in the mouse. Indeed, *snail2* is expressed in the premigratory neural crest in zebrafish[11], and although both *Snail* and *Slug* are expressed in the premigratory population in *Xenopus*, *Snail* is the first family member to be transcribed[86]. Experiments that are related to neural-crest development in the frog have been carried out only for *Slug*, so it will be interesting to analyse the effects of perturbing *Snail* function.

The mechanism that is responsible for the observed interchange is unknown. However, the inversion in expression sites between chicks and mice is not complete (some sites do not show this change), which indicates that swapping of regulatory modules, differential loss of tissue-specific *cis*-regulatory elements or differential availability of upstream regulators could occur.

Regardless of the mechanism, if *Slug* induces EMT in the chick and *Snail* is responsible in the mouse, are they functionally equivalent when ectopically expressed at the appropriate sites? It would be interesting to determine whether *Slug* can rescue the gastrulation phenotype of the mouse *Snail* mutant. However, there is some

VISCERAL ENDODERM
The extraembryonic cell layer that is involved in nutrient uptake and transport.

Box 2 | **The epithelial–mesenchymal transition in tumour progression**



The epithelial–mesenchymal transition (EMT) occurs not only during normal embryonic development, but also in pathological situations such as acquisition of the invasive phenotype in epithelial tumours, in which it constitutes the first step for the formation of metastasis. This pathological EMT has been associated with the downregulation of *E-cadherin* expression and the acquisition of migratory properties. Indeed, the loss of E-cadherin expression is crucial for the progression from adenoma to carcinoma[105].

The idea that pathological activation of *Snail* genes could be involved in tumour progression was proposed several years ago[6], and has been shown recently. Snail is a strong direct repressor of *E-cadherin* expression, and Snail transfection confers tumorigenic, invasive and migratory properties to otherwise normal epithelial cells[15,16]. An inverse correlation has been found between *Snail* and *E-cadherin* expression in mouse and human cell lines[15,16,106,107]. Furthermore, Snail is activated *in vivo* at the invasive front of chemically induced mouse skin tumours[15], and it is present in human breast carcinomas[108,109], in which it inversely correlates with the degree of differentiation and is associated with lymph-node metastasis[109]. As such, *Snail* can be now considered a marker of malignancy; this paves the way for the design of anti-invasive therapies and makes the search for endogenous or artificial regulators of exceptional interest.

functional equivalence, at least during embryonic development, both within and between species. Ectopic expression of chick and mouse *Snail* in the chick hindbrain induces an increase in neural-crest production, in a similar way to that of the endogenous gene, *Slug*[46]. But it is not clear whether this functional equivalence also occurs during tumour progression, as *Slug* is expressed in different carcinoma-derived cell lines regardless of their phenotype in terms of INVASIVENESS[15].

Snail superfamily and cell survival

Several lines of evidence point to a role for Snail superfamily members in regulating cell death or survival. In a particular population of *C. elegans* neurons, the protein involved in cell death, CES-2, represses CES-1 (scratch) function. This allows the cell-death activator EGL-1 to repress the survival gene *ced-9*, and allows the action of the cell-death proteins CED-4 and CED-3 (REF. 37; FIG. 5).

In some human leukaemias, a chromosomal translocation swapped the repression domain of HLF (hepatic leukaemic factor; a putative CES-2 homologue) for the E2A-positive transactivation domain. This leads to activation of a different family member, Slug, which in turn represses a partial homologue of EGL-1 (BH3) and renders the anti-apoptotic BCL-X_L protein active to promote survival, leading to leukaemia[25] (FIG. 5). So, both Scratch and Slug seem to function as anti-apoptotic agents, in agreement with data on the regulation of *Slug* during limb development in the chick[75,76]. Here, *Slug* is downregulated in the areas that are destined to die, and is proposed to act as a survival factor that maintains the undifferentiated mesenchymal phenotype.

Cell division and endoreduplication

During *Drosophila* gastrulation, the changes in cell shape that are associated with formation of the ventral furrow are accompanied by inhibition of mitosis. This links morphogenesis with cell division. This inhibition is mediated by Tribbles, a serine/threonine kinase that counteracts String, the homologue of the CDC25 phosphatase that is necessary for mitosis[87,88]. This inhibition

INVASIVENESS
The ability to degrade and migrate through the extracellular matrix.

