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The neural crest epithelial-mesenchymal transition in 4D: a 'tail' of multiple non-obligatory cellular mechanisms

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An epithelial-mesenchymal transition (EMT) is the process whereby epithelial cells become mesenchymal cells, and is typified by the generation of neural crest cells from the neuroepithelium of the dorsal neural tube. To investigate the neural crest EMT, we performed live cell confocal time-lapse imaging to determine the sequence of cellular events and the role of cell division in the EMT. It was observed that in most EMTs, the apical cell tail is retracted cleanly from the lumen of the neuroepithelium, followed by movement of the cell body out of the neural tube. However, exceptions to this sequence include the rupture of the neural crest cell tail during retraction (junctional complexes not completely downregulated), or translocation of the cell body away from the apical surface while morphologically rounded up in M phase (no cell tail retraction event). We also noted that cell tail retraction can occur either before or after the redistribution of apical-basolateral epithelial polarity markers. Surprisingly, we discovered that when an EMT was preceded by a mitotic event, the plane of cytokinesis does not predict neural crest cell fate. Moreover, when daughter cells are separated from the adherens junctions by a parallel mitotic cleavage furrow, most re-establish contact with the apical surface. The diversity of cellular mechanisms by which neural crest cells can separate from the neural tube suggests that the EMT program is a complex network of non-linear mechanisms that can occur in multiple orders and combinations to allow neural crest cells to escape from the neuroepithelium.

KEY WORDS: EMT, Asymmetric division, Chicken, Live cell imaging, Neural crest, Neural tube

INTRODUCTION

In multicellular organisms, cells are organized as either epithelial or mesenchymal tissues. Epithelial cells are typically assembled in a planar sheet and are apically and basolaterally polarized, with a basal surface that contacts the basal lamina and an apical domain demarcated by the adherens junctions and circumferential actin belt. By contrast, mesenchymal cells have greatly reduced areas of cell-cell contact and are embedded in a three-dimensional extracellular matrix (Hay, 2005). An epithelial-mesenchymal transition (EMT) is the process whereby epithelial cells become mesenchymal cells (Ahlstrom and Erickson, 2007; Hay, 2005; Thiery and Sleeman, 2006). EMTs occur normally throughout development (Shook and Keller, 2003) and misregulated EMTs can contribute to disease processes (Baum et al., 2008; Yang and Weinberg, 2008). As mesenchymal cells escape from an epithelium, cellular events include the loss of high affinity adherens junctions, the disruption of apical-basolateral polarity, the acquisition of mesenchymal motility and penetration of the basal lamina. We have used the avian trunk neural crest EMT (Duband, 2006; Duband et al., 1995; Kalcheim and Burstyn-Cohen, 2005; Le Douarin and Kalcheim, 1999; Morales et al., 2005; Sauka-Spengler and Bronner-Fraser, 2008) as a model to study a vertebrate EMT at high resolution in its native surroundings. We report the sequence of cellular events, the dynamics of apically and basolaterally distributed macromolecules and the role of cell division during this archetypical EMT.

The most widely accepted mechanistic explanation for the trunk neural crest EMT in amniotes (birds and mammals) proposes that the disruption of adherens junction-mediated adhesion regulates the emigration of neural crest cells from the neural tube (Coles et al., 2008; Nakagawa and Takeichi, 1998; Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985; Shoval et al., 2007). Another model for the neural crest EMT proposes that premigratory neural crest cells could generate sufficient tractional force to pull away from the adherens junctions [complete downregulation of the cell-cell adhesion complexes is not required (Bilozur and Hay, 1989; Erickson and Reedy, 1998)]. Alternatively, it has also been speculated that the cleavage furrow of an asymmetric mitosis in the neuroepithelium could produce a basal daughter cell, which would be separated from adherens junctions by the cleavage furrow and allowed to emigrate from the neural tube, whereas the apical daughter cell would still be tethered to the lumen by the terminal junctional complexes and remain as a neural tube progenitor cell (Duband, 2006; Erickson and Reedy, 1998; Gammill and Bronner-Fraser, 2002). There is also evidence that the timing of neural crest emigration is regulated by the cell cycle (Burstyn-Cohen and Kalcheim, 2002; Burstyn-Cohen et al., 2004). Finally, it might be that the EMT does not consist of a linear cascade of events, but rather several different mechanisms could be sufficient to allow the departure of neural crest cells from the neural tube (Duband, 2006; Newgreen and Minichiello, 1995; Newgreen and Minichiello, 1996; Shook and Keller, 2003). These EMT models need not be mutually exclusive. For example, the adherens junctions might become sufficiently reduced by enzymatic cleavage so that tractional forces are able to rip the adhesion complexes apart.

To discover precisely how the trunk neural crest EMT occurs, we tracked individual dorsal neural tube cells in slice culture with confocal time-lapse imaging and recorded the cellular events that accompany the EMT, including: (1) mode of detachment of the cell from the apical surface, (2) retraction of the apical cell tail, (3) translocation of the cell body out of the epithelium, (4) changes in the distribution of apical-basolateral polarity markers and (5) cell division. During most of our documented EMTs the order of the

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steps was (1), (2) and then (3): clean separation of the cell tail from the lumen during apical cell tail retraction followed by emigration of the cell body out of the neural tube boundaries, suggesting that downregulation of the apical adhesion complexes was the key controlling step. However, there were notable exceptions to this sequence, such as when the cell tail was ruptured during retraction and tail fragments remained behind near the apical surface [steps (2), (1) and then (3); the complete downregulation of the junctional adhesion complexes was not necessary]. Also, sometimes neural tube cells became separated from the apical surface while morphologically rounded up during mitosis so that cell division occurred at a non-apical location to produce neural crest daughter cells [(1), (5), (3); step (2) was not necessary]. Moreover, retraction of the cell tail could occur with or without the redistribution of epithelial apical-basolateral polarity markers [(4) happens at any point along the way]. We also discovered that the plane of cell division does not predict the generation of neural crest cells. In conclusion, our live cell imaging reveals that the trunk neural crest EMT consists of several non-obligatory cellular programs that can occur in a multiplicity of orders.

MATERIALS AND METHODS

Expression vectors

To generate membrane-localized EGFP (mem-EGFP) driven by a chicken β-actin promoter, an oligonucleotide encoding the human Harvey ras CAAX box (GTACAAGCTGAACCCTCCTGATGAGAGTGGCCCCGGCTG-CATGAGCTGCAAGTGTGTGCTCTCCTGA, Sigma-Proligo) was inserted at the C-terminal end of EGFP in the pCAβ-EGFPm5 plasmid [gift of Jon Gilthorpe (Yaneza et al., 2002)] using the 5′ *Bsr*GI and 3′ *Xho*I restriction enzyme sites. mCherry-tubulin and GFP-actin plasmids were from the laboratory of James Nelson (Stanford University, Stanford, CA, USA) and were a gift from Soichiro Yamada. The GFP-α-catenin plasmid was a gift from Robert Kypta (Giannini et al., 2000) and γ-tubulin-GFP was a gift from Frank McNally [made in the lab of Conly Rieder (Khodjakov and Rieder, 1999)].

In ovo electroporation

Fertile White Leghorn chicken eggs from the UC Davis Animal Science Department were incubated in a humidified 37°C incubator until they reached Hamburger and Hamilton (HH) stages 14-16 (Hamburger and Hamilton, 1992). mem-EGFP DNA (1-3 μg/μl) was injected into the lumen of the neural tube, and embryos were electroporated at the axial level of the segmental plate using a Gentronics BTX-T820 square wave generator with five 50-millisecond square pulses at 30 V (Itasaki et al., 1999; Kos et al., 2003). The other half of the neural tube was labeled by reversing the position of the electrodes and electroporating with one 50-millisecond pulse at 30 V. For co-electroporation studies, mCherry-tubulin (0.35 μg/μl) was co-electroporated with either GFP-actin (0.12 μg/μl), GFP-α-catenin (0.35 μg/μl) or γ-tubulin-GFP (0.2 μg/μl). The eggs were further incubated at 37°C for 16-19 hours to HH stages 17-19.

