Ets-1 Confers Cranial Features on Neural Crest Delamination

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INTRODUCTION

Neural crest cells (NCC) are a transient population of versatile cell types derived from the dorsal neural folds of the developing vertebrate embryo. Their ontogeny is a complex morphogenetic process which encompasses the delicate step of separation from the tight pseudostratified neuroepithelium. This crucial event, called delamination, is characteristic of the NCC population as other components of the early nervous system differentiate and remain confined within the neuroepithelium.

Induction of NCCs appears to take place as early as gastrulation, creating eventually a territory of presumptive NCCs in the dorsal neural tube, at the border between neural plate and epidermis [1]. The most dorsally located of these cells become migratory NCCs and undergo delamination. This latter process results from an epithelium to mesenchyme transition (EMT) [2,3], characterized by loss of cell-cell contacts, loss of polarity and acquisition of migratory capabilities [4]. After EMT, NCCs migrate into the periphery, where they differentiate in multiple cell types, prominently neurons and glia of the peripheral nervous system, as well as pigment-producing melanocytes of the skin [3,5,6]. In addition, cranial NCCs possess the capability to differentiate into cartilage, bone, connective tissue and smooth muscle, hence constituting the main source of craniofacial structures.

Cranial and trunk NCC delamination events are intrinsically different. Trunk neural tube is flanked by somitic mesoderm and cells emigrate individually in a dripping fashion over a long period of time, more than two days at any given axial level [3,7]. Recently, mechanisms of action that initiate trunk delamination have been partly unraveled. In the dorsal neural tube, the balance between BMP and its inhibitor Noggin triggers trunk delamination under control of signals coming from somites hence coordinating NCC emigration to somites segmentation [8,9]. BMP signaling pathway controls G1/S transition in trunk NCCs which delaminate mainly in S-phase [10]. This regulation is pivotal as blockage of G1/S transition prevents delamination from neural tube [11]. In contrast, in head, the neural tube is surrounded by loose mesoderm devoid of somites. Cells pour out as dense, multilayered bulges in a short time scale (approximately 10–15h). So far little is known about signalings that regulate cranial NCC delamination but mechanisms described for trunk are unlikely to apply as inhibition of BMP activity did not prevent cranial delamination [7].

In order to gain insights onto the regulation of cephalic emigration, we sought for genes with cranial-specific expression pattern. Among these, ets-1, the founding member of Ets family of transcription factors, is expressed by cranial NCCs just before the onset of emigration and is restricted to cells leaving the neural tube [12]. Furthermore, several lines of evidence implicate ets-1 in acquisition of cell mobility and invasiveness. During embryonic development, ets-1 is expressed in tissues exposed to cell movements and scattering such as sclerotome, dermatome and endothelial cells [13–18]. Moreover, it endows cells with the capacity to migrate through basement membranes and to invade interstitial space during embryonic angiogenesis and wound healing angiogenesis [19–21]. It regulates expression of numerous extra cellular matrix (ECM) components, ECM degrading enzymes and adhesion molecules ([22–24] and references therein). Ets-1 and members of Ets family have also been linked to leukemia, tumor progression and metastasis (for review, [24,25]). In addition, ETS-1 is also known to regulate genes involved in cell cycle progression such as p16 Ink4a, p21Waf1/Cip1 and cyclin-d1 [25–29]. In light of these properties and of its specific expression pattern, we thus aimed to investigate the

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role of ets-1 in NCC development, in particular in cranial versus trunk NCC emigration.

Overall, our studies indicate that in the chick embryo, gathered cranial NCCs delaminate as a multilayered cell population without subjecting to G1/S transition of the cell cycle. We demonstrate that the activity of the proto-oncogene ets-1, is required for their delamination. Moreover, its ectopic expression allows all neuroepithelial cells (including trunk NCCs) to massively pour out of the neural tube independently of being in S-phase hence mimicking cranial delamination. We show that ets-1 promotes massive cell recruitment and induces local degradations of the basal lamina, two separable events which are sufficient to initiate ectopic delaminations.

In contrast, ets-1 alone does not provoke epithelium-to-mesenchyme transition (EMT) but can cooperate with snail-2 (previously called slug) to this process. We thus conclude that ets-1 confers on NCCs their cranial specific kinetics of delamination.

RESULTS
Cranial Neural Crest Cells Delamination is not S-phase Dependent
In order to better document cranial NCCs delamination, we first analysed the neural tube organization at mesencephalon level using normal chick embryos between stage HH8+ and 10. During the first steps of the delamination process, cranial NCCs are massively gathered together forming a bulge in the most dorsal part of the neural tube (Figures 1A-1L). Noticeably, they continue to express N-Cadherin like neuroepithelial cells, but, its distribution does not coincide with phalloidin staining anymore (Figures 1C, 1G, 1K). This indicates that cell-cell junctions are lost and that cranial NCCs start to undergo EMT. As migration progresses, N-Cadherin expression is gradually lost (Figures 1E, 1G, 1K). This alone does not provoke epithelium-to-mesenchyme transition (EMT) but can cooperate with snail-2 (previously called slug) to this process. We thus conclude that ets-1 confers on NCCs their cranial specific kinetics of delamination.

