

Evidence for dynamic rearrangements but lack of fate or position restrictions in premigratory avian trunk neural crest

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SUMMARY

Neural crest (NC) cells emerge from the dorsal trunk neural tube (NT) and migrate ventrally to colonize neuronal derivatives, as well as dorsolaterally to form melanocytes. Here, we test whether different dorsoventral levels in the NT have similar or differential ability to contribute to NC cells and their derivatives. To this end, we precisely labeled NT precursors at specific dorsoventral levels of the chick NT using fluorescent dyes and a photoconvertible fluorescent protein. NT and NC cell dynamics were then examined *in vivo* and in slice culture using two-photon and confocal time-lapse imaging. The results show that NC precursors undergo dynamic rearrangements within the neuroepithelium, yielding an overall ventral to dorsal movement toward the midline of the NT, where they exit in a stochastic manner to populate multiple derivatives. No differences were noted in the ability of precursors from different dorsoventral levels of the NT to contribute to NC derivatives, with the exception of sympathetic ganglia, which appeared to be 'filled' by the first population to emigrate. Rather than restricted developmental potential, however, this is probably due to a matter of timing.

KEY WORDS: Chick, Neural tube, Neural crest, Trunk, Cell fate, Cell tracing, Photoconversion

INTRODUCTION

The neural crest (NC) is a uniquely vertebrate cell type that originates within the newly formed neural tube (NT), or presumptive central nervous system. 'Premigratory' NC precursors initially reside within the dorsal portion of the neuroepithelium along its entire length, with the exception of the anteriormost future olfactory placode region. These precursors then undergo an epithelial to mesenchymal transition (EMT), whereby they are liberated from the neuroepithelium and form migrating cells that move through adjacent tissues, following characteristic pathways.

NC cells undergo an orderly migration, such that they fill their derivatives in a ventral to dorsal progression (Weston and Butler, 1966) and then differentiate into derivatives as diverse as autonomic and sensory neurons, glial cells, facial cartilage and melanocytes (Le Douarin and Kalcheim, 1999). In the trunk of avian embryos (Krispin et al., 2010; Serbedzija et al., 1989; Weston and Butler, 1966), for example, labeling the NT early gives rise to labeled cells in the ventralmost derivatives such as sympathetic ganglia, whereas labeling NTs progressively later marks more dorsal derivatives, such that those cells migrating along the pigment pathway underneath the skin are the last to exit the NT.

One problem with labeling large populations of NT cells is that neither the site of origin within the NT nor the site of exit from the NT can be discerned. Moreover, the results of these vital labeling experiments cannot distinguish whether premigratory NC cells are fate restricted or multipotent. Microinjection of individual dorsal NT cells *in vivo* (Bronner-Fraser and Fraser, 1988) and clonal analysis

in vitro (Baroffio et al., 1988; Dupin et al., 2010; Stemple and Anderson, 1993) clearly show that single precursors can contribute to multiple NC derivatives, and that premigratory NC can form both NT and NC derivatives (Bronner-Fraser and Fraser, 1988). However, others have suggested that the first NC cells to emigrate are fate restricted as neurons or glial cells (Henion and Weston, 1997) and those migrating later are destined to become melanocytes (Henion and Weston, 1997; Reedy et al., 1998). Thus, there remains considerable controversy in the literature regarding whether some or all NC cells may be fate-restricted versus multipotent.

In an effort to resolve these issues, recent studies have used either DiI or green fluorescent protein (GFP) to label small numbers of NT cells *in vitro* (Ahlstrom and Erickson, 2009; Krispin et al., 2010). Using a semi-open book preparation, Krispin and colleagues (Krispin et al., 2010) raised the intriguing possibility that trunk NC cells may relocate within the NT in a ventral-to-dorsal direction; they suggested that this represents a spatiotemporal map within the NT that confers ventrodorsal fate restriction onto the premigratory NC. They further reported that NC cells emigrated only from the dorsal midline, without undergoing an asymmetric cell division, such that both progeny left the NT concomitantly. These results contrast with those of Ahlstrom and Erickson (Ahlstrom and Erickson, 2009) who, using transverse slice cultures, failed to note a ventral-to-dorsal relocation of cells within the NT and reported that trunk NC cells exited from any region of the dorsal NT and not solely from the midline. Moreover, the results of Krispin and colleagues are at odds with the finding from single cell lineage experiments *in vivo* (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988) showing that NC and NT progeny can arise from a single precursor.