Embryo slice culture

Brightly labeled and properly developed embryo trunks were dissected in Locke's saline (2.3 M NaCl, 80 mM KCl, 32 mM $CaCl_2$, 36 mM $NaHCO_3$ in ddH_2O, pH 7.4). Trunk segments were embedded in 2.5% low melting point (LMP) agarose (Invitrogen 15517-014) in Ham's F-12 (HyClone) at 37°C, with the pH adjusted with 10 mM HEPES (Gibco). A Vibratome (series 1000) was used to make transverse slices (300 μm) of trunk segments in a bath of 4°C Locke's saline and then the slices were transferred to a 35 mm glass bottom Petri dish (MatTek P35G-0-14-C). The slices were overlaid with 1-2.5% LMP agarose and positioned close to the coverslip with blunt tungsten needles. Slices were kept at 4°C for 40-60 minutes and then overlaid with Neurobasal medium without phenol red, supplemented with 2% B-27, L-glutamine (5 mM), penicillin/streptomycin (100 mg/l), gentamicin (50 mg/l) and 10 mM HEPES (all from Gibco). Light mineral oil (Fisher O121) was overlaid on top of the culture medium to prevent evaporation.

Slice imaging

Trunk slices were imaged with an Olympus FV1000 laser scanning confocal (inverted microscope) using an Olympus 60× PlanApo N oil immersion lens (NA 1.42). The imaging compartment was kept at 37°C with Reflectix thermal insulation (Kulesa and Fraser, 1999) and a Sage Instruments air curtain incubator (model 279). A metal nut weighing 3.3 g was placed on top of the hardened agarose to prevent the slices from lifting off the coverslip during imaging. Neural tube cells were imaged at least 20 μm past the cut surface and only if the ectoderm was in place over the dorsal neural tube. Stacks of confocal images spaced 1 μm apart (*z*-stacks of 30-77 μm) were captured at 7-minute intervals (1-2 minutes total exposure per slice) for up to 30 hours. Multiple slices were imaged sequentially using the Multi Area Time Lapse controller of Fluoview v1.6. Time-lapse imaging data sets were analyzed using Imaris 5.5 (Bitplane AG) and rendered as movies with Imaris 5.5 and QuickTime Pro.

Immunohistochemistry

Embryonic trunks were electroporated at HH stages 14-16 with mem-EGFP, harvested 16-19 hours after electroporation at HH stages 17-19 and cryosectioned at 30 μm. For antibody detection, cryosections were rehydrated in PBS, the cells permeabilized in –20°C acetone for 20 minutes, washed in PBS and then blocked in PBS containing 2% normal goat serum (Gibco), 1% BSA, 0.1% cold water fish skin gelatin (Sigma G7041), 0.1% Triton X-100 and 0.05% Tween 20 for 20 minutes. Blocking solution was removed and cryosections were incubated overnight at 4°C with both mouse IgM anti-α-tubulin (1:500, Abcam ab7749) and rabbit IgG anti-phospho-Histone H3 (1:100, Upstate Biotechnology 06-570) diluted in PBS containing 1% BSA and 0.1% cold water fish skin gelatin. After several washes in PBS, the secondary antibodies, Cy3-conjugated goat anti-rabbit IgG (1:300, Jackson ImmunoResearch 111-165-003) and Cy5-conjugated goat anti-mouse IgM (1:300, Jackson ImmunoResearch 115-175-020), were applied for 30 minutes in PBS with 300 μg/ml Hoechst 33342 (Sigma B2261). Images were captured using confocal microscopy as described above.

Analysis of cleavage plane orientation and nuclear position

The time-lapse imaging data were viewed using Imaris 5.5 and snapshots of all neural tube cell divisions were captured. The angle of each cleavage plane was scored as being 60°-90° (perpendicular), 30°-60° or 0°-30° (parallel) relative to the plane of the epithelium. To evaluate the position of the nucleus of neural tube cells prior to an EMT across several experiments, images were sized to match a grid and values assigned based on a relative *x* and *y* position.

RESULTS

Imaging the trunk neural crest EMT in slice culture

To identify the cellular mechanisms that constitute the neural crest EMT and test prevailing EMT models, we performed live cell confocal time-lapse imaging on trunk dorsal neural tube cells in slice culture. Recently, fluorescent labeling combined with slice culture of avian embryonic tissue has proved useful for studying previously unobservable developmental events (Gros et al., 2005; Roszko et al., 2006; Wilcock et al., 2007). Although slice culture cannot support development indefinitely, this technique does allow for a time interval when normal development can be observed. Our criteria for judging 'normal development' during live cell imaging were that the neural tube maintained an epithelial architecture with a well-defined lumen, the neural tube cells remained spindle shaped and interkinetic nuclear migration and mitosis occurred regularly. In addition, care was taken to image slices at least 20 μm past the cut surface, and only where the ectoderm was intact over the dorsal neural tube. Imaging was performed on HH stage 17-19 chicken embryos at the trunk axial level between 5 and 15 somites anterior from the last-formed somite (either epithelial somite or dissociated somite axial levels; Fig. 1A). Altogether, from 21 imaging sessions, there were 54 slices that maintained normal neural tube architecture and cell morphology for at least 10 hours and some up to 29 hours of imaging. We defined an EMT to be when a neural tube cell

