Snail genes at the crossroads of symmetric and asymmetric processes in the developing mesoderm

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ABSTRACT

Retinoic acid signalling ensures that vertebrate mesoderm segmentation is bilaterally synchronized, correcting transient interferences from asymmetric left-right (L-R) signals involved in organ lateralization. *Snail* genes participate in both these processes and while they are expressed symmetrically in the presomitic mesoderm, *Snail1* transcripts are asymmetrically distributed in the L-R lateral mesoderm. We show that the alteration of the symmetric *Snail* expression in the presomitic mesoderm induces asynchronous somite formation. Furthermore, in the absence of retinoic acid signalling the normal asymmetric *Snail1* expression in the lateral mesoderm is also extended to the presomitic mesoderm, desynchronizing somitogenesis. Thus, Snail1 appears to be the first cue corrected by retinoic acid in the presomitic mesoderm to ensure synchronized bilateral segmentation.

INTRODUCTION

The bilateral symmetry in the body-plan of vertebrate embryos is readily apparent in the somites, aligned in rows on either side of the neural tube. The periodic segmentation of the presomitic mesoderm (PSM) generates each pair of epithelial somites. The periodicity of this segmentation is reflected in regular pulses in the expression of components of the Notch and Wnt signalling pathways (Pourquie, 2003). These cycles of expression are symmetric in the left and right PSM, although how they are bilaterally synchronized remains unclear. In the absence of retinoic acid (RA), transient asymmetry is observed in vertebrate somite formation (Vermot *et al*, 2005; Vermot & Pourquié, 2005; Kawakami *et al*, 2005). Thus, during a short temporal window, the “interference

period", symmetric somitogenesis is protected from left-right (L-R) asymmetric patterning cues by RA. However, the cues that RA buffers have not been yet identified.

Members of the Snail superfamily of transcription factors are expressed in distinct mesodermal territories where they fulfil different roles (Nieto *et al*, 1994; Sefton *et al*, 1998; Carver *et al*, 2001). Due to a high degree of modularity and reshuffling displayed between *Snail* family members during vertebrate evolution (Sefton *et al*, 1998; Locascio *et al*, 2002), murine *Snail* and chicken *Slug* are the members expressed in the PSM (renamed *Snail1* and *Snail2*, respectively: Barrallo-Gimeno & Nieto, 2005). They seem to be functionally equivalent (Del Barrio & Nieto, 2002) and the participation of one or the other in a particular process is driven by tissue-specific enhancers in each species (Locascio *et al*, 2002). In addition to their symmetrical expression in mesoderm territories, the right hand lateral plate mesoderm (LPM) transiently expresses higher levels of *Snail1* than the left hand side both in chick and mouse embryos (Sefton *et al*, 1998), reflecting its role in generating L-R asymmetry (Isaac *et al*, 1997).

Here we show that the temporal window of L-R asymmetric expression of *Snail1* in the LPM coincides with the "interference period". *Snail* genes are expressed cyclically in the PSM where they integrate the Notch, Wnt and FGF signalling pathways and control somite epithelialization (Dale *et al*, 2006). We show here that their equivalent L-R levels in the PSM are necessary to maintain synchronic somitogenesis. Our data also demonstrate that RA blocks the asymmetric expression of *Snail1* in the PSM, preventing desynchronization and helping to discriminate between the territories in which Snail fulfils different roles.

RESULTS AND DISCUSSION

Asymmetric Snail1 expression coincides with the "interference period"

Snail genes encode pleiotropic proteins that fulfil different functions during embryonic development and indeed, they are simultaneously expressed in different mesodermal territories (Sefton *et al*, 1998). The expression of these genes in the PSM is cyclical, almost synchronous with genes of the Notch pathway and out of phase with *Axin2*, a cycling gene from the Wnt pathway (Dale *et al*, 2006). In addition, *Snail1* is transiently expressed asymmetrically in the LPM of chick and mouse embryos where it influences organ lateralization (Isaac *et al*, 1997; Sefton *et al*, 1998). The bilateral synchrony of somitogenesis is protected from the influence of organ lateralization during a short developmental window, the "interference period". We show that the transient L-R asymmetric expression of *Snail1* in the LPM occurred at the 4 to 11 somite stage (HH8-HH10+) in chicken and mouse embryos (Fig. 1A-D and data not shown), coinciding with the period in which RA offers protection from asymmetric signals.