To resolve these discrepancies, we have performed experiments *in vivo* and in slice culture in which we label cells with high precision and reproducibility at specific dorsoventral depths within the avian trunk NT. We use fluorescent dyes, photoconvertible fluorescent proteins and two-photon microscopy to highlight optically single nuclei in small subpopulations of the dorsal

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NT/premigratory NC cells in the trunk. By following single cell behaviors within the NT and examining sites in the periphery to which their progeny migrate, we find that cells from all subregions in the dorsal-quadrant of the NT have the ability to contribute NC cells to diverse dorsoventral locations. Moreover, we show that there is significant ventrodorsal movement of precursor cells within the NT that move as a cohort to the dorsal midline. Some precursors tend to remain resident in the dorsal midline, perhaps generating a stem cell ‘niche’ from which emigrating NC cells arise.

MATERIALS AND METHODS

Embryo preparation

Fertilized White Leghorn chicken eggs (Phil’s Fresh Eggs, Forreston, IL, USA) were incubated at 38°C in a humidified incubator until Hamburger and Hamilton (HH) stages 8-11 (Hamburger and Hamilton, 1951). Eggs were rinsed with 70% ethanol and 5 ml of albumin was removed before windowing the eggshell. A solution of 10% India ink (Pelikan Fount; www.mrart.com, Houston, TX) in Howard Ringer’s solution was injected below the area opaca to visualize each embryo.

Microinjection and electroporation delivery of fluorescent reporters

A solution of psCFP2 (Evrogen, PS-CFP2-N vector, #FP802, Moscow, Russia), or H2B-psCFP2 (Kulesa et al., 2009) was microinjected into the lumen of the chick NT in embryos at HH stages 10-11. Electroporation delivery used platinum electrodes and Electro Square Porator ECM 830 (BTX, Holliston, MA, USA) with 20 volts of current and 50 millisecond pulses at 500 millisecond intervals. For double label experiments, a cocktail of 2.5 µg/µl of both psCFP2 and Gap43-TagRFP was used. To create the membrane-localized TagRFP, pTagRFP-C (Evrogen, pTagRFP-C vector, #FP141) and pGap43-CFP (Clontech, Mountain View, CA, USA) were both digested with *AleI* and *BstI* and appropriate fragments were ligated.

In vivo cell marking in the dorsal NT by two-photon photoconversion

After reincubation for 16-24 hours, embryos (HH14-18) were prepared for selective cell marking within one side of the dorsal 25% (quadrant) of the NT, using targeted photoconversion. Selected subregions of the NT were photoconverted using a Plan-Apochromat 20 (0.8 NA or 1.0 NA) objective with two-photon excitation at 800 or 825 nm on either an LSM-510 or -710 (Carl Zeiss MicroImaging, Germany). The psCFP2-labeled non-photoconverted cells were visualized with ~5% laser power and cyan fluorescent protein (CFP) emission filters. Photoconverted psCFP2-labeled cells were visualized with 488 nm laser light and GFP emission filters. The Gap43-TagRFP was visualized with 543 nm laser light and red fluorescent protein (RFP) emission filters. Typically, a region of the trunk NT that was well labeled (approximately near somite 18 of 24-32 somite embryos) was selected and photoconversion was performed, as described.

Photoconverted cells were categorized by their depth from the dorsal midline of the NT in either Zone 1 (Z1; 0-15 µm), Zone 2 (15-30 µm) or Zone 3 (30-50 µm). From transverse cryosections of embryos at the same developmental stage, the NT dorsoventral length was measured to be ~200 µm, such that Z1-Z3 were up to 25% of the dorsal NT.

For *in ovo* photoconversion experiments (technique described in Stark and Kulesa, 2007), only a single plane was photoconverted in a subregion ~80 µm along the NT. The movement of the embryo resulted in a 20 µm band on average in the NT within the desired zone. Resulting structures that contained photoconverted cells were scored based on position within the transverse section using the nonconverted cells (which labeled most NC cells) and brightfield images to aid in classification. If a cell was unable to be classified based on position, it was not counted.

Fixation and tissue sectioning

After reincubation, photoconverted embryos were fixed with 4% paraformaldehyde for 16 hours at 4°C. Trunk regions of approximately six somites long were dissected out and mounted in optimal cutting temperature (OCT) media and cryosectioned in 20 µm transverse sections, then mounted in PBS on glass coverslips. Cryosections were visually screened in brightfield and imaged with a confocal microscope (Zeiss 510).

Scoring cell positions

Photoconverted cell positions were recorded as depth from the dorsal midline of the NT (using the nonconverted cells and outline of the NT from the differential interference contrast brightfield images) and lateral distance from the dorsal NT midline. The width of the NT from the midline at the depth of the photoconverted cell was also recorded so that every cell’s position could be scaled to a NT template to account for different NT sizes in each embryo. For cryosections, the length of the NT was also measured for scaling, but the total length of the NT could not be seen at the time of photoconversion because of its depth. A representative NT transverse section at HH14 and 3 days was used as templates to fit onto all measurements of individual photoconverted cells. Software from AIM (Zeiss) and Imaris (Bitplane) were used for measurements.