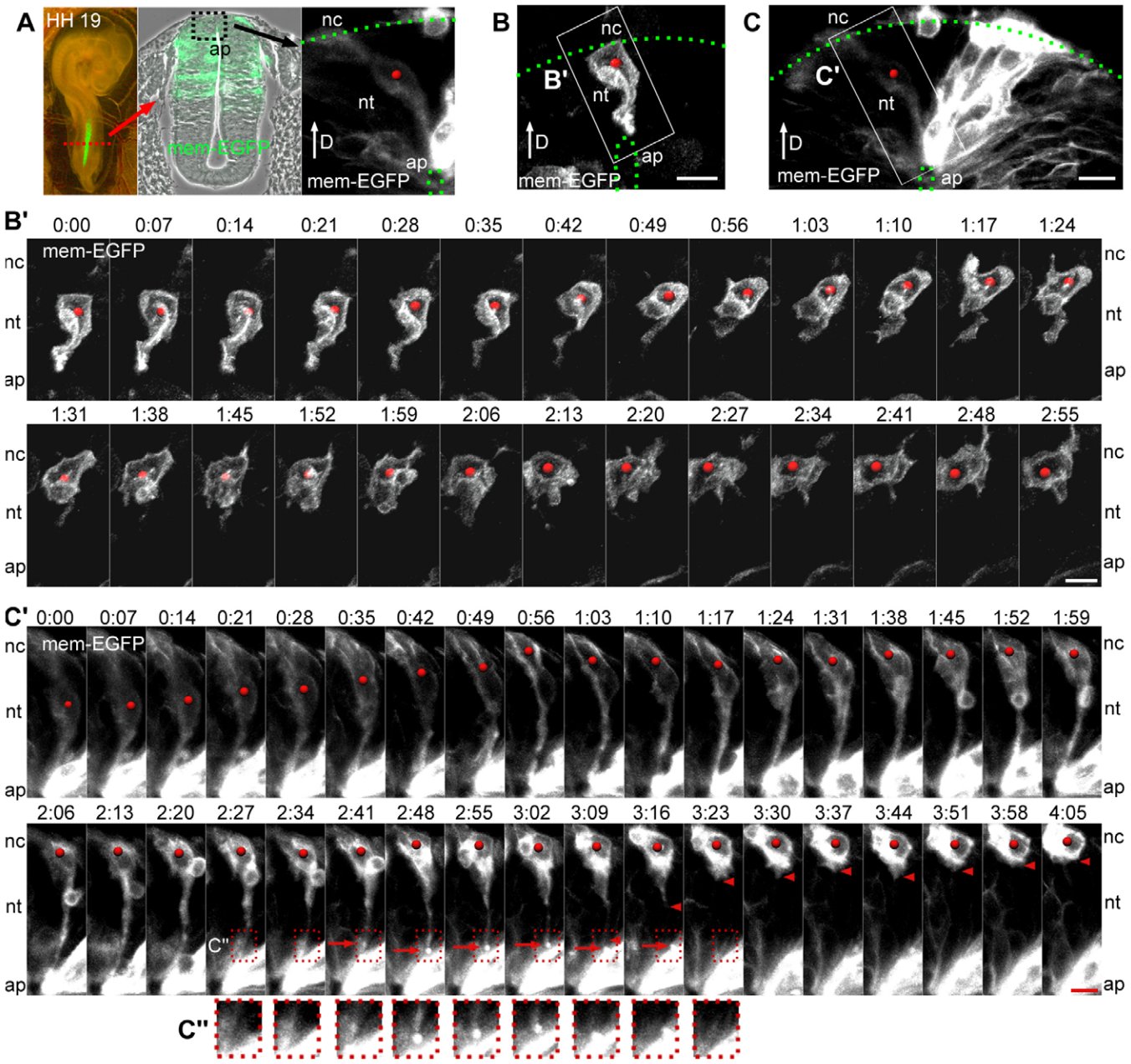

Fig. 1. Retraction of the neural crest cell tail occurs most often after complete detachment from the apical surface, but sometimes the cell tail ruptures. (**A**) A chicken embryo (HH stage 19) electroporated in ovo 18 hours earlier. Imaging was performed on transverse slices at the trunk axial level (red dotted line) to visualize individual cells within the dorsal neural tube (region in black dashed box). (**B**,**C**) The mem-EGFP labeled dorsal neural tube cells within the white boxes are shown in the time-lapse series below. The green dotted lines indicate the apical and basal boundaries of the neural tube. (**B′**) Complete detachment: mem-EGFP labeled cell (red dot) detaches from the apical surface without leaving behind any detectable debris. Time (hours:minutes) is indicated along the top of the series. (**C′**) Ruptured cell tail: mem-EGFP labeled cell (red dot) ruptures its cell tail (arrowhead), leaving cellular fragments behind (arrow). A more detailed confocal analysis of the cell tail rupture is shown in Fig. S1 in the supplementary material. (**C″**) Magnified view of the cellular fragment (bright spot) that was part of the cell tail but was left behind during cell tail retraction. Scale bars: 10 µm. D, dorsal; nt, neural tube; nc, neural crest; ap, apical.

becomes detached from the apical surface and the cell body moves mostly out of the boundary of the dorsal neural tube (as inferred by its position relative to other labeled neural tube and neural crest cells). In total, of the 1567 dorsal neural tube cells that were labeled with fluorescent constructs and initially visible, 132 of these cells (8%) were recorded undergoing an EMT during the 10-29 hours of imaging (see Table S1 in the supplementary material).

The neural crest cell tail is most often withdrawn cleanly from the apical surface, but sometimes the cell tail is ruptured

To investigate how the EMT occurs, we began by characterizing the following cellular events: (1) detachment of the cell from the apical surface (loss of adherens junction-mediated adhesion), (2) retraction of the apical cell tail and (3) translocation of the cell body out of the

epithelium. Just hours prior to most of the neural crest EMTs, the main cell body was positioned at the basal-most domain of the neuroepithelium and the cell was attached to the lumen by a thin tail, giving the appearance of a bottle-shaped cell with its tail under tension. For most of the EMTs (95/132 EMTs or ~72%), the cell body emigrated out of the boundaries of the neural tube only after the tail retracted. Where we could clearly view the separation of neural crest cell tails from the apical domain of the neuroepithelium (n=28), most often the cell tail was withdrawn cleanly from the apical surface of the neural tube without leaving behind any detectable fluorescent fragments (25/28, as in Fig. 1B′; see also Movie 1 in the supplementary material). This retraction behavior, which we term 'clean' cell tail retraction, is consistent with the idea that the enzymatic degradation or downregulation of adherens junctions located near the apical domain controls the timing of neural crest cell departure from the neural tube. However, in a few examples (3/28), fluorescent debris remained when the tail retracted, suggesting that separation of the cell from the lumen was accomplished by physical rupture of the cell tail (as in Fig. 1C′, and in more detail in Fig. S1 and Movie 2 in the supplementary material). The actual point of rupture varied from very close to the lumen to partway along the cell tail. Breakage of the cell tail during retraction is consistent with the possibility that tractional forces can overcome adherens junction-mediated cell-cell adhesions and rip the neural crest cell out of the neural epithelium. In summary, cell tail retraction occurs primarily after complete separation from the apical surface [steps (1), (2), (3)], but tail retraction can also occur before disruption of the adherens junctions is complete [steps (2), (1), (3); complete dissolution of the adherens junctions not necessary].

Neural crest cells can detach from the apical surface while morphologically rounded up in mitosis

In the pseudostratified epithelium of the dorsal neural tube, cells underwent interkinetic nuclear migration and cell division occurred most of the time (414/441 or ~94% of divisions) at the apical surface (Frade, 2002). However, sometimes the apically localized rounded mitotic cells would detach from the lumen of the neural tube and undergo mitosis within the neuroepithelium at a non-apical position (27/441 mitoses, as in Fig. 2A′; see also Movie 3 in the supplementary material). The daughter cells from these non-apical mitoses were never seen to re-establish a cellular connection with the apical domain of the neural tube and all the daughter cells that could be tracked eventually became neural crest cells (37 neural crest cells from 27 non-apical mitoses; see Table 1). These non-apical mitoses generated 37/132 (28%) of the neural crest cells recorded in this study (see Table S1 in the supplementary material).

To confirm that non-apical mitotic cells could be found in the dorsal neural tube of intact embryos and that they were not an artifact of slice culture, cryosections of uncultured mem-EGFP labeled embryos fixed 18 hours after electroporation were examined for non-apical mitotic neural tube cells. Even though the existence of a non-apical mitotic cell is a transient and rare event (seen in 27/1567 or 2% of labeled cells during time-lapse imaging), we were able to detect cells positive for phospho-Histone H3 (mitosis marker) that were within the dorsal neuroepithelium (seen with α-tubulin), and not attached to the lumen (seen with mem-EGFP; Fig. 2B). These 'non-surface' divisions have been reported previously in the mammalian cortex (Miyata et al., 2004) and within the lateral domains of the chicken neural tube (Wilcock et al., 2007). Verifying that non-apical mitotic dorsal neural tube cells are found in uncultured fixed embryos supports our live cell observations that some pre-neural crest cells undergo detachment and initial translocation towards the basal surface during M phase.

The behavior of some neural crest cell tails is indecisive

One surprising observation was the occasionally irresolute behavior of neural crest cell tails. For example, the neural tube cell in Fig. 3A′ (see Movie 4 in the supplementary material) partially withdraws its cell tail, then projects it back towards the lumen and finally retracts it completely as the cell body moves out of the neural tube. Additionally, the neural crest cell in Fig. 3B′ (see Movie 5 in the supplementary material) is already outside of the neural tube but extends a cell process all the way to the apical surface before retracting it again and migrating out of the field of view. This 'reinsertion into the epithelium' behavior of neural crest cells is not consistent with the premise that adherens junctions are the only force keeping neural tube cells polarized within the neuroepithelium. Rather, these data suggest that there are additional cellular mechanisms, such as those that regulate apical-basolateral polarity, that also control how cells position themselves within the epithelium.

Markers for the adherens junctions and the circumferential actin belt are not always lost during detachment and retraction of the neural crest cell tail

Another facet of an EMT is the loss of epithelial apical-basolateral polarity. In the neuroepithelium, the adherens junctions [including N-cadherin, β-catenin and α-catenin (Pokutta and Weis, 2007)] and the circumferential actin belt are found at the interface between the apical and basolateral domain (near the lumen). To study the dynamics of apical-basolateral polarity during the neural crest EMT, neural tube cells were co-electroporated with mCherry-tubulin (cytoskeletal marker) and either GFP-α-catenin (adherens junction component) or GFP-actin (circumferential actin belt marker). These GFP markers showed strong localization to the apical domain at the lumen, which is where the adherens junctions and circumferential actin belt are found. In our studies using GFP-α-catenin or GFP-actin, 14 slices were suitable for analysis, and of the 248 neural tube cells that were initially visible, 28 cells subsequently underwent an EMT. In 9/14 instances of neural crest cell tail retraction imaged with GFP-α-catenin (data not shown) and 8/14 cell tail retractions of cells labeled with GFP-actin (as in Fig. 4A′; see also Movie 6 in

Table 1. The fate of neural tube cells after mitosis

	60°-90°	30°-60°	0°-30°	Non-apical	Totals
Total number of neural tube divisions recorded	321 (73%)	21 (5%)	72 (16%)	27 (6%)	441
Number of times neural tube division then one neural crest cell	10	2	11	3	26
Number of times neural tube division then two neural crest cells	1	0	2	17	20

The cleavage planes of dorsal neural tube divisions were scored as being either 60°-90° (perpendicular), 30°-60°, or 0°-30° (parallel) relative to the plane of the epithelium. 'Non-apical' refers to mitoses that occurred within the neural tube, but at a non-apical location.