In both species, the territories expressing *Snail1* are complementary to those with RA activity (Hochgreb *et al*, 2003; Vermot *et al*, 2005). Indeed, *Snail1* is expressed asymmetrically in regions devoid of RA activity (LPM) where it is required for organ lateralization. Conversely, *Snail* genes expression is bilaterally symmetric in the anterior PSM where RA signalling is active. The inverse correlation between the sites of *Snail1* expression and RA signalling suggests that RA may regulate *Snail1* expression.

RA prevents asymmetric *Snail1* expression in the PSM

To determine whether RA signalling regulates asymmetric *Snail1* expression, chicken embryos were exposed to RA or disulphiram (DSM; a Raldh2 inhibitor) at stages when organ lateralization cues emanate from the node (Raya & Izpisua-Belmonte, 2004). When analyzed just before the interference period (4 somite stage; HH8), the asymmetric L-R expression of *Snail1* was lost in nearly 70% of the embryos treated with RA (18/26: Fig. 1E to 1H) indicating that RA signalling regulates *Snail1* asymmetric expression. However, this asymmetry in *Snail1* transcription was maintained in the presence of DSM (Fig. 1I, J) and as previously described, no alterations in bilateral synchronization were observed at this stage (Vermot & Pouquié, 2005).

Interestingly, when the embryos were analyzed at the period of maximum interference (HH10), RA continued abolishing asymmetric L-R *Snail1* expression in the LPM (4/7; Fig. 1K-N; Q, R) without affecting the PSM (Fig. 1U). In contrast, downregulation of RA signalling by DSM provoked the appearance of asymmetric L-R *Snail1* expression in the anterior PSM (3/7, Fig. 1O, P, S) without affecting its expression in the LPM. Thus, RA administration exerted a strong influence in the LPM, a tissue devoid of endogenous RA signalling (Fig. 1M, N, R), and DSM had a clear impact on the anterior PSM, a site of endogenous RA activity (Fig. 1O, P, S). In DSM treated embryos, both *Snail2* and *Lfng* (a cycling gene from the Notch pathway: McGrew *et al*, 1998) continued to cycle although their expression was asymmetric (Fig. 1V-X). Indeed, diminished RA activity led to asymmetric somitogenesis as previously described (Fig. 1X: Vermot & Pourquie, 2005). Thus, our data show that RA signalling

regulates *Snail1* expression and that when signalling is blocked, *Snail1* is expressed asymmetrically in the PSM and asynchronous somitogenesis occurs.

Unilateral Snail1 expression in the PSM delays somitogenesis

We next investigated whether asymmetric *Snail* expression in the L-R presomitic mesoderm was sufficient to induce asynchronous somitogenesis. We misexpressed *Snail1* in one side of the chicken PSM through *in ovo* electroporation and, as when RA signalling was abolished in chick embryos (Vermot & Pourquié, 2005), increased expression of Snail1 on one side desynchronized somite formation (n=15; Fig. 2A-H). *Snail2* continued cycling (not shown) and somitogenesis was delayed on the side with increased *Snail1* expression in two thirds of the embryos as assessed by morphology and *Uncx4.1* expression (10/15; Fig. 2E-H) while somite formation progressed synchronously in embryos electroporated with control constructs (n=8; Fig. 2A-D). These data indicate that bilateral asymmetric expression of Snail1 is sufficient to induce desynchronization and thus, it should be avoided in the chick PSM to maintain bilateral synchrony.