Slice culture time-lapse imaging

Embryo slices were prepared as described in Shiau et al. (Shiau et al., 2011). Briefly, after psCFP2 microinjection and electroporation delivery into cells, embryos were reincubated for 24 hours. Transverse sections were cut at a thickness of 300 µm using a microknife. Each slice was placed on a glass plate and embedded in collagen at a final concentration of 2.3 mg/ml (Type I rat tail, BD Biosciences, 354236) including L-15 medium (Gibco) with final concentrations of 0.02% acetic acid and ~0.25-0.35% of sodium bicarbonate to polymerize the collagen. Slice cultures were equilibrated to 38°C with 5% CO₂ level for 1 hour. Then selected cells were photoconverted (as described above) within Z3.

Some slice cultures were selected for time-lapse imaging to follow the movement of the photoconverted cells ($n=6$; Zeiss LSM-510). Time-lapse images were collected in z-stacks of 60 µm, 10 minutes apart for at least 13 hours. Photoconverted cells were tracked with the spots function and detailed statistics about the cells were exported and analyzed using Imaris (Bitplane).

Whole embryo time-lapse imaging

In a typical embryo, the trunk region of the NT of 15 somite embryos (before NC emigration) were labeled by injection and electroporation with a mixture of DiI and an H2B-eGFP plasmid. After additional incubation, 25-27 somite embryos were mounted on a semi-solid agar/albumen media (Chapman et al., 2001) either dorsal or ventral side up, depending on the microscope used. Images were collected in a single track, 7 minutes apart for 7 to 12 hours. Images were concatenated and aligned in ImageJ using either the 3D drift or the descriptor based registration (Preibisch et al., 2010) plugins. Cells were manually tracked using Imaris (Bitplane).

Molecular profiling

Fluorescent cells of interest were isolated by contact-free laser capture microdissection (LCM) using a protocol developed in the Kulesa Laboratory (Morrison et al., 2012). Specifically, we analyzed trunk NC cells: (1) shortly after emigration from the dorsal midline of the NT; (2) along the medioventral sympathetic ganglia (SG) pathway; or (3) along the dorsal root ganglia (DRG) pathway. Specific transcripts were linearly pre-amplified using Ambion’s Cells-to-Ct kit (Applied Biosystems/Ambion Austin, TX, USA, #AM1729M). RT-qPCR was performed by the Fluidigm Genetic Analysis Facility of the Molecular Genetics Core Facility at Children’s Hospital Boston on Fluidigm’s BioMark HD system (Fluidigm, South San Francisco, CA, USA). Raw Ct data was analyzed with Integromics’ RealTime StatMiner bioinformatics software.

RESULTS

NC cells at the trunk level migrate along two pathways: a ventral pathway followed by precursors to sensory and sympathetic ganglia as well as adrenomedullary cells; and a dorsolateral pathway followed by precursors to melanocytes. In chick, NC cells first follow the ventral pathway, with the dorsolateral pathway opening approximately 1 day later. Moreover, they are thought to fill their derivatives in a ventral to dorsal order, such that the most ventral (sympathoadrenal) locations are filled first, followed by progressively more dorsal derivatives (Krispin et al., 2010;