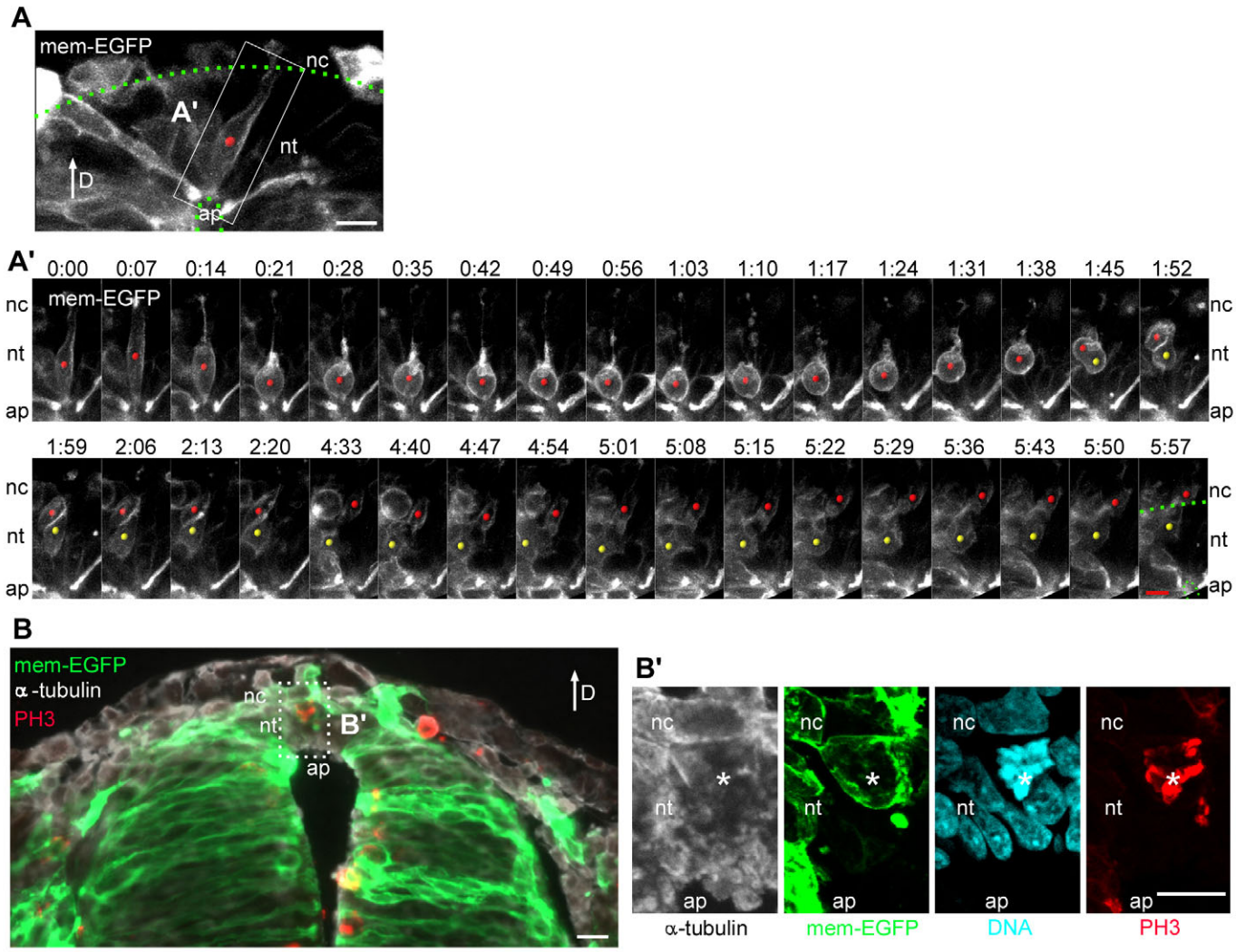

Fig. 2. The neural crest EMT can also occur by a non-apical mitosis. (**A**) The mem-EGFP labeled neural tube cell within the white box is shown in the time-lapse series below. (**A′**) Non-apical mitosis: mem-EGFP labeled neural tube cell (red dot) rounds up for mitosis, separates from the lumen and divides at a non-apical location. By the end of the time series the daughter cell with the red dot is mostly out of the neural tube. (**B**) Non-apical mitotic cells are found in uncultured embryos: mem-EGFP labeled embryos were fixed 18 hours after electroporation and cryosectioned. Mitotic cells were detected with an antibody against PH3 (red), the boundaries of the neural tube were visualized with an antibody against α-tubulin (white, labels all cells) and the mem-EGFP signal (green) was used to verify that the non-apical PH3-positive cells within the dorsal neuroepithelium were rounded and not attached to the apical surface. (**B′**) Magnified view and separate fluorescent channels for the non-apical mitotic cell (*) from the white dashed box in B. Scale bars: 10 μm.

the supplementary material), the apical signal was notably reduced before the cell tail detached and retracted from the lumen, which is consistent with the idea that the disassembly of adherens junctions and loss of apical-basolateral polarity occurred before neural crest cells withdrew from the apical surface. However, in several other instances, the polarized signal remained at the tip of the cell tail as it detached from the lumen and was retracted [4/14 instances visualized with GFP-α-catenin (as in Fig. 4B′; see also Movie 7 in the supplementary material) and 6/14 instances visualized with GFP-actin (data not shown)], suggesting that neither the adherens junctions nor apical-basolateral polarity were completely disrupted prior to the retraction of the neural crest cell tail. Finally, in one instance the GFP-α-catenin signal remained behind at the lumen even after the neural crest cell body exited the neural tube (as in Fig. 4C′ and in more detail in Fig. S2 and Movie 8 in the supplementary material), further demonstrating that neural crest cell tails are sometimes ruptured distal to the adherens junction, and that the adherens junctions do not need to be completely disassembled in order for tail retraction to occur. In summary, it appears that the complete dissolution of the adherens junctions or circumferential actin belt can happen either before or after retraction of the cell tail.

The centrosome remains at the end of the cell tail during retraction

The position of the centrosome (seen with γ-tubulin-GFP) was used as another marker for a polarized epithelium. We predicted that during the neural crest EMT the centrosome would depart from its epithelial location at the lumen (where the cilium is nucleated) and reorient to the basal side of the nucleus in the direction of cell migration, as occurs for many (Gomes et al., 2005), but not all (Pouthas et al., 2008), migratory cell types. During time-lapse imaging the γ-tubulin-GFP signal often did