In the mouse, *Snail1* is also asymmetrically expressed in the LPM during the "interference period" (Sefton *et al*, 1998) but unlike in the chick, it is the family member that synchronically cycles in the L-R PSM (Dale *et al*, 2006). Thus, in the mouse, the expression of the *Snail* genes in the LPM and PSM is associated to *Snail1*. The asynchronous somitogenesis observed in the absence of RA signalling in the mouse (Vermot *et al*, 2005) would suggest that the requirement for bilateral symmetrical *Snail1* expression is evolutionary conserved. However, since this remains to be

demonstrated, we generated a transgenic mouse carrying a tamoxifen-inducible form of Snail1 to study this (Snail1 transgenic hereafter; see Methods and Supplementary Figure). We cultured bisected caudal regions of 10.5 dpc Snail1 transgenic embryos (n=70: Fig. 2I-N). When the two halves of these embryos were cultured in medium alone (n=21), we observed asymmetric *Lfng* expression in less than one third of the embryos (6/21) (Fig. 2I, J, O) and there were no discrepancies in somite number in any of them. In contrast, when one half was cultured in the presence of tamoxifen and the other half was maintained in control medium (n=49), somitogenesis was delayed in the tamoxifen-treated half (35/49; 71%; Fig. 2O) and there was one somite less compared to the control half (Fig. 2K-N). Out of the 49 embryo tails in which Snail1 was specifically activated in one half, 25 were right halves (brown in Fig. 2O) and 24 were left halves (pale blue in Fig. 2O). Snail1 overexpression in either left or right sides produced similar results (Fig. 2O). These data indicate that this effect was not lateralized and confirm that as in the chick, increasing the levels of Snail1 on one side of the PSM causes a delay in somite formation.

Equivalent L-R *Snail* expression required for symmetric somitogenesis

As previously discussed and in contrast to the mouse, *Snail2* is the Snail family member expressed in the chick PSM. Since they are though to be functionally equivalent when expressed in similar territories, we checked whether increasing the levels of Snail2 in one side of the PSM would induce the same effects than Snail1. When control vectors were electroporated in one side of the embryonic PSM (n=10; Fig. 3A, B) or when similar levels of Snail2 were misexpressed on both sides of the PSM (n=18; not shown) there was no clear effect on synchronization. In contrast, unequal misexpression of

Snail2 in the left or right somitic mesoderm disrupted somite alignment (17/34), with 66% of these embryos developing fewer somites on the side with higher levels of Snail2 (11/17: Fig. 3F, G). We analyzed these asymmetries by defining the expression of *Lfng* and *Hairy2* (another cycling gene from the Notch pathway: Jouve *et al,* 2000) in embryos overexpressing Snail2 or a dominant-negative Snail2 construct lacking the zinc-fingers (Δ*Zf-Snail2*: Aybar *et al,* 2003). Expression of the cycling genes was always bilaterally symmetric in control embryos electroporated (10/10 for *Lfng* and 7/7 for *Hairy2*: Fig. 3C, D). In contrast, this symmetry was disrupted by unequal L-R expression in the PSM of either *Snail2* (72%, 18/25 for *Lfng* and 64%, 7/11 for Hairy2; Fig. 3H, I) or its dominant-negative form (61%, 14/23 for *Lfng* and 47%, 8/17 for *Hairy2*; Fig. 3M, N). Like Snail1 overexpression in the mouse, delayed expression occurred on the side exhibiting higher *Snail2* expression (83%, 15/18 for *Lfng* and 86%, 6/7 for *Hairy2*). The delay in segmentation was also similar to that obtained after increasing Snail1 expression (Fig. 2A-H), again reflecting that the two Snail proteins are functionally equivalent when expressed in similar territories (Del Barrio & Nieto, 2002; Bolos *et al,* 2003). Interestingly, somitogenesis was more advanced on the side of the PSM with higher level of *ΔZf-Snail2* (81%, 18/22), and an extra somite developed in one third of these embryos (Fig. 3K-N). Thus, the phenotype induced by expressing a Snail2 dominant-negative form was complementary to that found after Snail2 overexpression.