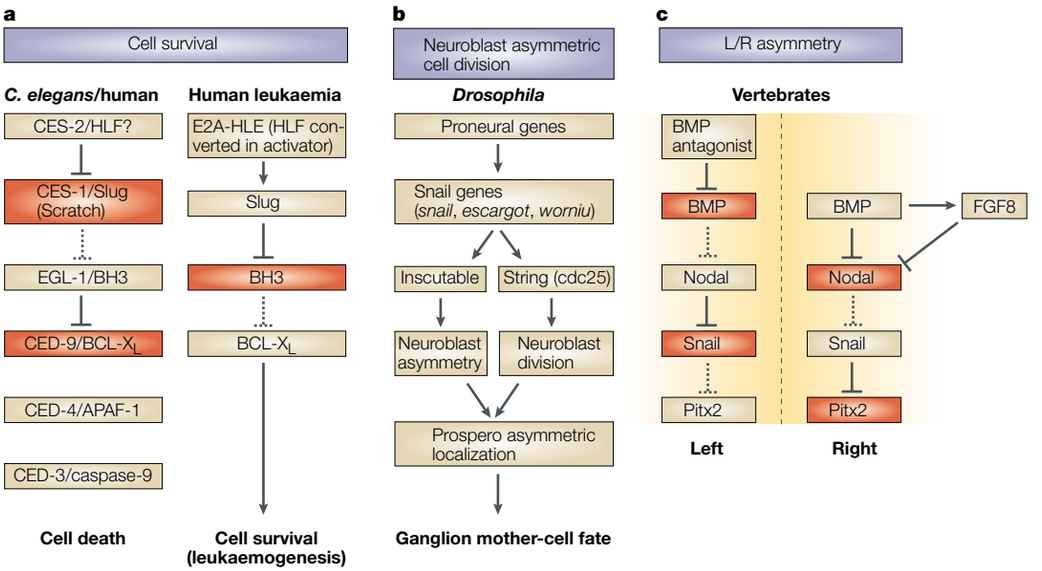


Figure 5 | **Different genetic pathways involving Snail function.** In addition to triggering the epithelial–mesenchymal transition, Snail function has been described in several genetic pathways that lead to **a** | cell death or survival, **b** | asymmetric cell division and **c** | left–right (L/R) asymmetry. In all cases, arrows indicate the flow of the pathway, not direct transcriptional repression or activation. Although function as transcriptional activators cannot be fully excluded, Snail proteins have been described as transcriptional repressors in all the species analysed so far. To follow the sequence of active proteins in the corresponding pathway, genes that are repressed or inactive are shown in red, and the inactive regulatory steps are shown as dotted lines. HLF, hepatic leukaemic factor; BMP, bone morphogenetic protein; FGF, fibroblast growth factor.

depends on Snail function[88], which, therefore, might act as a mitotic inhibitor. This is in agreement with the low proliferation rate that is observed in *Snail*-transfected epithelial cells compared with control cells (S. Vega and M.A.N., unpublished observations). It seems reasonable that cells that undergo massive cytoskeletal reorganization associated with changes in cell shape or active migration are prevented from undergoing cell division.

Other members of the Snail family — including mouse Snail itself — have been associated with mitosis in two processes. Indeed, Escargot and mouse Snail are involved in the control of polyploidy in several tissues, including IMAGINAL DISCS cells in *Drosophila*[24,89], mouse TROPHOBLAST cells[27] and human megakaryocytes[90]. Both proteins inhibit ENDOREDUPLICATION, and therefore induce progression of the cell cycle to mitosis. The molecular mechanism could be related to the activation of String, as this protein is involved in the control of mitosis coupled to the process of ASYMMETRIC CELL DIVISION in *Drosophila*[91] (FIG. 5). Certainly, a deficiency in the three Snail-family members (*snail*, *escargot* and *worniu*) leads to an inappropriate activation of Inscutable, which controls the subcellular localization of Prospero, a key protein in determining the GANGLION MOTHER CELL fate[91,92]. So, depending on the cellular process, at least in *Drosophila*, Snail seems to act as an inhibitor or an activator of String. Snail transcription factors have been shown to act as repressors[9,14–16,24,26,27,29,30], which indicates that Snail-mediated activation could be the result of an indirect regulation. However, the possibility that they act as activators cannot be excluded at the moment[18].