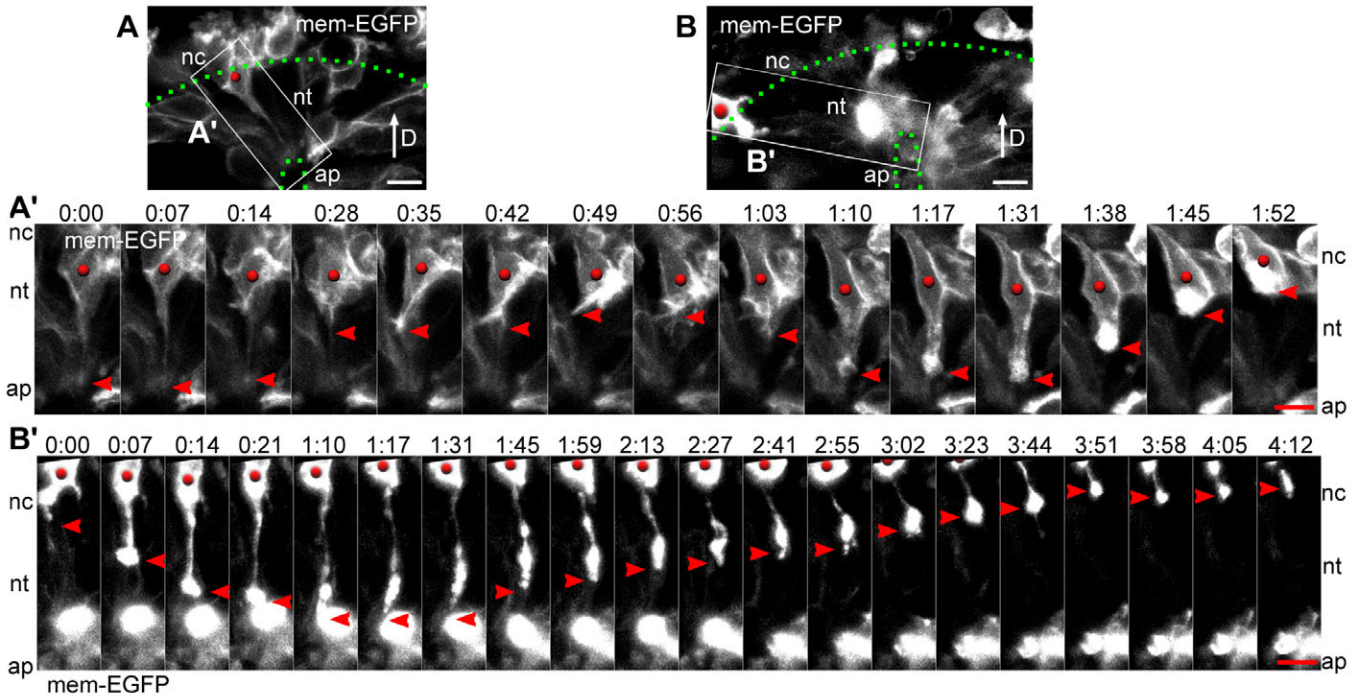

Fig. 3. The behavior of some neural crest cell tails is indecisive. (**A**,**B**) The mem-EGFP labeled neural tube cells within the white boxes are shown in the time-lapse series below. (**A′**) A mem-EGFP labeled neural tube cell (red dot) detaches from the apical surface (arrowhead) and then redeploys a cell process (arrowhead) to the lumen before withdrawing the cell tail completely. (**B′**) A mem-EGFP labeled neural crest cell (red dot) that is already outside the boundaries of the neural tube sends a cell process to the apical surface (arrowhead) and then retracts it and migrates out of the field of view. Scale bars: 10 μm.

leave the apical domain temporarily, but never prior to an EMT. Rather, during interkinetic nuclear migration in the basal to apical direction, the γ-tubulin-GFP signal would leave its luminal position, meet the nucleus at a non-apical position, and they would then travel apically together until they reached the lumen and mitosis occurred (Fig. 5A′; see also Movie 9 in the supplementary material). Contrary to our hypothesis, during an EMT the γ-tubulin-GFP signal remained at the apical-most end of the cell tail as it detached and retracted (Fig. 5B′,C′; see also Movies 10 and 11 in the supplementary material). These observations are similar to those reported for retraction of zebrafish retinal ganglion cells (Zolessi et al., 2006). We also recorded dorsal neural tube cells that were transporting γ-tubulin-GFP puncta intracellularly from the nucleus towards the lumen, and this apically directed transport of γ-tubulin-GFP continued until the very moment of tail retraction from the apical surface (Fig. 5C′; see also Movie 11 in the supplementary material). Therefore, we conclude that the complete disruption of epithelial polarity is not required prior to cell tail retraction.

The orientation of the mitotic cleavage plane does not predict neural crest cell fate

It has been proposed that neural crest cells may be produced by an asymmetric mitosis, because a horizontal (parallel) mitotic cleavage plane in neuroepithelial cells would result in the separation of the basal daughter from the luminal adherens junctions (Duband, 2006; Erickson and Reedy, 1998; Gammill and Bronner-Fraser, 2002). To test this model we tracked the fate of all neural tube daughter cells following mitosis and looked for a correlation between the orientation of the plane of cell division and the subsequent generation of neural crest cells. To visualize mitosis we utilized either a membrane-tagged EGFP or the microtubule marker mCherry-tubulin. Overall, of 1567 dorsal neural tube cells that were initially visible during live cell imaging, there were 414 cell divisions recorded that occurred at the apical domain (lumen) of the dorsal neural tube. The orientation of the cleavage plane was scored (see Fig. S3 in the supplementary material) as either: (1) perpendicular to the lumen of the neural tube (60°-90°), (2) inbetween (30°-60°) or (3) parallel to the plane of the epithelium (0°-30°). Neural crest EMTs were recorded after only 26/414 (6%) of these dorsal neural tube apical mitoses (Table 1). After 11 of the 60°-90° (perpendicular) mitotic divisions that later produced a neural crest cell, 10/11 resulted in one of the daughter cells becoming a neural crest cell, whereas the other remained within the neural tube (until it could no longer be tracked); in 1/11 cases both daughter cells eventually left the neural tube. From the 0°-30° (parallel) divisions preceding an EMT, there were 11/13 examples in which the basal-most daughter cell left the neural tube and the apical daughter remained within the neural tube for the duration of the imaging session (as in Fig. 6A′; see also Movie 12 in the supplementary material), consistent with the asymmetric mitosis model for generating neural crest cells. However, we recorded 2/13 examples of 0°-30° (parallel) neural tube mitoses in which both daughter cells became neural crest cells. Therefore, we conclude that the orientation of the cleavage plane does not predict neural crest cell fate, and that asymmetric mitoses cannot account for the creation of all neural crest cells.

Rounded mitotic cells in the neural tube retain a thin membranous attachment to the basal surface (Fishell and Kriegstein, 2003; Wodarz and Huttner, 2003). When an EMT followed mitosis at the apical surface (recorded in only 26/414 or 6% of apical neural tube

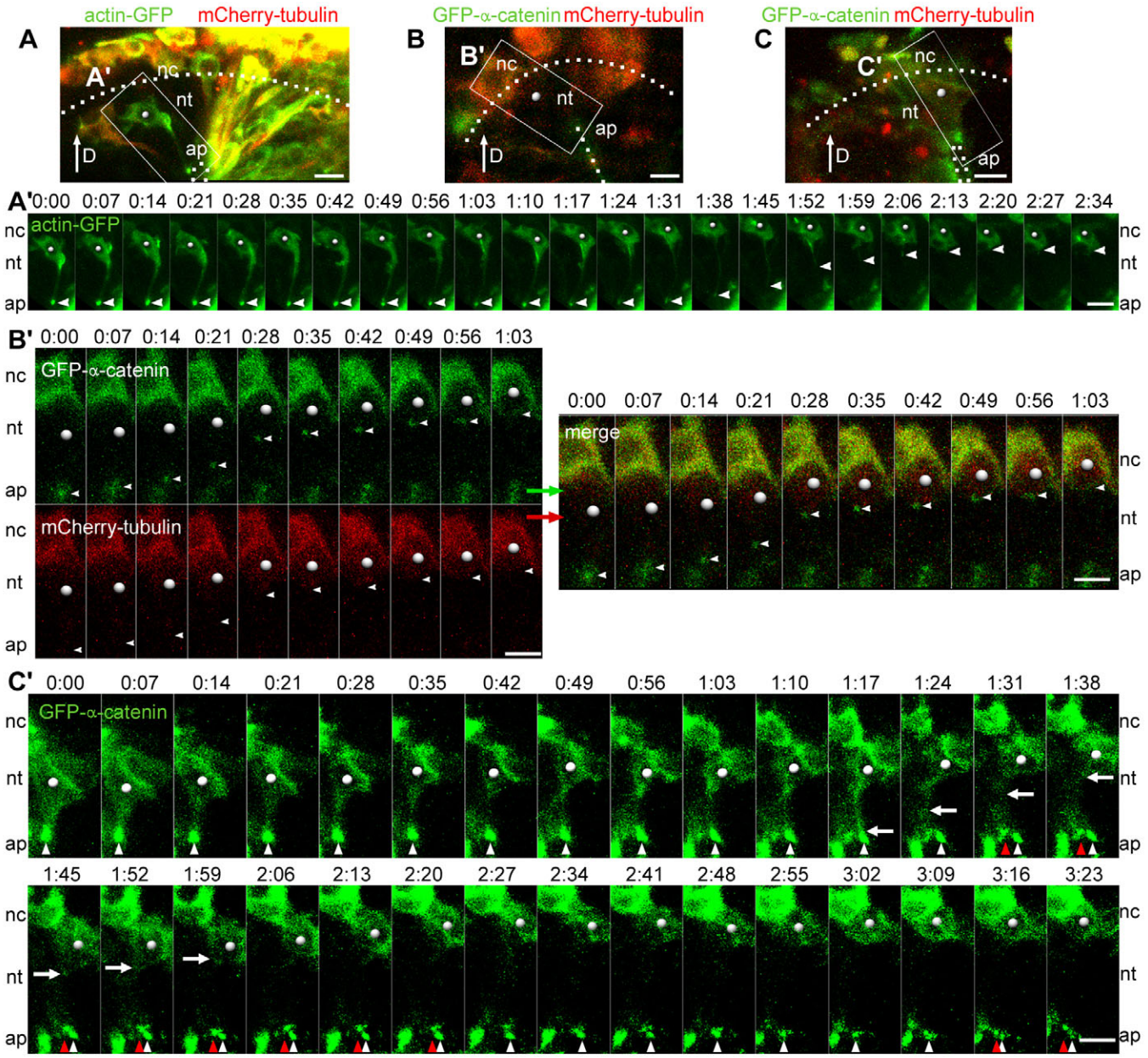

Fig. 4. Time-lapse imaging of dorsal neural tube cells with the circumferential actin belt marker GFP-actin or the adherens junction marker GFP-α-catenin. (**A-C**) The neural tube cells within the white boxes are shown in the time-lapse series below. (**A′**) The apical GFP-actin signal (arrowhead) becomes reduced until the cell tail detaches and retracts (arrowhead) from the lumen. (**B′**) Time-lapse imaging with GFP-α-catenin (green) and mCherry-tubulin (red), showing both the individual and merged channels. When this cell (dot) detaches from the apical surface, the apical GFP-α-catenin signal travels with the end of the cell tail as it retracts (arrowhead). (**C′**) When this GFP-α-catenin labeled cell (dot) retracts its cell tail (white arrow), the apical GFP-α-catenin signal (white arrowhead) remains behind at the lumen in close proximity to a different apical GFP-α-catenin signal (red arrowhead), not associated with the same cell. A more detailed confocal analysis of the cell tail rupture is shown in Fig. S2 in the supplementary material. Scale bars: 10 μm.