The *EphA4* receptor is a marker of somite epithelialization and boundary formation (Barrios *et al,* 2003) and its symmetric expression (10/11; Fig. 3E) was also disrupted by these constructs. One stripe was lost on the side with higher *Snail2* expression (7/13; Fig. 3J) while an extra stripe appeared on the side with higher levels

of *ΔZf-Snail2* (3/9; Fig. 3O). Our data in chick and mouse confirm that *Snail* genes regulate somite boundary formation and that an excess in Snail activity delays epithelialization in agreement with recent data showing that the downregulation of *Snail* genes in the anterior PSM determines the time of epithelialization (Dale *et al*, 2006) and with the role of Snail in maintaining the mesenchymal phenotype of undifferentiated cells (reviewed in Nieto, 2002).

Significantly, we observed the same phenotypes in embryos electroporated at different developmental times (up to the 30 somite stage; HH16). Thus, regardless of the role of the cyclical expression of *Snail* genes in the posterior mesoderm, and in epithelialization, equivalent L-R levels of *Snail* expression in the PSM are necessary to maintain synchronous bilateral segmentation all throughout somitogenesis in both chick and mouse. This reflects the requirement that the L-R asymmetric Snail1 expression is excluded from the anterior PSM and also explains the temporal coincidence of the "interference period" with the asymmetric L/R *Snail1* expression in the embryo. Before this period, the inhibition of RA signalling does not have any effect on somite synchronization nor Snail1 expression. At the period of maximal interference, when RA signalling is inhibited, asymmetric *Snail1* expression develops in the anterior PSM and induces desynchronization. In conclusion, endogenous RA activity in the anterior PSM acts as a barrier that prevents the entry of the asymmetric Snail1 expression directed by the L-R patterning signals in the region where somite boundaries form, thereby ensuring bilateral symmetry during somitogenesis (Fig. 4).

The Snail gene family is a good example of pleiotropic genes that may cause interferences when the different developmental processes in which they are involved occur simultaneously in adjacent or overlapping regions. In these circumstances, correction mechanisms are required when the processes conflict, such as bilaterally symmetric somitogenesis and asymmetric organ lateralization.

METHODS

Embryo dissection and in situ hybridization. Both chicken and mouse embryo staging, and the method for caudal bilateral dissections and explant culture are described in Morales *et al*, 2002). Whole-mount in situ hybridization was performed as described in Sefton et al (1998) using the chick *Snail1*, *Snail2*, *Lfng* and *Lfng* intronic, *Hairy2*, *EphA4* and *Uncx4.1* and the mouse *Snail1*, *Lfng* and *Uncx4.1* riboprobes (Sefton *et al*, 1998; Morales *et al*, 2002; Jouve *al*, 2000; Irving *al*, 1996; Uncx4.1, BBSRC chicken clone ChEST47F8). In some cases, 40µm vibratome sections were used from gelatin-embedded embryos.

Plasmids. The EGFP expression vector (pCX-dEGFP) contains a destabilized EGFP construct with a half-life of ca. 1 hour (d1EGFP, Clontech) in the pCAAGS expression vector (Niwa *et al*, 1991). The full length chicken *Snail1* and *Snail2* coding sequence (Sefton *et al*, 1998) and a truncated *Snail2* construct (a.a. 1 to 134) were cloned into the pCAAGS expression vector (pCX-*Snail1/2* and pCX-ΔZf-*Snail2*, respectively). These pCX plasmids were electroporated at concentrations of 3µg/µl with 1µg/µl of pCX-EGFP. The empty expression vector and pCX-dEGFP was electroporated into control embryos. The fusion protein between Snail1 and a modified human estrogen receptor (pCMVSnail1-ERT2) was generated by cloning the mouse Snail1 coding region into the pCre-ERT2 expression vector (Feil *et al*, 1997: a generous gift from by Dr. Pierre Chambon) before transferring it into pcDNA3.