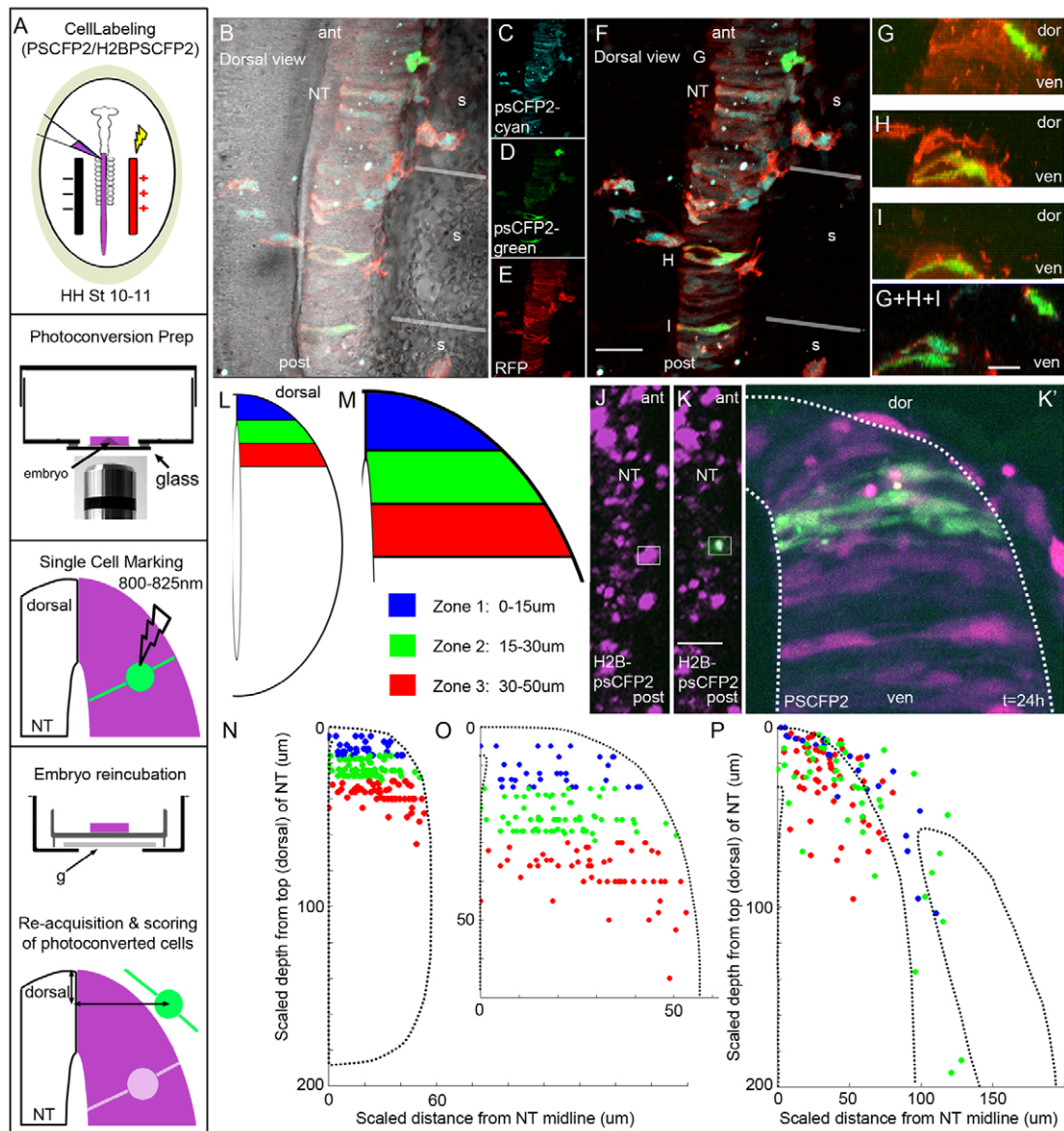


Fig. 1. Photoconversion of cells in the dorsal quadrant of the NT *in vivo*. (A) Experimental schematic. (B) Typical multicolor labeled embryo (Gap43-TagRFP and psCFP2) with brightfield image and four photoconverted cells in the NT. (C-E) Individual fluorescence channels of the image in B. (F) Same image as shown in B, but without the underlying brightfield image. The photoconverted cells in G-I are marked. (G-I) Cropped sections of the NT rotated 90° around the x-axis to view the individual cells from F. (J,K) A typical HH St 14 embryo labeled with an area selected for photoconversion (box) and (K) two-photon converted cell. (K') A typical cryosection of the trunk of an embryo 24 hours post-photoconversion. (L,M) Schematic of NT, highlighting zones within the dorsal quadrant of the NT. (N) Locations of all photoconverted cells at the time of photoconversion mapped onto a template NT. The boundary of the NT is marked with dotted line. Cells are color-coded by zone. (O) The location of cells at the time of photoconversion. (P) Locations of all observable photoconverted NC cells after 16-24 hours of incubation. The boundary of the NT and DRG is indicated by the dotted line. Scale bars: 40 μm in F,K; 20 μm in G-I.

Serbedzija et al., 1989; Weston and Butler, 1966). Here, we use sophisticated imaging techniques to accurately label NT precursors at precise dorsoventral levels in the dorsal quadrant of the NT to determine their ability: (1) to translocate within the NT before emigration; (2) to contribute to migrating NC cells; and (3) to contribute to various NC derivatives.