divisions), the daughter cell that inherited the original basal attachment was always the first neural crest cell to leave the neural tube (26/26), regardless of the orientation of the cleavage plane (perpendicular or parallel). The remaining daughter cell that had to form a new basal attachment either left the neural tube a few hours later (3/26), or remained within the neural tube for as long as it could be tracked (23/26). Hence, when an EMT follows mitosis, an attachment to the basal surface influences the timing of neural crest cell emigration.

Daughter cells that are separated from the lumen of the neural tube by the plane of cell division predominantly remain within the neural tube

As mentioned above, 72/441 (17%) of all the dorsal neural tube mitoses at the apical surface had a cleavage plane angle of 0°-30° (parallel), thereby separating the basal-most daughter cell entirely from the adherens junctions found near the apical surface. According to the asymmetric mitosis model for the neural crest EMT, these 72 basal-most daughter cells following the 0°-30°

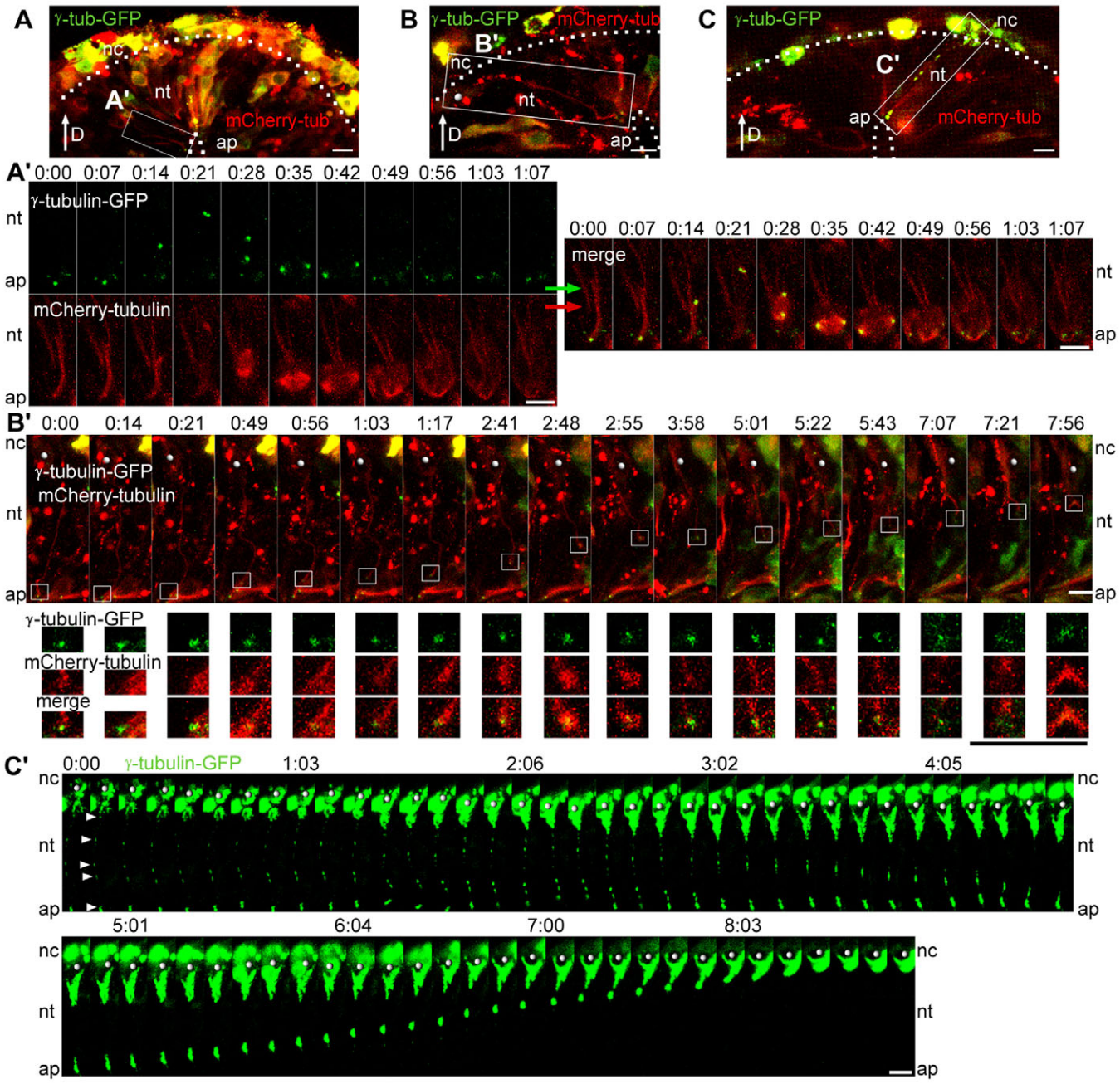

Fig. 5. Time-lapse imaging of dorsal neural tube cells with the apical polarity marker γ-tubulin-GFP (marking the centrosome). (**A-C**) The neural epithelial cells labeled with γ-tubulin-GFP (green) and mCherry-tubulin (red) within the white boxes are shown in the time-lapse series below. (**A′**) As this cell undergoes interkinetic nuclear migration and apical mitosis, the centrosome (γ-tubulin-GFP signal) leaves the apical surface and travels basally. Minutes later the nucleus moves towards the apical domain. The nucleus meets the centrosome in a non-apical location, and then both the centrosome and the nucleus travel together to the apical surface, where mitosis occurs. (**B′**) As this neural tube cell (dot) retracts its tail from the lumen, the γ-tubulin-GFP signal remains at the apical-most tip of the cell tail (white box; corresponds to magnified view below showing γ-tubulin-GFP, mCherry-tubulin and merged channels) as the cell tail is retracted. (**C′**) In a cell labeled with γ-tubulin-GFP, puncta of γ-tubulin-GFP (arrowheads) are transported from the nucleus towards the apical domain of the epithelium (0-5 hours) until the cell tail detaches from the lumen (5-8 hours). Scale bars: 10 μm.

mitosis should become neural crest cells. However, in only 13/72 (18%) of these 0°-30° divisions did the basal-most (n=11) or both (n=2) daughter cells become neural crest cells during the imaging session (see Table 1). In 59/72 (82%) of the 0°-30° cell divisions, the basal-most daughter cell re-established an apical connection to the neuroepithelium and remained within the neural tube for the duration of the imaging session (as in Fig. 6B′; see also Movie 13 in the supplementary material), which further discredits the asymmetric mitosis model for the neural crest EMT. Even after the 0°-30° mitosis depicted in Fig. 6A′, when a neural crest cell was eventually produced, the basal-most daughter cell projected a thin process to the apical surface before retracting it to become a neural