In ovo electroporation and chicken embryo culture. Stage HH5 embryos were electroporated as described previously (Dubrulle et al. 2001). A train of electric pulses (6 pulses, 30 volts, 50 msec) was applied using a square wave electroporator (Intracel TSS20). Embryos were left for 20 to 40 hours (mostly 30 hours) and assayed for dEGFP expression. Embryos with a normal overall morphology and good levels of EGFP expression in the PSM were processed for *in situ*

hybridization. Chicken embryos were explanted at stage HH4 and cultured as described in Chapman et al (2001). Where appropriate, embryos were exposed to 100µl of retinoic acid (RA, 100µM) and disulphiram (DSM, 800µM) in PBS, and the treated embryos were processed for *in situ* hybridization.

Transgenic mice, PSM culture and tamoxifen induction. A transgenic mouse for pCMV-Snail1-ERT2 was generated (Hogan et al. 1994) whereby the constitutively expressed protein is only functional when translocated into the nucleus upon tamoxifen administration. (See Supplementary Figure). Bisected 10.5 dpc caudal halves (PSM plus 3 somites) were cultured for 15 hours as described in Morales et al (2002), with 50 µg/ml of gentamycin (Gibco) in the culture medium. In culture, 6 to 7 new somites were formed and where appropriate, the cultured halves were exposed to 600 nM 4-OHTamoxifen (Sigma; Feil et al. 1997).

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Figure Legends

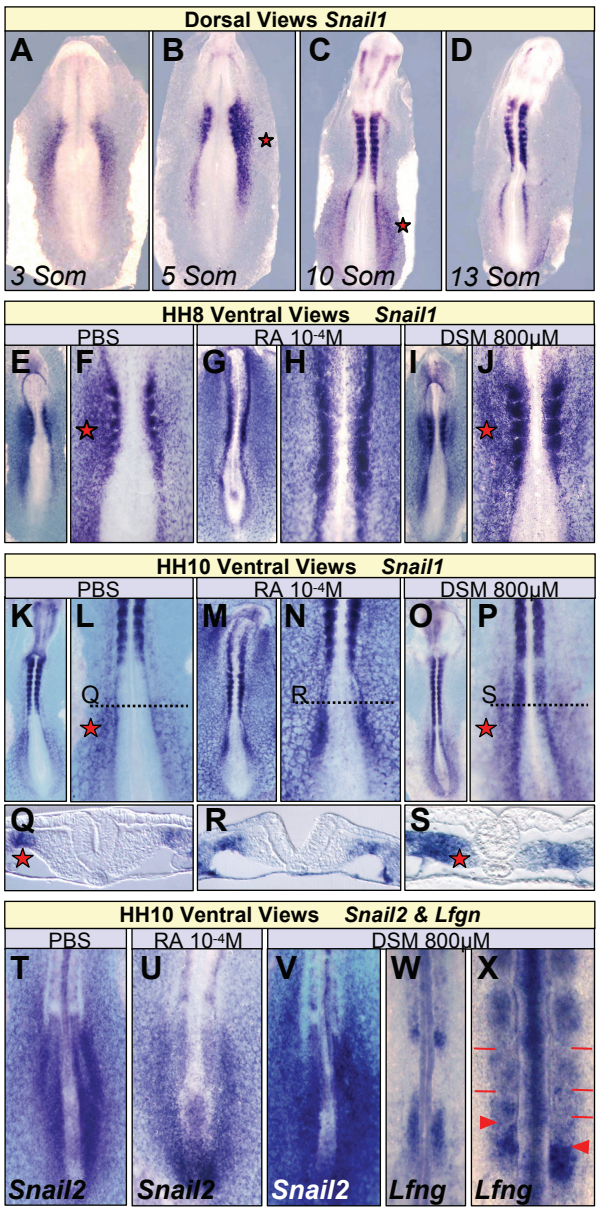
Fig 1. Retinoic acid (RA) signalling prevents asymmetric *Snail1* expression in the anterior presomitic mesoderm (PSM). (A-D) Transient asymmetric L-R *Snail1* expression in the lateral plate mesoderm (LPM) of 4 to 11 somite chicken embryos (see also Isaac *et al,* 1997). (E-X) Embryos incubated with PBS, RA or DSM and analyzed at the 4 (HH8; E-J) or 10 somite stage (HH10; K-X). Q, R and S are sections at the levels indicated in L, N and P, respectively. Embryos were hybridized for *Snail1* (A-S), *Snail2* (T-V) and *Lfng* (W, X). Exposure to RA abolishes asymmetric left-right (L-R) *Snail1* expression in the lateral plate mesoderm (LPM: G, H, M, N) without affecting *Snail2* expression in the PSM (T, U). Asymmetric L-R *Snail1* expression invades the anterior PSM in HH10 embryos treated with DSM (O, P, S), which delayed segmentation on the side of highest *Snail1* expression (X). Asymmetric L-R expression in the LPM is not affected in these embryos (P), where both *Snail2* and *Lfng* continue cycling in the PSM (V-X). Red stars indicate asymmetric L-R expression; red bars the somite boundaries and red arrowheads newly-formed somite boundaries.