Ets-1 is Sufficient to Provoke a Cranial-Like NCCs Delamination in Dorsal Trunk Neural Tube
In order to test whether ets-1 expression plays an important role in conferring specific cranial NCCs delamination features, we misexpressed the human form of ets-1 (h-ets-1) in dorsal trunk neural tube in stage HH10+ embryos. At 15hpe, we observed more sox-10 expressing NCCs emigrating from the electroporated side compared to the control side (Figures 4A-4D; n = 7). Moreover, at more caudal level, where the endogenous delamination has not started yet, we observed a premature exit of sox-10 positive NCCs in the electroporated side, in contrast with the control side where sox-10 expression is barely detectable (Figures 4A, 4E). Electroporation of an inactive form of ets-1 (w375r) in the same conditions has no effect (data not shown). This shows that ets-1 expression can prime NCCs delamination and in addition increase the flow of delaminating cells. This effect is associated with a strong decrease of cadherin-6B expression (Figures 4O-4P; n = 5), which is normally lost by the NCCs leaving the neural tube (31). Expression of w375r does not affect it (Figures 4Q-4R). Therefore, this suggests that ets-1 forces the dorsal premigratory NCCs to massively leave the neural tube. At 24hpe, the trunk NCCs...
Figure 1. Description of the Cranial Neural Crest Cells Delamination. (A–P) Transversal cryosections (10 μm) of normal chick embryos at stages 6s (A–D), 8s (E–H), 10s (I–L) and HH14 (M–P) at cranial (A–L) and trunk (M–P) levels. Sections were assayed for N-Cadherin expression by immunofluorescence (A, C, E, G, I, K, M, O). The actin microfilaments and the nuclei were stained by Phalloidin (B, C, F, G, J, K, N, O) and DAPI incorporation (D, H, L, P) respectively. During delamination of cranial NCCs there is a massive accumulation of cells in the dorsal part of the neural tube (A–D). In this cell population, colocalisation of N-Cadherin and Phalloidin is lost indicating that they undergo an EMT (C, G, K). By contrast, during trunk delamination, NCCs emigrate one by one. No particular distortion of the dorsal neural tube is detectable (M–P, arrow heads). (Q–V) Analysis of BrdU incorporation in cranial neural tube during and after NCC delamination. Transversal cryosections (5 μm) of stages HH8–9 embryos, during and after delamination, labeled by immunofluorescence using anti-BrdU antibody (Q, R, T, U). Nuclei are stained by DAPI. Percentages of BrdU positive cells in the different zones of the neural tube are represented in diagrams (S,V). Cranial NCCs are not synchronized in S-phase during delamination (Q–R) or migration (T–U). del, delaminating cells; sur, surrounding region; mid, midline region; mig, migrating cells.

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emigration is enlarged as evidenced by a thick flow of 
sox-10 (Figures 4F–4J, arrow heads; n = 4) and HNK1 (Figures 4K–4N; n = 2) positive cells delaminating from the dorsal neural tube. At rostral trunk level, NCCs delamination persists whereas the endogenous delamination is already completed on the control side (Figures 4H–J, asterisks; n = 4). However, at 48hpe, cells which leave the neural tube under 
ets-1 expression do not display NCCs characteristics anymore. Delaminating electroporated cells are organized as a tongue at the top of the dorsal neural tube (Figures 4S–4U; n = 7). They express high level of N-Cadherin (Figures 4Z–4a) whereas its specific repression is required for trunk NCCs departure [30] and fail to express NCCs markers such as sox-9 (Figures 4V–4W), sox-10 (Figures 4X–4Y) or HNK1 (Figures 6S–6T). Altogether these results indicate that 
ets-1 expression leads to precocious, enhanced and prolonged delamination of trunk NCCs which delamate gathered as a multilayer stripe of cells instead of one by one progressively. It thus raised the question of whether these cells are still subordinate to successful G1/S transition to delamate, consequently the delamination rate is enhanced. Overall, our data show that 
ets-1 is sufficient to convert the slow dripping delamination shaped by subjection to the cell cycle and characteristic of trunk NCCs into a massive cranial-like emigration emancipated from links to G1/S transition.