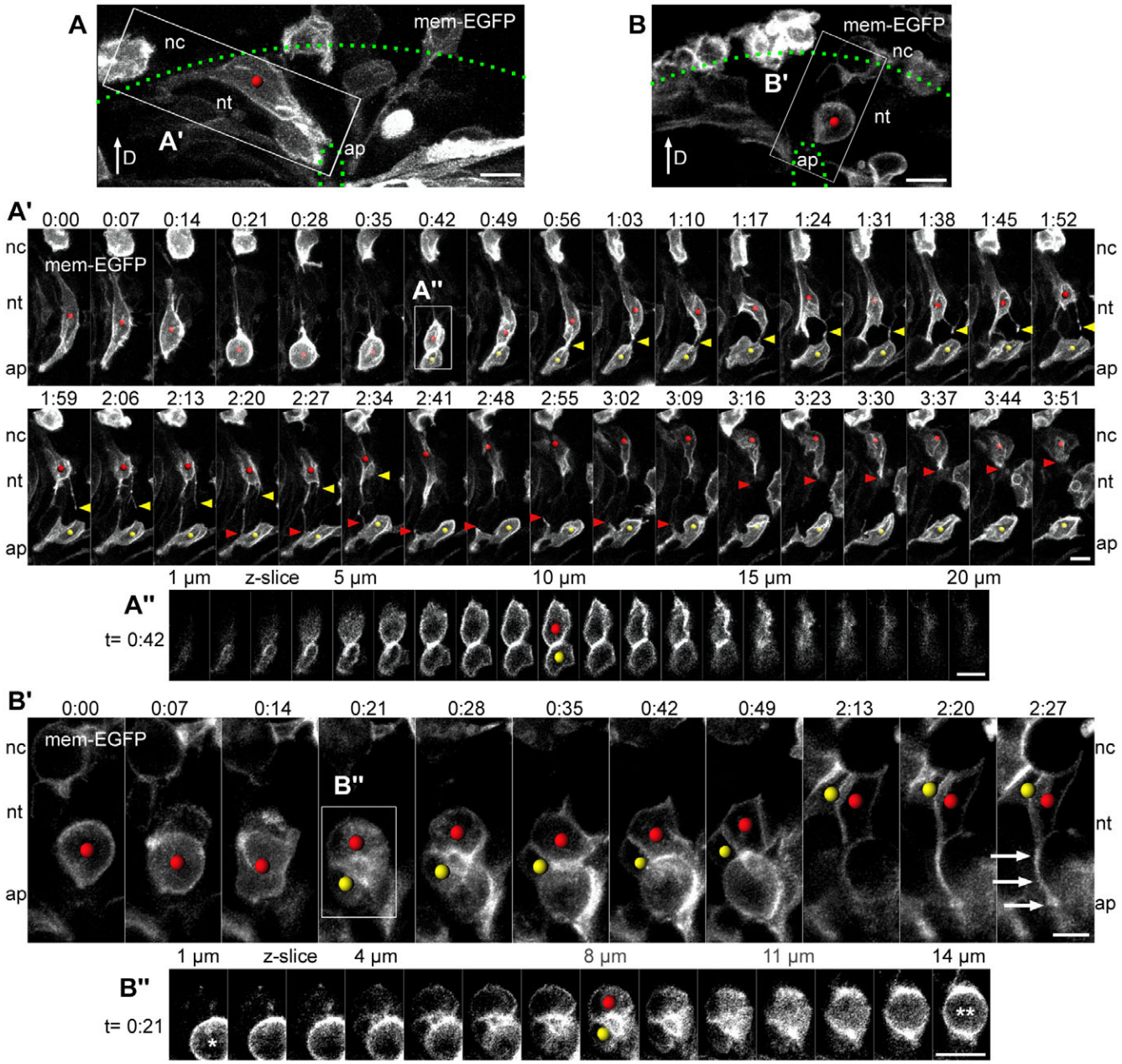

Fig. 6. The fate of neural tube cells that are separated from the apical surface by a 0°-30° division. (**A**,**B**) The mem-EGFP labeled neural tube cells within the white boxes are shown in the time-lapse series below. (**A′**) A mem-EGFP labeled cell undergoes an apical cell division where the cleavage plane completely separates the basal-most daughter cell (red dot) from the apical surface. The basal-most daughter cell retains a midbody attachment (yellow arrowhead) to the apical daughter cell (yellow dot) for a time, and then the basal-most daughter cell extends a cell process to the lumen (red arrowhead), but retracts this process (red arrowhead) as the cell exits the neural tube. (**A″**) Confocal *z* slices from time frame 0:42 show that the basal-most daughter cell (red dot) is completely separated from the apical surface by the plane of cell division. (**B′**) A mem-EGFP labeled neural tube cell (red dot) undergoes an apical cell division that separates the basal-most daughter cell (red dot) from the apical surface. By the end of this time series, the basal-most daughter cell has clearly re-attached to the lumen (arrows) and remains within the neural tube for the duration of the imaging session. The attachment of the other daughter cell (yellow dot) was out of the confocal viewing field, but the cell body (yellow dot) remains in the neural tube for the duration of the imaging session (not shown). (**B″**) Confocal *z* slices of the cell division from time frame 0:21, showing that the basal-most daughter cell (red dot) is completely separated from the apical surface, which can be seen at z=8 µm, even though the dividing cell is bound on either side by mitotic cells at z=1 µm (*) and at z=14 µm (**). Scale bars: 10 µm.

crest cell, suggesting that epithelial identity was not completely lost even though the daughter cell had severed contact with the luminal adherens junctions. Therefore, the separation of a neural tube daughter cell from the adherens junctions by the plane of cell division is not usually sufficient to create a neural crest cell.

The position of cells within the neural tube prior to an EMT

Although it is generally believed that neural crest cells are derived from the entire dorsal neural tube, there is speculation that the EMT might occur in a more limited region, such as the dorsal midline

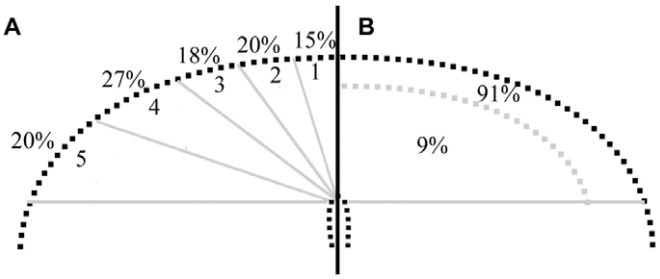

Fig. 7. The surface position and apical-basolateral location of neural tube cell nuclei prior to an EMT. (**A**) Diagram of the dorsal neural tube showing the percentage of neural crest cells that emigrated from a given region of the neural tube. (**B**) The percentage of neural tube cell nuclei within a given apical-basal domain 1 hour prior to cell emigration.

(Burstyn-Cohen and Kalcheim, 2002). It was therefore of interest to discover the precise region of the avian dorsal neural tube that produces neural crest cells. By determining from all our recorded EMTs the location along the basal surface from which neural crest cells exit, we found that trunk neural crest cells emigrate from any region of the dorsal neural tube, and not solely from a discrete region, such as the dorsal midline (Fig. 7A). The domain that is competent to produce neural crest cells is coincident with neural crest markers such as Snail2 [formerly Slug (Locascio et al., 2002)], FoxD3 (Kos et al., 2001) and Cad6B (Nakagawa and Takeichi, 1998).

Additionally, there is evidence that neural crest cells preferentially undergo the EMT during S phase of the cell cycle, during which the position of the nucleus is most likely to be basal (Burstyn-Cohen and Kalcheim, 2002; Burstyn-Cohen et al., 2004). Consistent with these reports, our live cell imaging data showed that the nucleus for most pre-neural crest cells was positioned at a very basal position of the neuroepithelium within hours of neural crest emigration (Fig. 7B; Table 2). However, the production of neural crest cells following non-apical mitoses (37/132 EMTs) cannot be explained by the 'S phase' model for the EMT, because these cells detached and translocated towards the basal domain of the neural tube while morphologically rounded up in M phase of the cell cycle. Therefore, not all neural crest cells undergo the EMT while in the 'S phase' of the cell cycle.

DISCUSSION

Epithelial-mesenchymal transitions [originally termed 'transformations' (Greenburg and Hay, 1982)] occur normally throughout development (Hay, 2005; Shook and Keller, 2003; Thiery and Sleeman, 2006) and abnormally in many disease states (Baum et al., 2008; Yang and Weinberg, 2008). This study is the first to report the sequence of cellular events, the dynamics of apical-basolateral markers and the role of cell division during the trunk neural crest EMT by direct observation, and our results challenge several prevailing hypotheses about EMTs.