Fig 2. Unilateral Snail1 overexpression delays somite formation in chick and mouse embryos. Expression of dEGFP (A, C, E, G), the morphology (B, F) and *Uncx4.1* expression (D, H) in chicken embryos electroporated with pCX-dEGFP (A-D) or pCX-Snail1 (E-H). Caudal halves of 10.5dpc Snail1-ER transgenic mouse embryos cultured for 15 hours in the presence (K, N) or absence (I, J, L, M) of 4'-OH-tamoxifen and analyzed for *Lfng* (blue)and *Uncx4.1* (orange) expression. Red bars indicate somite boundaries and red arrowheads newly-formed somite boundaries. (O) Diagram quantifying the percentage of embryos that showed synchronous or asynchronous

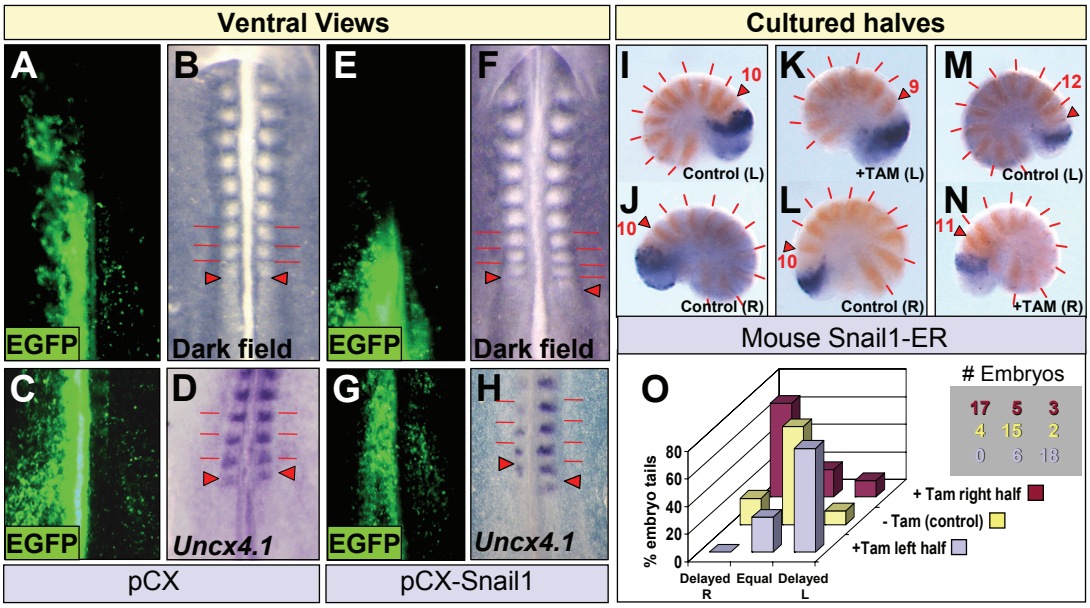
somitogenesis after the different conditions in culture. Delayed R: embryos with delayed somitogenesis on the right side; Equal: embryos showing synchronic bilateral somitogenesis; Delayed L embryos with delayed somitogenesis on the left. The number of embryos represented are also shown in a table that maintains the same colour code and relative position.