Ets-1 Promotes Ectopic Cells Emigrations from the Neuroepithelium without Inducing Neural Crest Fate

To better appreciate the capabilities that 
ets-1 could confer on the neuroepithelial cells autonomously, we decided to analyse the effects of its ectopic expression within the intermediate to ventral parts of the neural tube, regions which cannot produce NCCs in normal conditions. As early as 12hpe, both at cranial and trunk levels, h-ets-1 misexpression leads to small ectopic clusters of cells emerging from the neural tube (n = 13, not shown). At 24hpe and 48hpe, the phenotype is amplified and compact heaps of clustered cells are detected bulging out through the extracellular matrix or towards the lumen (Figures 6A–6D, 24hpe, n = 26, 48hpe, n = 18). Misexpression of u375r has no effect indicating that the observed phenotype is due to specific transcriptional activation of 
ets-1 targets (n = 21, not shown). At 24hpe and 48hpe, these ectopic bulges of cells are associated with local degradations of the basal lamina (Figures 6E–J, arrow heads, n = 8) hence confirming the initiation of a delamination process. Given that delamination is a trademark of NCCs compared with others neuroepithelial cells, we assessed whether ectopic delaminating cells induced by h-ets-1

Figure 2. 
ets-1 Expression Occurs Later and in a More Restricted Area than Those of FoxD3 and AP2. (A–O') Whole in situ hybridization of normal chick embryos at stages 4s–13s using c-ets-1 (A–H), foxd-3 (F–J') and ap-2 (K–O') probes. (F–H) Embryos were cut along the rostro-caudal axis. The left and right sides of the neural tube were treated independently with c-ets-1 and foxd-3 probes. C-ets-1 expression (A–B, F–G) begins after foxd-3 (F–G) and ap-2 (K–L) expressions. It is restricted to cells leaving the neural tube (C–D, insets) and cranial region (compare E–E’ to J–J’ and O–O’). * indicates 
ets-1 expression in the sclerotome. r, rhombomere.
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Figure 3. Ets-1 Activity Is Required for Cranial but not Trunk Delamination. (A–L) Analysis of cranial NCCs delamination in HH7-9 chick embryos electroporated by c-ets-1 DBD and harvested at 15 hours post electroporation (hpe). (A–D; G–J) Whole mount in situ hybridization using ap-2 probe. GFP expression in (A) and (G) indicates the electroporation zone. (C–D) and (I–J) are vibratome sections (30 μm) of embryos presented in (B) and (H) respectively. (E; F; K–L) Transversal cryosections (14 μm) labeled by immunofluorescence using anti-HNK-1 antibody and nuclear-stained by DAPI incorporation. Electroporated cells are detected by GFP expression. Pink broken lines in (L) outline the NCCs. (M–N) Analysis of NCC migration in embryos electroporated in the rhombencephalon at stage HH10 when the delamination is in progress and harvested at 15hpe (stage HH14). (O–T) Analysis of NCC specification in chick embryos electroporated by c-ets-1 DBD at stage HH7-9 and harvested at 6hpe. GFP expression in (O), (Q) and (S) respectively. (U–W) Analysis of trunk NCCs delamination in chick embryos electroporated by c-ets-1 DBD harvested at 24hpe. Transversal cryosections (10 μm) were labeled by immunofluorescence using anti-HNK-1 (U), anti-Pax-7 (V) and anti-Pax-6 (W) antibodies. Expression of c-ets-1 DBD in the head leads to a decrease or a lack of ap-2 (B–D, H–J) and HNK-1 expression (E, K). At cellular level, there is either a reduction of the size of the NCC stream on the electroporated side (L) or even a lack of NCCs between the ectoderm and the neural tube (E; F, green staple) while on control side NCCs are normally localized (B–D, ap-2 staining; F, pink arrow heads; L, left pink lasso). Data shown in A–D, E–F, G–J, K–L come from four distinct embryos respectively. This effect is restricted to the delamination step since inhibition of ETS-1 activity after the delamination does not affect the migration (M–N). Finally, c-ets-1 DBD does not prevent NCC specification as snail-2 (O–P), foxd-3 (Q–R) and sox-9 (S–T) remain expressed in the neural folds on both electroporated and control sides. At trunk level, misexpression of c-ets-1 DBD has no effect on dorso-ventral patterning, or on NCCs delamination and migration (U–W), r, rhombomere.

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at intermediate to ventral level of the neural tube did also express NCCs markers. At 24hpe, at both cranial and trunk levels, h-ets-1 does not induce expression of snail-2 (Figures 6K, 6O, n = 8), foxd-3 (Figures 6L, 6P, n = 8), ap-2 (Figures 6M, 6Q, n = 8), or sox-10 (Figures 6N, 6R, n = 10). Similarly, we never detected HNK-1 immunoreactivity in transfected neural tubes or in intermediate delaminating electroporated cells at 24 or 48hpe (Figures 6S–6X, n = 7). To turn down the possibility of transient induction of NCC markers, we analysed snail-2 and sox-10 expressions at earlier time points and did not find any upregulations (data not shown).

Therefore, els-1 expression within the neural tube initiates ectopic delamination independent of NCCs fate.