Several dorsoventral levels of the trunk NT give rise to emigrating NC cells *in vivo*

We first asked whether their dorsoventral position within the NT contributes to their ability to give rise to progeny that emigrate

from the NT as NC cells. To this end, we fluorescently marked small numbers (1-5) of NT cells at different dorsoventral levels in the trunk at the wing bud level (between somites 14 and 18) of HH stage 13-17 embryos. At this stage, NC specifier genes *FoxD3*, *Snail2* and *Sox9* are expressed in the dorsal portion of the NT just before and during early NC emigration (supplementary material Fig. S1). Embryos were electroporated with a photoconvertible fluorescent protein (psCFP2 or nuclear localized H2B-psCFP2) at HH stages 10-11 to precisely label NT cells (Fig. 1A-K'; supplementary material Movie 1). Cell locations were mapped onto a template NT and three zones within the dorsal quadrant of the NT

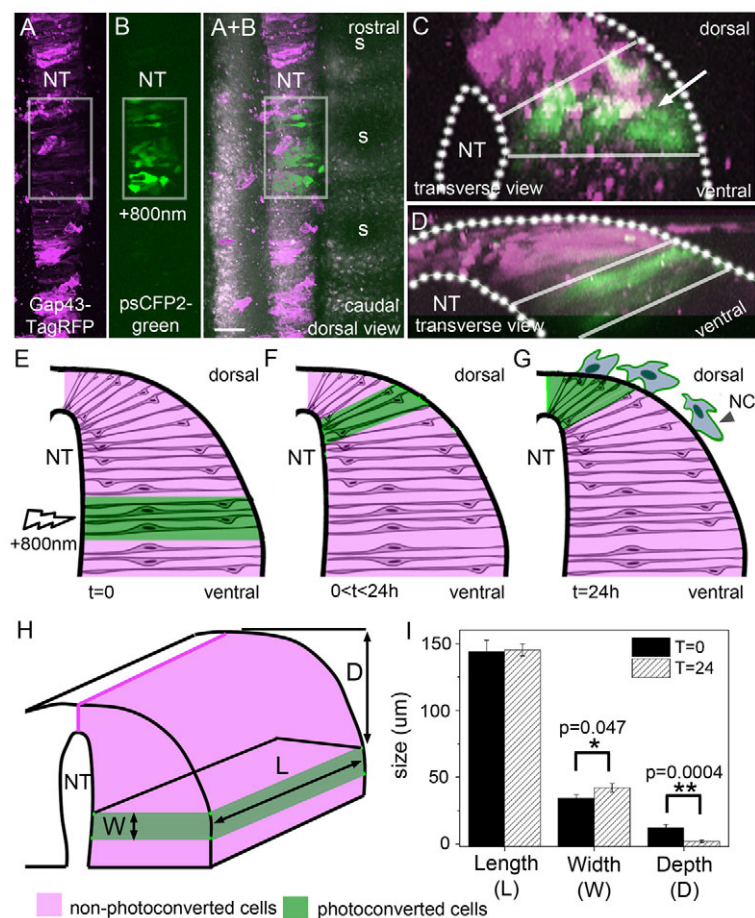


Fig. 2. Photoconversion of a band of cells in the dorsal quadrant of the NT and 24 hours of reincubation.

(A) Gap43-TagRFP image after photoconversion (box). (B) psCFP2 image. (A+B) overlay of green and red channels with brightfield. (C) Transverse view of converted (box; arrow) and nonconverted psCFP2 cells. (D) Same embryo 24 hours later. (E-G) Representation of NT cell movements based on the photoconversion data. (H,I) Measurement of the changes in volume for photoconverted cell clusters. The depth of the top of the band, the width of the band and the length in the anterior/posterior direction were measured at the time of photoconversion and after 24 hours of reincubation ($n=17$).

were defined (Fig. 1L-O). Photoconverted cells were categorized by their depth from the dorsal midline of the NT in either Zone 1 (Z1; 0-15 μm), Zone 2 (15-30 μm) or Zone 3 (30-50 μm). Cells below 50 μm were not converted because it became difficult to image accurately below this level. Thus, NC precursor cells potentially could arise even from below Zone 3. However, we used this level for comparison, as it compares with that examined in previous studies (Krispin et al., 2010).

Embryos were incubated for an additional day, allowing sufficient time for emigration of many NC cells. After fixation, the locations of photoconverted nuclei were mapped onto a template NT and pseudo-colored according to their position of origin in Z1 ($n=25$), Z2 ($n=46$) or Z3 ($n=60$) (Fig. 1P). Cells from all levels (Z1-Z3) contributed to the migrating NC population by 24 hours post-labeling. Given that NC cells emerge from the dorsal portion of the NT, it is not surprising that a larger fraction of labeled cells emerged from dorsal rather than ventral levels. Whereas over half (56%) of the labeled cells from Z1 had emigrated from the NT, 46% had emigrated from those labeled at Z2 and 18% from Z3 (Fig. 1P). In all cases, the rest of the labeled cells remained in the NT (Fig. 1P).