The cellular events that constitute the EMT include: (1) detachment from the lumen, (2) retraction of the cell tail, (3) translocation out of the epithelium, (4) loss of apical-basolateral polarity and (5) potentially cell division. Our data clearly demonstrate that the sequence of events does not always occur in the same order, nor are all the steps obligatory for every EMT. In most cases, neural crest cells retract their cell tail cleanly from the apical surface after adherens junctions are downregulated, followed by movement of the cell body out of the neural tube [steps (1), (2), (3)]. However, sometimes the neural crest cell tail ruptured and tail fragments were left behind at the lumen, indicating that contractile events were activated before cell-cell adhesions were lost [steps (2), (1), (3); complete loss of the adherens junctions not necessary]. Additionally, neural crest cells can leave the neural tube either before or after the redistribution of markers for the adherens junctions or circumferential actin belt, suggesting that loss of apical-basal polarity is not completed at a fixed time [(4) at any step]. Not only can these cellular events occur in a different order, some steps can be omitted. For example, an asymmetric mitosis (0°-30°) can generate a neural crest cell from a basal daughter cell without either downregulating adhesion or forcefully retracting a cell tail [(5), (3); steps (1) and (2) omitted]. In other situations, mitotic cells detach from the neural tube and finish division in a non-apical location, whereupon both cells become neural crest cells [steps (1), (5), (3); no obvious step (2)]. The EMT model most consistent with our observations is that the neural crest EMT does not comprise an invariant series of linear checkpoints, but that any of several non-obligatory cellular programs can bring about the neural crest EMT, as has been proposed previously (Duband, 2006; Newgreen and Minichiello, 1995; Newgreen and Minichiello, 1996; Shook and Keller, 2003). This model is additionally supported by previous studies showing that the neural crest EMT can be stimulated in a variety of ways, by either the disruption of junctional complexes with light collagenase treatment or by Ca^{2+}-free medium in vitro (Newgreen and Gooday, 1985), by the inhibition of atypical protein kinase C in vitro (Minichiello et al., 1999), or from expression of a constitutively active RhoB in vivo [together with Sox9 to prevent apoptosis (Cheung et al., 2005)]. Furthermore, the different types and combinations of growth factors and transcription factors required to bring about the full EMT illustrate the complexity of this process and the flexible interconnections between these multiple regulatory events (Duband, 2006; Sauka-Spengler and Bronner-Fraser, 2008; Thiery and Sleeman, 2006).

Although our study definitively answers several long-standing questions about the neural crest EMT, there are still many issues that need to be resolved. For example, it is not known what dictates which individual cells will detach and when. There is evidence that not every dorsal neural tube cell will become a neural crest cell, e.g. fate mapping studies whereby an individual dorsal neural tube cell sometimes only gives rise to neuroepithelial clones (Bronner-Fraser and Fraser, 1988). This idea is further confirmed by our observation that very few (~8%) of the labeled neural tube cells undergo an EMT during the 10-29 hours of imaging. Why, then, do only some dorsal

Table 2. Position of the nucleus prior to an EMT

Position of nucleus in neural tube	–1 hour	–2 hours	–3 hours	–4 hours	–5 hours	–6 hours	–7 hours	–8 hours	–9 hours
Basal neural tube domain	91%	69%	48%	44%	38%	39%	34%	35%	39%
Inner neural tube domain	9%	31%	52%	56%	62%	61%	66%	65%	61%
Total nuclei counted	132	121	108	96	85	72	64	48	41

The apical-basolateral position of the cell body (nucleus) of cells that later underwent an EMT, tracked as far back in time as possible. For the demarcation of 'basal neural tube' versus the 'inner neural tube' domains, see Fig. 7B.

neural tube cells become neural crest cells and the rest do not? Are there cellular markers that will predict when cell detachment and emigration will occur? We evaluated many cellular behaviors for their ability to predict neural crest cell fate, such as changes in cell shape, membrane behavior at the basal leading edge, downregulation of the adherens junctions, loss of apical-basolateral cell polarity, the position of the centrosome, orientation of the mitotic cleavage plane, the amount of time cells spend in mitosis (see Fig. S3 in the supplementary material), the length of the cell cycle (see Table S2 in the supplementary material) (Wilcock et al., 2007), rates of interkinetic nuclear migration (see Table S3 in the supplementary material) and total basal nuclear distance traveled (Del Bene et al., 2008). For all of these, there was no highly significant difference between neural tube and pre-neural crest cells. It might be that molecular events such as the translocation of β-catenin (de Melker et al., 2004), N-cadherin cleavage products (Shoval et al., 2007) or Snail2 (Yang et al., 2005) from the cytoplasm into the nucleus will predict neural crest cell fate, but this remains to be determined.

It is also not known whether the cellular mechanisms of the neural crest EMT are the same for all axial levels or for other stages of development. This study focused on the first wave of trunk neural crest cell migration (Le Douarin and Kalcheim, 1999). However, at the vagal or cranial axial levels or during later waves of emigration at the trunk level, the EMT is likely to be distinct (Duband et al., 1995; Theveneau et al., 2007). It might also be that certain cellular mechanisms correspond to distinct subpopulations of trunk neural crest, as it is known that many neural crest cells are specified at the time of emigration (Henion and Weston, 1997; Reedy et al., 1998).

Furthermore, although multiple molecular mechanisms are required for the EMT, we do not know what cellular events they regulate. Numerous studies have identified signaling pathways [BMP, Wnt, FGF and Notch (Kalcheim and Burstyn-Cohen, 2005; Morales et al., 2005)] and transcription factors [including Snail2, FoxD3 and Sox9 (Duband, 2006; Sauka-Spengler and Bronner-Fraser, 2008)] that are important for the EMT. However, until now there has not been a cellular context in which to interpret these molecular data. In future studies, the combination of live cell imaging with molecular perturbations will be a powerful approach to identify and study EMT mechanisms.

In conclusion, our direct visual analysis of the dynamic trunk neural crest cell EMT has solved several issues about the cellular mechanisms of the EMT, such as the sequence of events, modes of detachment and the role of cell division in generating neural crest cells. Our studies show that the order and combination of cell behaviors that accompany the EMT are quite variable, suggesting a model whereby multiple independent signaling pathways are loosely interconnected to regulate the escape of neural crest cells from the neuroepithelium.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/cgi/content/full/136/11/1801/DC1

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Table S1. Live cell imaging summary

Imaging sessions	Normal slices	Hours per session	Total # nt cells	EMTs recorded	EMTs: cell tail retraction	EMTs: non-apical mitosis
21	54	13-29	1567	132	95 (C=25, R=3)	37 (div=27)

The 'Total # nt cells' column represents only those dorsal neural tube cells that were labeled and initially visible in the confocal field (not all neural tube cells are labeled by electroporation, resulting in a mosaic pattern of fluorescence expression). In the 'EMTs: cell tail retraction' column, 'C' indicates the number of cell tail retractions that were complete, or 'clean', detachments, and 'R' indicates the number of cell tail retractions that had 'ruptured' cell tails. nt, neural tube; div, divisions.

Table S2. Cell cycle analysis

Cleavage plane	Average time (hr:min) cell cycle neural tube cells (*n*)	Average time (hr:min) cell cycle pre-neural crest cells (*n*)	*P* value
60-90°	9:53 ±2:38 (7)	(0)	–
30-60°	(0)	(0)	–
0-30°	10:06 ±1:46 (5)	4:56 ±1:04 (2)	0.19

The length of the cell cycle was determined for cells that underwent two successive divisions, with either both cell divisions within the neural tube (neural tube cells), or the first division inside the neural tube and the second division outside the neural tube as a neural crest cell (pre-neural crest cells). '60-90°' refers to a perpendicular cleavage plane, '30-60°' is inbetween, and '0-30°' is a parallel mitotic cleavage plane. *P* values were determined by one-way ANOVA with alpha=0.05. Averages are given (± s.e.m.). hr, hour; min, minute.

Table S3. The velocity of nuclear migration and the maximum basal distance attained during interkinetic nuclear migration

	Neural tube cells (*n*=12)	Pre-neural crest cells (*n*=26)	*P* value
Average velocity from basal to apical surface (μm/hr)	66 (±12)	54 (±9)	0.44
Average velocity after mitosis to basal position (μm/hr)	8 (±3)	21 (±3)	0.03
Average time (hr:min) in mitosis	1:00 (±0:21)	1:01 (±0:12)	0.96
Maximum basal position (% of nt total distance)	66% (±5%)	70% (±3%)	0.47

The position and velocity of the nucleus was tracked in neural tube cells during interkinetic nuclear migration. The velocity of nuclear migration and total basal distance traveled were compared between neural tube cell progenitors (neural tube cells that underwent two subsequent divisions within the neural tube) and pre-neural crest cells (neural tube cells that undergo a neural tube division followed by an EMT). *P* values were determined by one-way ANOVA with alpha=0.05. Values are given (± s.e.m.). hr, hour; min, minute; nt, neural tube.