**Ets-1 Induces Cell Mobilization but not Cell Dispersion**

Ets-1 is known to be involved in tumorigenesis. Therefore, to better understand the nature of the ectopic clusters and investigate their possible tumor-like nature, we decided to analyse the impact of h-ets-1 expression on neural cells proliferation. Despite an
at 48hpe, we observed accumulations of cell nuclei between the non-proliferating region of the neural tube and the basal lamina, an area normally largely deprived of nuclei (Figures 7M–7P, n = 12). This led us to hypothesize that cells movements within the neural tube might occur under ets-1 expression. To test out this hypothesis, we used regionalized markers such as Pax-6 (Figures 7Q–7S, n = 6) and Pax-7 (Figures 7T–7V, n = 6). At 48hpe, Pax-6 and Pax-7 positive cells can be detected within the ectopic clusters but only in register with their endogenous region of expression. It hence gives rise in some cases to mixed Pax-6-low/high expressing clusters or mixed Pax-7-expressing/non-expressing clusters (see high magnifications in Figures 7S and 7V). These results show that Pax-6 and -7 are not ectopically induced in ectopic clusters and that regionalization of the neural tube is conserved in them. We thus conclude that ets-1 induces massive cell movements along apico-basal axis of the neuroepithelium and recruits neuroepithelial cells for subsequent delamination initiated by local disruption of the basal lamina. It is interesting to note that cell mobilization and basal lamina degradation are two separate events since if we use a non-phosphorylatable form of ets-1, cell mobilization occurs without a systematic degradation of the basal lamina (n = 9, not shown).

Since the ectopic clusters of electroporated cells remain close to the neural tube and no migrating electroporated cells are detected far away, it seems that ets-1 does not promote acquisition of migratory capabilities. Migrating cells are normally individually surrounded by ECM. In contrast, clusters of delaminating electroporated cells are completely devoid of Fibronectin (head not shown, trunk Figures 8A–8D, n = 7). In addition, these cells retain a strong expression of N-Cadherin (head not shown, trunk Figures 8I–8L, n = 7), similar to the adjacent neuroepithelium, which suggests cohesive relationships between them. Accordingly, the bulges are formed of a high density of cells (Figures 8E–8F, 8M–8N) comparable to the density observed in the neuroepithelium. In order to test out whether the N-Cadherin was involved in functional structures, we analysed the distribution of the actin microfilaments by Phalloidin staining. Within the ectopic bulges, there are some hot spots of N-Cadherin expression (Figure 8K, arrows, n = 7). This particular distribution of N-Cadherin correlates with a specific organization of the electroporated cells around the hot spots (Figures 9A–9C, 9G–9J) which coincides with the distribution of the actin microfilaments (Figures 9D–9F, 9K–9L, n = 8). These results indicate that, in the electroporated cells, N-Cadherin is still involved in functional cell-cell junctions and strongly argue against EMT and migration abilities. This observation is confirmed by the fact that electroporated cells do not upregulate various molecules involved in NCC EMT or expressed by mesenchymal cells such as RhoB ([32]; n = 12, not shown), Cadherin-7 ([31]; n = 7, not shown), activated β1-Integrin ([33]; n = 8, not shown), Tenascin ([34]; n = 4, not shown) and β3-Integrin ([35]; n = 3, not shown). All these data indicate that the ectopic delamination process initiated by ets-1 occurs without an EMT.

**Ets-1 and Snaill-2 Cooperate to Achieve a Full Delamination Process**

As the cranial NCCs perform an EMT during their normal development, we looked for gene able to achieve ectopic delaminations initiated by ets-1. Interestingly, snail-2 has been described to increase the total amount of emigrating cranial NCCs

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**Figure 5. Ets-1 Misexpression Emancipates Trunk NCC Delamination from Subordination to Successful G1/S Transition.** (A–N) Analysis of the effects of h-ets-1 misexpression in trunk dorsal neural tube on cell cycle assayed at 15hpe. Immunofluorescence labeling using anti-BrdU antibody of transversal cryosections (5 μm). Nuclei are stained by DAPI incorporation. Dotted lines in (C), (F), (I) indicate delaminating transfected area as defined by GFP expression (B, E, H). Trunk NCCs emigrating precociously from dorsal electroporated neural tube opposite segmental plate (A–C) or the first epithelial somite (D–F) are not synchronized in S-phase. Similarly, opposite dissociating somites (G–I), h-ets-1 misexpression in the dorsal neural tube leads to increased NCC delamination of a mix of BrdU positive and negative cells. In contrast, trunk NCCs are predominantly in S-phase when h-ets-1 misexpression does not target the most dorsal territory (J–K, arrow heads) or when NCCs are transfected by w375r (L–N, arrow heads). doi:10.1371/journal.pone.0001142.g005
but, strikingly, does not affect trunk NCCs [36]. Moreover, in contrast to ets-1, snail-2 is unable to induce neuroepithelial cells from intermediate to ventral level of the neural tube to delaminate [36,37]. These results suggest that snail-2 could be only active in cells expressing ets-1. Therefore, we analysed its ability to cooperate with ets-1 to promote accomplished delamination of trunk neuroepithelial cells including mesenchymalisation. We hence coelectroporated h-ets-1 and snail-2 at trunk level in intermediate to ventral neural tube and analysed the effects at 48hpe. Local degradations of the basal lamina are detected in association with ectopic delaminations as expected from h-ets-1 ectopic expression (Figures 10A–10B, n = 4). However, in contrast...
to h-ets-1 electroporation alone, coelectroporated cells do not express N-Cadherin either within or outside the neural tube (Figures 10C–10F, see arrow heads in E and F, n = 3) and the ectopic delaminating cells invade the ECM as a population of dissociated cells (Figures 10E–10F, n = 4). Furthermore, electroporated cells strongly express HNK1 both in the neural tube and during migration (Figures 10G–10H). The amount of departing cells is very high which as a result massively reduces the size of the neural tube on the electroporated side. These data show that coelectroporation of h-ets-1 and snail-2 is sufficient to induce EMT followed by massive dispersion of migratory NCCs from the intermediate part of the neural tube. To further characterize the nature of the specific cooperation between ets-1 and snail-2, we analysed the effect of snail-2 electroporation alone in the neural tube at 48hpe. Interestingly, snail-2 is able to induce ectopic neural crest fate as shown by ectopic HNK1 staining (Figures 10M–10N, n = 6) but fails to provoke ectopic delamination or EMT and has no effect on N-cadherin expression or localisation (Figures 10I–10L, n = 4). Those results indicate that ectopic NCC fate in ets-1 and snail-2 coelectroporated embryos is due to snail-2 alone. However, the massive ectopic EMT that occurs in coelectroporated embryos is specific to ets-1 and snail-2 cooperation as it is never detected when either ets-1 or snail-2 are independently electroporated.