Sites of localization of NC cells one day after photoconversion

At 24 hours post-photoconversion, NC cells from Z1-Z3 were mostly localized in dorsal regions of the embryo, suggesting that their migration was still in progress (Fig. 1P). This was particularly true for Z3, where labeled cells had only recently emigrated and some were found close to the dorsal NT midline (Fig. 1P).

Interestingly, however, even at this early time point, we found numerous cells labeled from all zones (Z1-Z3) on the presumptive SG and DRG ventral pathways (Fig. 1P).

Photoconversion of subgroups of NC precursors reveals cell dynamics in the dorsal NT

To analyze cell movements within the dorsal trunk NT, small clusters of NT cells were photoconverted at the dorsoventral levels of Z2 or Z3 (Fig. 2A-C; supplementary material Movies 2, 3). Embryos were allowed to develop for a further 24 hours and the positions of photoconverted cells were examined by two-photon microscopy. We observed that clustered, labeled cells remained together as a band (Fig. 2D-G) as cells traveled from ventral to dorsal positions. Non-photoconverted cells did not infiltrate the photoconverted cluster of cells in a wholesale manner, but one or two nonconverted cells were observed within a cluster (Fig. 2D-G).

Measurements of the shape of the photoconverted cluster of cells revealed that its length did not change appreciably over 24 hours (1% increase), indicating that the cells did not spread in the anterior/posterior direction while the embryo grew (Fig. 2H,I). However, the width (in the dorsoventral axis) grew 23% larger on average from its original size (Fig. 2H,I). In addition, the depth of the top of the cluster decreased over time by 84%, indicating that the cells moved dorsally in the NT (Fig. 2I). Because NC cells had already exited the NT at 24 hours after photoconversion in some embryos, this depth change may be more significant than represented. We did not measure whether there were changes in individual cell volume over time.