Snail in left–right asymmetry

A striking asymmetric and transient Snail expression in the right-hand lateral mesoderm of the chick embryo led Cooke and colleagues[84] to investigate a possible role for this gene in the establishment of LEFT–RIGHT ASYMMETRY. Incubation of early chick embryos with antisense oligonucleotides to Snail led to a randomization of the HEART SITUS[84]. Further experiments[93,94] established that *Snail* lies in the genetic cascade that gives rise to bilateral body asymmetries. Snail is downstream of the signal that is generated by the TGF-β superfamily member Nodal, and upstream of the transcription factor Pitx-2 — a bicoid-type homeobox protein that is responsible for activating the left-side-specific differentiation programme (FIG. 5). Inhibition of the BMP signal that inactivates Nodal on the left side of the embryo leads to the repression of Snail, which in turn cannot repress Pitx-2. The left–right asymmetric expression of *Snail* is also observed in the mouse embryo, which constitutes one of the few sites of mesodermal expression that have not been interchanged between chick and mouse at these early stages[50]. The transient nature of this asymmetric expression, particularly in the mouse, might explain why it has not been detected in *Drosophila* or other vertebrates. It would be interesting to re-analyse other species, as a left–right asymmetric expression has also been found in the limpet *Patella vulgata* for one of the two *Snail* genes isolated, *sna2* (REF. 32). This conservation indicates that this might be an ancient function that is associated with the Snail family.

CDC25
A family of protein phosphatases that dephosphorylate cyclin-dependent kinases during cell-cycle progression.

IMAGINAL DISCS
The primordia of different adult structures that are present in the larvae of insects with complete metamorphosis.

TROPHOBLAST
The extraembryonic epithelial tissue that is crucial for formation of the placenta.

ENDOREDUPLICATION
The process by which the cells pass to rounds of DNA duplication in the absence of a mitotic division.

ASYMMETRIC CELL DIVISION
A process by which a cell gives rise to two different descendants after division.

GANGLION MOTHER CELL
One of the daughters of a *Drosophila* neuroblast after asymmetric cell division. It divides once more to give rise to two post-mitotic neurons.

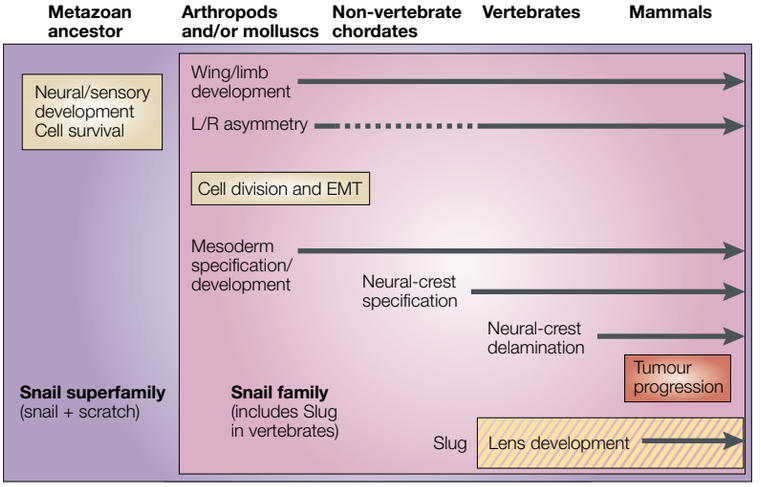
LEFT–RIGHT ASYMMETRY
The differences along the left–right axis of the body.

HEART SITUS
The position of the heart with respect to the left–right axis of the body.

Box 3 | **Proposed ancestral and derived functions of the Snail superfamily**

Phylogenetic and expression studies together with functional analyses in different model organisms allow ancestral and acquired functions to be proposed for the different Snail superfamily groups in metazoan evolution. An ancestral function in the development of sensory and/or neuronal structures is proposed for the whole superfamily[36], which includes both the *Snail* and *Scratch* genes. An additional ancestral function in the control of cell death/survival is also proposed[25,37,75,76].

The role in epithelial–mesenchymal transitions (EMTs) seems to be exclusively associated with the members of the Snail family, with representatives analysed in Lophotrochozoans, ECDYSOZOANS and deuterostomes. This role in EMT has been co-opted for cell migration during mesoderm and neural-crest formation and tumour progression, when these processes emerged[36]. In vertebrates, Snail and/or Slug proteins participate in this process depending on the species. Further roles in the development of appendages[74–76,99] and cell division[24,27,88–92] are associated with particular members of the Snail family in different groups. Finally, a still-uncharacterized role in lens development[50] has been specifically proposed for Slug subfamily members, which seem to participate neither in cell division nor in the definition of left–right (L/R) asymmetry. None of the known functions has been specifically associated with the Scratch family or the vertebrate Snail subfamily.