DISCUSSION
Here, we more precisely describe the delamination of the cranial NCCs and find that these cells are first massively gathered at the dorsal part of the neural tube before the onset of migration and that their following delamination is characterized by emergence of great numbers of cranial NCCs in multilayered streams of cells (Figure 11A). Importantly, we show that these cells, in contrast to trunk, are not synchronized in S-phase (Figures 11A–11B). Therefore, the kinetic features of the cranial delamination are

Figure 7. Ets-1 Misexpression Leads to Massive Cell Movements within the Neuroepithelium. (A–V) Analysis of the effects of h-ets-1 misexpression in intermediate to ventral neural tube at 24hpe (A–D) and 48hpe (E–V). (A) Vibratome section (30 μm) of whole mount in situ hybridization using cyclin-d1 probe. (B–L, Q–V) Immunofluorescence on cryosections (10 μm) with anti-BrdU (B), anti-phosphohistoneH3 (C–F), anti-β3-Tubulin (G–I), anti-Lim-1/2 (J–L), anti-Pax-6 (Q–S), anti-Pax-7 (T–V) antibodies. (M–P) Nuclei are stained with DAPI. H-ets-1 misexpression leads to ectopic activation of cyclin-d1 expression without affecting equilibrium between cell proliferation (B–F) and cell differentiation (G–L). Ectopic h-ets-1 expression provokes cell accumulation close to the basal side of the neural tube (M–P). Interestingly, cell recruitment is detectable even when the phenotype is not strong enough to lead to ectopic delamination (M–N). These cell movements of neuroepithelial cells occur along the apico-basal axis of the neural tube and do not disturb dorso-ventral patterning (Q–V). fp, floor plate; lum, lumen.

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characterized by great number of cells delaminating at the same time and absence of S-phase subjection. We provide evidence that Ets-1, which is specifically expressed by cranial NCCs, holds a pattern perfectly matching early phases of cranial delamination and plays a central role in this process.

Ets-1 is necessary for proper cranial NCCs delamination since inhibition of its activity in cephalic neural tube results in a great reduction or even prevention of cranial crest delamination. At trunk level, NCCs delaminate one after the other with the restriction they have successfully achieved their G1/S transition ([11]; Figure 11B). Strikingly, Ets-1 misexpression in the dorsal trunk is sufficient to convert the parcellous outflow of isolated NCCs in S-phase into massive cranial-like delamination of unsynchronized cells (Figure 11C). We also show that ectopic Ets-1 electroporation in intermediate to ventral regions of trunk neural tube, a region normally unable to produce NCCs, leads to massive mobilization of neuroepithelial cells along the apico-basal axis of the neural tube, associated with local degradations of the basal lamina and initiation of ectopic delamination (Figures 11C–11D). These phenomena occur without changing original identity of the cells or their proliferation rate. Electroporated cells do not undergo EMT and thus hold no migratory capabilities. Alone, Ets-1 is therefore able to perform cell sorting and to induce selected cells to disrupt the basal lamina. These events are sufficient to initiate but not complete the delamination process. In addition, we show that Ets-1 can cooperate with other genes to achieve full delamination since when coelectroporated with snail-2 in the trunk neural tube, coelectroporated cells acquire migratory NCCs identity, massively leave the neural tube by an EMT process and migrate away.

Altogether, these results lead us to conclude that Ets-1 is necessary and sufficient to confer cranial features to NCCs delamination independently of neural crest induction and suggest that Ets-1 and snail-2 cooperate to achieve the cranial NCC delamination (Figure 12).