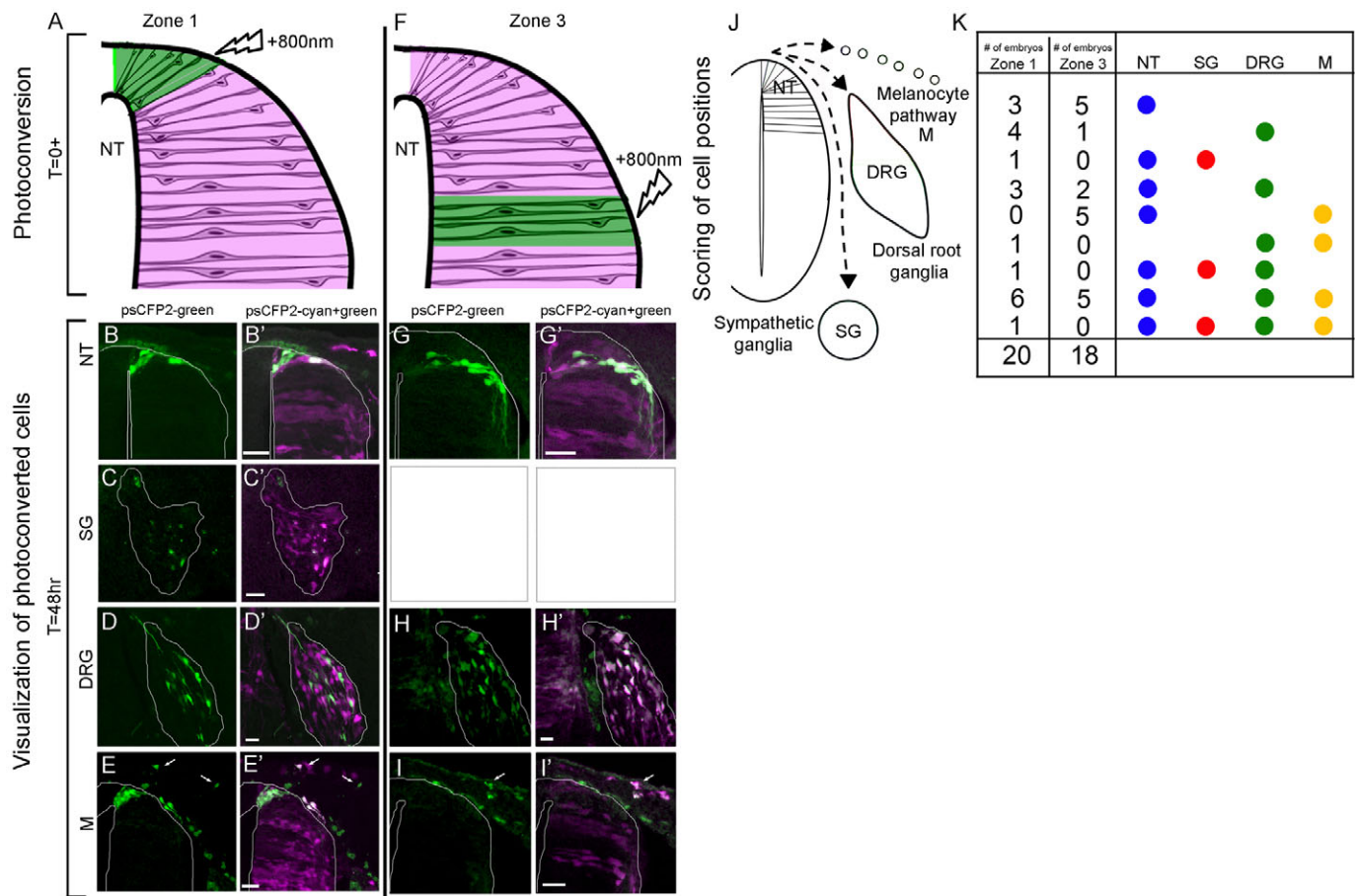


Fig. 3. *In ovo* photoconversion of a band of cells in the dorsal quadrant of the NT and 48 hours of reincubation. (A-I') Cells in either zone 1 (A) or zone 3 (F) were photoconverted *in ovo*. (B-E', G-I') Resulting locations of the photoconverted cells. Autofluorescence of blood cells in the GFP channel should not be confused with NC cells, which appeared in both the GFP and CFP channels. The scored structures are: NT, SG, DRG and melanocyte (M) pathway. (J) Schematic of the trunk NC cell migratory pathways and the target locations. (K) Table showing the number of embryos with photoconverted cells in various target locations after 48 hours of reincubation.

NC cells from Zones 1 and 3 contribute to NC derivatives on both dorsal and ventral pathways

The above results suggest that precursors from Z1, Z2 and Z3 may all contribute to emigrating NC cells. However, cells from Z3 are just exiting the NT by 24 hours post-photoconversion. To allow sufficient time for these cells to migrate to prospective derivatives, we performed *in ovo* photoconversion and reincubated embryos for longer times, up to 48 hours (4.5 day embryo) (Fig. 3). It has been shown previously that chick NC cells cease NT exit at around 3.5-4 days (Serbedzija et al., 1989) so at this developmental stage, most if not all NC cells would have exited the NT.

The results show that labeled cells emerging from both Z1 (Fig. 3A-E') and Z3 (Fig. 3F-I') migrated to various sites in the embryo, including both the ventral and the dorsolateral pathways. Z3 cells contributed both to presumptive melanocytes on the dorsolateral pathway and DRG on the ventrolateral pathway (Fig. 3F-K). Similarly, cells emerging from Z1 contributed to melanocytes, DRG as well as SG (Fig. 3A-E', J, K), a derivative not occupied by cells emerging from Z3. This is not surprising and agrees with previous cell-labeling studies that show that this ventral derivative becomes 'filled' by the first migrating NC cells (Serbedzija et al., 1989; Weston and Butler, 1966).

Interestingly, the overwhelming majority of embryos not only generated labeled cells in NC derivatives, but also had sister cells

in the NT (Fig. 3B, B', G, G', K). In fact, only six embryos (five from Z1 and one from Z3) gave rise exclusively to NC cells that localized in the DRG or melanocyte (Fig. 3K). This suggests that there remains a 'resident' population of NC cells in the dorsal midline that gradually contributes to emigrating NC cells. This would explain why cells from Z1 also contribute to melanocytes. However, in some cases, the photoconverted cells had long protrusions away from the NT and appeared to contribute to sensory neurons that projected axons from the NT. Thus, these did not contribute to NC cells but rather to precursors of dorsal sensory neurons that must have been mixed in with NC precursors at the time of photoconversion (Fig. 3G).

Molecular profiling reveals trunk NC cells have distinct expression patterns depending on their selection of migratory pathway

To examine the molecular profile of trunk NC cells in a spatiotemporal manner, newly emerged NC cells (EMT), as well as NC cells on the presumptive SG and DRG pathways were isolated and used as templates for RT-qPCR (Fig. 4; supplementary material Table S1). NC cells on both the presumptive SG and DRG pathways (Fig. 4A) had molecular profiles that were significantly different from those cells that had recently emigrated from the NT (Fig. 4B). We found that genes