Fig 3. Delayed or accelerated somite formation provoked by Snail2 overexpression or dominant negative-Snail2 expression in the chick. Chicken embryos electroporated with pCX-dEGFP plus pCX (A-E), pCX-Snail2 (F-J) or a dominant negative form of *Snail2* (pCX- ΔZf-Snail2; K-O) showing dEGFP expression (A, F, K), their morphology (B,G,L) and the expression of *Lfng* (C, H, M), *Hairy2* (D, I, N) and *EphA4* (E, J, O). High levels of ectopic expression were observed in the right presomitic mesoderm (PSM) of the embryos. Brackets indicate the progress of PSM expression. The asymmetric phase of the cycling genes represented in this figure corresponds to embryos in which the differences were more apparent. Red bars indicate somite boundaries, red arrowheads newly-formed boundaries and the black arrowhead indicates an extra band of *EphA4* expression.

Fig. 4. Inhibition of RA signalling provokes asymmetric *Snail1* expression in the PSM inducing desynchronization in somite formation. Diagramme depicting RA signalling activity and *Snail1* expression in the wild type embryo and after inhibiting RA signalling. Endogenous RA prevents the invasion of the L-R asymmetric Snail1 expression in the PSM and ensures synchronic somitogenesis.



Morales Fig. 1



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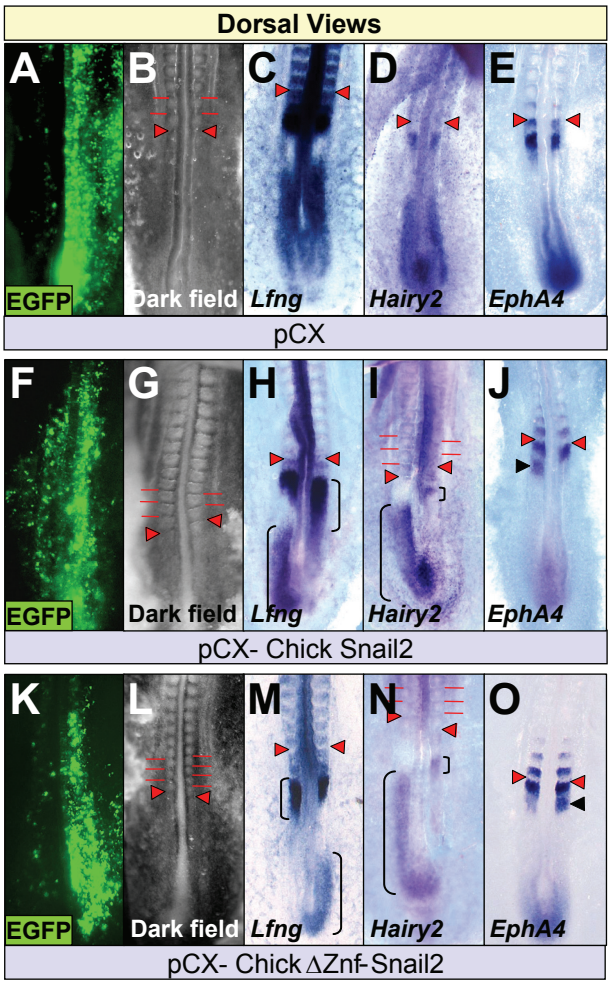