**Ets-1 Acts Independently of Neural Crest Cells Induction**

Here, we show that electroporation of the dominant negative c-Ets-1 DBD leads to dramatic reduction of cranial emigrating NCCs.
which do not delaminate. Conversely, when misexpressed in trunk dorsal region of the neuroepithelium, h-ets-1 dramatically enhances trunk NCC delamination by anticipating their departure, increasing their number and extending duration of their exit. This raised the question of whether ets-1 would play a role in NCCs induction with consequences on delamination or only regulates delamination.

NCC ontogeny proceeds in sequential steps including specification of NCC precursors territory, acquisition of premigratory NCC identity, delamination from the neural tube and migration in the periphery. Previous data have shown that some of these events are independent from each other. For instance, foxd-3 ectopically expressed in intermediate to ventral neural tube induces NCCs markers but this induction is not followed by EMT [37,38]. Also, blockade of delamination does not interfere with NCC specification [8,10]. This seems also to be the case here as inhibition of delamination by c-ets-1 DBD happens without affecting NCCs markers but this induction is not followed by EMT [37,38]. Also, blockade of delamination does not interfere with NCC specification [8,10]. This seems also to be the case here as inhibition of delamination by c-ets-1 DBD happens without affecting NCC specification [8,10]. This seems also to be the case here as inhibition of delamination by c-ets-1 DBD happens without affecting NCC specification [8,10]. This seems also to be the case here as inhibition of delamination by c-ets-1 DBD happens without affecting NCC specification [8,10]. This seems also to be the case here as inhibition of delamination by c-ets-1 DBD happens without affecting NCC specification [8,10]. This seems also to be the case here as inhibition of delamination by c-ets-1 DBD happens without affecting NCC specification [8,10]. This seems also to be the case here as inhibition of delamination by c-ets-1 DBD happens without affecting NCC specification [8,10].

N-Cadherin DAII GEP Phalloidin DAII GEP

Figure 9. Ectopic Electroporated Cells Are Still Attached by Functional Cell-Cell Junctions. (A–J) Analysis of the effects of h-ets-1 misexpression in intermediate to ventral neural tube at 48hpe. Transversal cryosections (10 μm) labeled by immunofluorescence with anti-N-Cadherin antibody (A–C, red; G–J, blue). Actin microfilaments and nuclei are stained with Phalloidin (D–I) and DAPI incorporation (A–F) respectively. Electroporated cells detected by GFP expression (A, D, G) are organized around dots of high N-Cadherin expression (A–C, red dots, white arrows) or high Phalloidin staining (D–F, red dots, white arrows). N-Cadherin and Phalloidin perfectly match to each other (G–J, arrow heads) indicating that N-Cadherin expressed by the electroporated cells is involved in functional cell-cell junctions.

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Ets-1 Expression Abolishes Requirement of Successful G1/S Transition During Trunk NCCs Delamination

Ets-1 electroporated trunk NCCs delaminate into massive streams independently of the S-phase of the cell cycle. This is in contrast to normal situation where trunk NCCs delaminate one by one and where it has been shown that only NCCs in S-phase are able to exit the neural tube [11]. This process is under the regulation the Bmp/Wnt pathway [10]. It could be argued that S-phase is the most favorable phase to delaminate as S-phase nuclei are located at the basal side of the neuroepithelium from which cells exit [11]. In agreement with this argument, since premigratory NCCs are not synchronized in S-phase prior delamination, the outflow of trunk NCCs is restrained as few cells are in the appropriate phase of the cell cycle. However, when the G1/S transition is blocked in vivo, no nucleus-free zone can be detected at the border of the neural tube [10,11]. Moreover, cranial NCCs which naturally express ets-1 delaminate at high rate without being in S-phase. Similarly, when they misexpressed h-ets-1, trunk NCCs delaminate massively, even when they are not in S-phase. Therefore, these
results suggest that the position of cells within the neuroepithelium is not the sole explanation for S-phase requirement. Interestingly, it has been previously described that some promoters are only accessible for transcription factors during S-phase thanks to the loose chromatine organization during the DNA replication [39]. In trunk NCCs, promoters of some targets of the Bmp4/Wnt1 cascade might be only accessible during S-phase. This would explain the unique ability of these cells to leave the neural tube. When they misexpress \textit{ets-1}, they are able to bypass the S-phase condition and delaminate.

Altogether, these data raise the question of the putative subjection to G1/S transition of the remaining cranial NCCs when ETS-1 activity is inhibited. There are numerous genes and mechanisms involved in the control of G1/S transition during trunk NCCs delamination including in particular Bmp/Wnt signaling pathway ([10,30], for review see [7]). This regulation is strongly dependent of specific interactions occurring between trunk neural tube and somites which are lacking in cranial regions. In addition, expression patterns and identified roles of the members of Bmp and Wnt pathways are different from those known at trunk level [40-44]. Therefore, G1/S subjection of cranial NCCs when endogenous ETS-1 activity is inhibited seems unlikely.