The Snail superfamily in neural development

Although the four *Drosophila* genes that have been analysed so far — *snail*, *escargot*, *worniu* and *scratch* — are prominently expressed in the nervous system, individual mutants do not show a strong neural phenotype. However, double mutants of *scratch* and the HLH protein *deadpan* show loss of neurons[33]. Similarly, deletion of *snail*, *escargot* and *worniu* leads to the loss of central nervous system determinants[95]. The identification of two additional *scratch*-related genes in *Drosophila*[36] indicates that the three *scratch* genes could collaborate, as the Snail members do, and a strong neural phenotype might be expected for the triple mutant.

Interestingly, the *C. elegans* scratch homologue *ces-1* (REF. 37) is essential for the formation of neurons, and the mouse *Scratch* gene is neural specific and induces neuronal differentiation in P19 embryonal carcinoma cells[96]. In addition, a *Scratch* homologue is specifically expressed in the primary neurons of the zebrafish embryo (M. J. Blanco and M.A.N., unpublished observations), which indicates a neuronal-specific function for both the invertebrate and vertebrate Scratch family. However, this function is not unique for this family, as vertebrate *Snail* and *Slug* are expressed in the nervous system at later developmental stages (F. Marin and M.A.N., unpublished observations), indicating that, probably, neuronal differentiation might be a function that is associated with both the Snail and Scratch families (BOX 3).

Cooperativity and antagonism

What is the relationship between different members of the Snail superfamily when they act in the same biological process or on similar targets? Interestingly, there are examples of both cooperativity and antagonism.

With respect to cell differentiation, chick *Slug* and mouse *Snail* and *Slug* have been proposed to maintain the mesenchymal phenotype and repress differentiation[15,75,76]. Similarly, *Drosophila snail* and *escargot* also maintain the undifferentiated phenotype — they antagonize neurogenesis by competing with bHLH proteins[97]. So, they seem to antagonize the role of *scratch* in promoting neural differentiation in *Drosophila*[33], *C. elegans*[37] and mouse[96].

Chick and human *Slug* are associated with cell survival[25,75,76], whereas chick *Snail* has been associated with the apoptotic programme in the developing limb[74]. With regard to target genes, *Snail* represses *E-cadherin* expression during mesoderm formation in *Drosophila*[54] and mammals[15,16]; by contrast, *escargot* activates *cadherin* expression during tracheal development in the fly.

In some cases, different family members cooperate, such as the three *Drosophila snail* genes in neurogenesis[95] and asymmetric cell division[91,92]. Moreover, *snail* and *escargot* cooperate in wing development[99] in the fly, and the vertebrate *Snail* and *Slug* genes might also cooperate in triggering EMT and maintaining the mesenchymal phenotype during neural-crest development[13–15].

Finally, a striking example is the regulation of String by *Drosophila snail*, which seems to activate it during asymmetric cell division[91] and inhibit it during gastrulation[88].

Perspectives

Although we now have invaluable information on the different processes in which the Snail superfamily proteins are involved — both during development and in some pathological situations — we are a long way from fully understanding their functions and mutual relationships. Further work will take advantage of the completed genomes and of the new imaging approaches that allow cell movements to be followed in the living embryo.

As *Snail*-mutant mice die at gastrulation, spatiotemporal, conditional *Snail*-mutant mice are needed to study the participation of Snail in later processes such as formation of the neural crest or differentiation of tissues and organs, including the mesoderm. In terms of the role of Snail in the appearance of the neural crest during evolution, experiments that are similar to those carried out for the Hox genes — in which regulatory sequences from non-vertebrate chordates are introduced in transgenic mice[100] — will help to challenge the genetic programme that is already present in the proposed precursor

ECDYSOZOANS
One of the important groups within the animal kingdom, it includes arthropods and nematodes.

population[44]. Obviously, characterization of the regulatory sequences that drive specific spatio-temporal expression of the different members in different tissues and species is a long-term goal that has to be approached systematically.

From a more biochemical point of view, we have little information on the mechanism that is used by Snail for transcriptional regulation. We do not know whether *Snail* genes can act as activators, and have little information on the proteins that induce or repress their expression, the targets they regulate or the nature of the transcription complex. Competition with bHLH transcription factors for binding to E boxes will depend on relative affinities that might need the participation of different co-regulators, and cooperation with bHLH proteins or other unidentified partners could provide additional degrees of complexity for the patterning and differentiation of specific cell types.

The description of Scratch as a new family offers unexplored territory for the study of new functions in the different species. And finally, the implication of the Snail family in pathology challenges the use of amenable systems to identify specific repressors that can be used to develop new therapeutic strategies.

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