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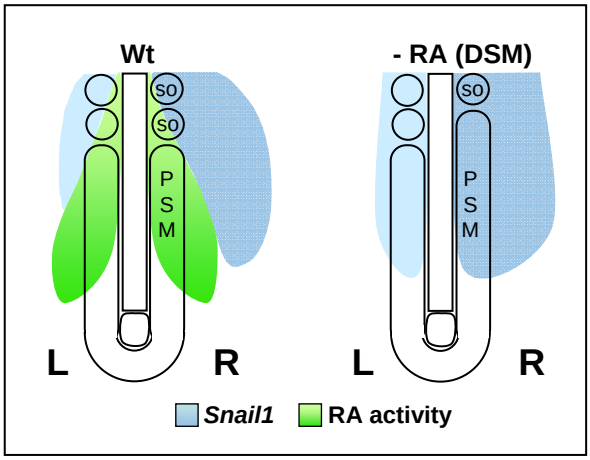
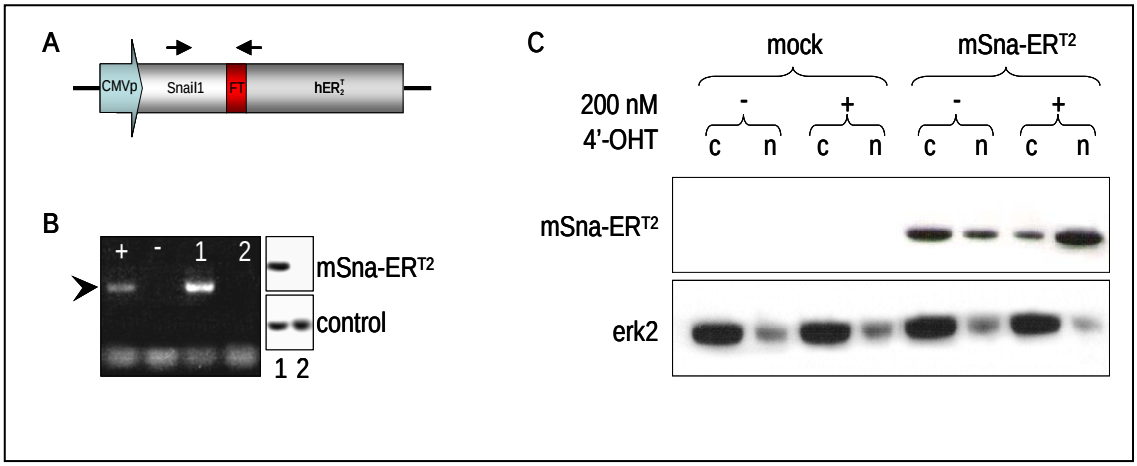


Morales Fig. 4



Analysis of transgenic mice expressing Snail inducible protein. (A) Schematic representation of the transgene construct. FT, Flag tag. (B) PCR of genomic DNA expression in Snail1-ER^{T2} mouse lines. +, plasmid positive control; -, negative control; 1, Snail1tg/tg; 2: wild type. Right panel, Western blot analysis of protein expression in caudal regions of 9.5 dpc embryos. (C) Nuclear translocation of the fusion protein in stable transfected cells upon treatment with 200 nM 4'-OHT. Immunoblotting of cytoplasmic total erk2 was used as a control for cytoplasmic content. n, nuclear extract; c, cytoplasmic extract.

Stable transfections and Western blots

Transfection of MDCK cells was carried out as described (Cano et al., 2000). Six independent clones were isolated from both pcDNA3-Snail-FT-ERT2 (mSna-ERT2) and control pcDNA3 transfections (mock). Cells were cultured in the absence or the presence of 200 nM 4'-OH-Tamoxifen for 48 h to activate the fusion protein.

Following treatment, the cells were scraped off the plates after washing with cold PBS, and lysed at 4ºC in hypotonic cytosolic lysis buffer (10 mM Hepes-K at pH 7.9, 10 mM KCl, 10 mM MgCl2, 0.5 mM DTT, 1mM NaF, 1 mM β-glycerophosphate, 5 mM NaPPi, 5 mg/ml leupeptin, 1 mM sodium o-vanadate and 1 mM PMSF). After isolating cytosolic proteins, the pellet was diluted in hypertonic nuclear lysis buffer (20 mM hepes-K at pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 1mM NaF, 1 mM β-glycerophosphate, 5 mM NaPPi, 5 mg/ml leupeptin, 1 mM sodium o-vanadate and 1 mM PMSF) to isolate nuclear proteins. The presence of the Snail1-ER fusion protein was assed by Western blotting using a human estrogen receptor antibody (Santa Cruz; 1:100).