**Ets-1 is Responsible for the Particular Kinetics of Cranial Delamination**

We show that \textit{ets-1} is sufficient to initiate ectopic delamination process by recruiting massively neuroepithelial cells and by inducing a cranial-like departure of trunk NCCs. Conversely, inhibition of endogenous ETS-1 activity after \textit{c-ets-1 DBD} misexpression abolishes the massive delamination of cranial NCCs. These results strongly indicate that \textit{ets-1} is responsible for
the particular kinetics of the cranial delamination. However, one could hypothesize that c-ets-1 DBD blocks other members of the ETS family in addition to ETS-1. Nevertheless, the inhibitory effect of c-ets-1 DBD on NCCs delamination is restricted to the cranial region where ets-1 is the sole member of ETS family known to be expressed at the time of NCCs departure. Moreover, co-electroporation of c-ets-1 DBD and h-ets-1 perfectly reverses the phenotype induced by h-ets-1. Then, we argue that the blockade of the cranial NCCs delamination, caused by the c-ets-1 DBD electroporation, is due to the lack of endogenous ETS-1 activity.

Our results indicate that when misexpressed in the neuroepithelium, h-ets-1 induces ectopic delaminations of packed clusters of transfected cells barely mingling with non-electroporated cells. This process is characterized by nuclei accumulation on the basal side of the neural tube without increase of cell proliferation or loss of cell original identity. At the contrary, cells transfected with an
inactive mutant form (wt375r) are spaced out and randomly distributed within the neuroepithelium. Therefore, ets-1 holds the ability to sort out cells from a population. It is also able to initiate their delamination since the massive recruitment of cells is associated with local degradation of the basal lamina. Our results suggest that ets-1 might act by modifying the expression of cell surface adhesion molecules mediating cell-cell recognition and cluster formation. Indeed, we observe reorganization of the neuroepithelium with loss of pseudostratified layout of the neural tube, downregulation of cadherin-6B expression and perturbed distribution of N-cadherin. We also detect bi-directional emigration of the transfected cells, both towards the lumen and the basal lamina, reminiscent of a defect of apicobasal polarity of the neural tube obtained by an increase or a decrease of cadherin expression [45]. However, ets-1 alone does not allow cells to undergo EMT and does not bestow them with migratory capabilities. The ectopic bulges remain attached to the neural tube and delamination is not completed. Therefore, ets-1 cannot summarize all the aspects of cranial delamination. Here, we have shown that ets-1 and snail-2 cooperate to induce ectopic EMT. Similarly, a cooperation between ets-1 and an other member of the Snail family (snail-1) has been previously described in human squamous carcinoma cells [46]. All these results suggest that ets-1 expressed in cranial NCCs might also synergize with snail-2 to induce full delamination process (Figure 12). Mechanisms which support this cooperation remain to be elucidated.

Altogether, our results show that, at cranial level, delamination is the result of two separable cellular events: (i) a massive mobilization of premigratory NCCs orchestrated by ets-1 that enables them to sort themselves out within the neuroepithelium and to acquire the ability to delaminate massively and (ii) a proper mesenchymalization controlled by multiple genes.

**MATERIALS AND METHODS**

**Embryos**

Fertilized eggs from Fasso strain chickens (brown eggs) were incubated at 38°C for appropriate times, then windowed and staged according to Hamburger and Hamilton [47].

**Plasmid constructs, in ovo electroporation, cell death and BrdU labeling**

Full-length human ets-1 cDNA (kindly provided by J. Ghysdael) was inserted downstream of adenovirus enhancer and RSV promoter in pAdRSV expression plasmid [48]. Integrity of the sequence was verified by restriction maps and sequencing. Level of expression and molecular weight of the encoded protein was checked by immunoblots performed on extracts of transiently transfected 293 cells. Nuclear localization of the protein was also asserted by immunocytochemistry in the same cells using a polyclonal anti-ETS-1 (gift of J. Ghysdael). An inactive mutant, h-ets-1 wt375r, unable to bind DNA and to transactivate expression [49,50] was generated by transforming tryptophan in position 375 of h-ETS-1 into arginine by PCR mutagenesis. A non-phosphorylatable form of ets-1 (h-ets-1 E38u) [51] in which threonine in position 38 is replaced by alanine was created by PCR mutagenesis. A dominant negative form, c-ets-1 DBD, was created by inserting the chick ets-1 DNA-binding domain corresponding to amino acids 306 to 423 into pAdRSV by PCR amplification. The resulting protein binds target DNA but, lacking its transactivation domain, does not transactivate expression [52]. We checked efficiency of c-ets-1 DBD by testing its ability to inhibit ectopic delaminations induced by h-ets-1. After, coelectroporation of c-ets-1 DBD and h-ets-1 in the trunk of stage HH14 embryos, we did not find any ectopic delaminations at 48hpe (n = 5, data not shown). Plasmid driving full length chick snail-2 expression was provided by J. Briscoe and M. Cheung. Embryos were electroporated between stages HH7 and HH10 for head and HH10+ and HH14 for trunk and collected as indicated. Plasmids encoding h-ets-1, h-ets-1 wt375r or c-ets-1 DBD were co-electroporated with a plasmid encoding enhanced GFP (pCAG-EGFP; gift of J. Gilthorpe) at respectively 2 µg/µl and 1 µg/µl in 12% sucrose solution containing 0,1% Fast-Green (Sigma). Plasmid solution was mouth pipetted into the lumen of the neural tube with a stretched glass capillary, anteriorward from the level of approximately the third somite for head and last somite for trunk. Electrodes (CUY610 platinum-coated, NEPA Gene) were applied on vitelline membrane on each side of the tube at level of the neural tube obtained by an increase or a decrease of cadherin expression [45]. However, ets-1 alone does not allow cells to undergo EMT and does not bestow them with migratory capabilities. The ectopic bulges remain attached to the neural tube and delamination is not completed. Therefore, ets-1 cannot summarize all the aspects of cranial delamination. Here, we have shown that ets-1 and snail-2 cooperate to induce ectopic EMT. Similarly, a cooperation between ets-1 and an other member of the Snail family (snail-1) has been previously described in human squamous carcinoma cells [46]. All these results suggest that ets-1 expressed in cranial NCCs might also synergize with snail-2 to induce full delamination process (Figure 12). Mechanisms which support this cooperation remain to be elucidated.

Altogether, our results show that, at cranial level, delamination is the result of two separable cellular events: (i) a massive mobilization of premigratory NCCs orchestrated by ets-1 that enables them to sort themselves out within the neuroepithelium and to acquire the ability to delaminate massively and (ii) a proper mesenchymalization controlled by multiple genes.

**Immunohistochemistry**

Immunohistochemical detections of proteins were performed on cryosections of embryos fixed 1 hour at room temperature (RT) or overnight at 4°C in 4% PFA with following primary antibodies
applied on cryosections for 15 minutes. In some cases, sections
were incubated with Triton X-100. DAPI (Sigma) and Phalloidin-TRITC (Sigma) used
blocking and antibodies incubations were carried out in 0.25%
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with chick-specific probes
or both DIG- and FITC-labeled RNA probes for double labeling
Whole mount in situ hybridization
Whole mount in situ hybridization was performed using either non-radioactive digoxigenin (DIG) probe for single labeling,
or both DIG- and FITC-labeled RNA probes for double labeling
with chick-specific probes ap-2 [J. Richman], cad-6B and cad-7 [M. Takeichi], cyclinD-1 [J. Lahiti], ets-1 (B. Vandenbunder), β3-integrin [35], foxd-3 (C. Erickson), rhob [Y. de Curtis], snail-2 (A. Nieto), sax-2 [P. Sharpe], sax-9 [J. Briecoe], sax-10 [P. Scolding] and human-specific
tests-1 [J. Ghysdale]. Reaction was carried out essentially as
described by Wilkinson 1992 [53] except that proteinase K steps
were omitted. Dark staining was obtained using NBT/BCIP
inhibitors (Boehringer Mannheim) whereas light blue staining
was obtained using BCIP alone. Specimens were refined using 4% FFA
prior to storing or sectioning. For sectioning, embryos were
infiltrated with 15% sucrose and embedded in 20% gelatin
solution in PBS. Blocks were refixed 24 hours in 4% PFA/0.1%
glutaraldehyde transversely sectioned on a vibratome (Leica) at
30 µm and further cleared in 60% glycerol/PBS. For whole
mounts, images were collected on Nikon SMZ1500 and Leica
MZFL III stereomicroscopes equipped with diascopic stand and
Nikon DMX 1200 camera. For sections, Nikon Eclipse E800
microscope with Nikon DMX 1200 F camera was used.

Measurements of cell proliferation
To establish the ratio of neuroepithelial cells in S-phase out of the
total number of cells, DAPI and BrdU positive cells were counted
on 5 µm cryosections in fields of at least 1600 µm2. In each case, at least 3 embryos and 3 non-adjacent sections per embryo were
used for analysis. In electroporated embryos, cells were scored in
the GFP area and in corresponding area on control side in order to
compare electroporated with non-electroporated regions (h-ets-1:
n(embryos) = 3, n(transfected cells) = 1036, n(control cells) = 1198;
w375r: n(embryos) = 3, n(transfected cells) = 1173, n(control
cells) = 1257). In normal embryos, cells were counted in regions
corresponding to endogenous chick ets-1 expression during (n = 9;
null cells) = 1479; n(sur cells) = 1904) and after delamination
(n = 6; n(del cells) = 624; n(mid cells) = 659; n(sur cells) = 1057)
of cranial NCs. In these embryos, surrounding regions of the neural
tube were used as reference.

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Author Contributions
Conceived and designed the experiments: MA ET JD. Performed the
experiments: MA ET. Analyzed the data: MA ET. Contributed reagents/
materials/analysis tools: MA ET. Wrote the paper: MA ET.

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