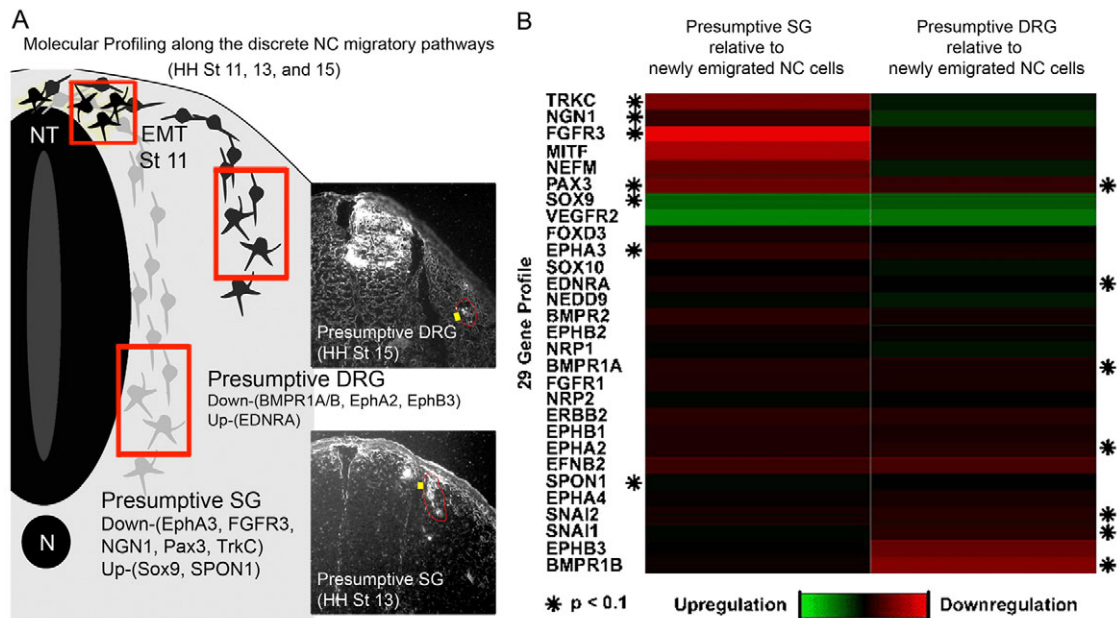


Fig. 4. Trunk NC cell molecular profiles are distinct and depend on cell position along different migratory pathways. (A) Schematic representation of the three locations from which trunk NC cells were isolated (red boxes). NC cells were isolated via LCM (red ring) shortly after EMT at HH St 11, along the medioventral pathway at HH St 13, and along the ventral pathway at HH St 15. (B) Heat map of changes in gene expression of NC cells on the ventral pathway compared with EMT expression.

characteristic of sensory neural precursors (*EPHA3*, *FGFR3*, *NGN1*, *PAX3* and *TRKC*) were significantly downregulated in NC cells on the presumptive SG pathway, compared with those recently emigrated (Fig. 4B). *SOX9* and *SPON1* were significantly upregulated (Fig. 4B). By contrast, genes such as *BMPR1A*, *BMPR1B*, *EPHA2*, *PAX3*, *SNAIL2* and *SNAIL1* were all significantly downregulated in NC cells on the presumptive DRG pathway, when compared with those cells recently emigrated from the NT (Fig. 4B). *EDNRA* was significantly upregulated (Fig. 4B). These results suggest that after exiting the NT, NC cells alter their molecular profiles, depending on their migratory pathway. More specifically, guidance receptor expression is altered (BMP receptors and EphAs) depending on the pathway, suggesting that different combinations of guidance cues are used along the different pathways. Furthermore, following EMT, those NC cells that follow the presumptive SG pathway express markers characteristic of a neuronal fate, suggesting that these cells begin differentiation earlier than cells on the DRG pathway (Fig. 4B).

NC precursors move as a cluster but exit from precocious locations in slice culture explants

Previous time-lapse imaging analyses in chick trunk slice culture explants focused on NC EMT (Ahlstrom and Erickson, 2009). However, due to the short length of the time-lapse imaging (<6 hours) and observation of only superficially located NT cells (cell depths at less than 20% of the 300 μ m slice thickness), it is unclear as to the precise cell behaviors during ventral to dorsal rearrangement of the NT cells, as we have described above from static 3D imaging.

To better visualize NT cell dynamics, we used an improved tissue slice culture explant preparation that allowed us to observe chick trunk NT cells for over 13 hours (Fig. 5; supplementary material Movie 4). We found that NT cells moved in a ventral to

dorsal manner as a cluster, which agreed with our static data (Figs 2, 3). However, NC precursors exited at precocious locations along the basal side of the dorsal NT, rather than at a common exit point near the dorsal midline (Fig. 5E,F; supplementary material Movie 4). Cell tracking revealed that some photoconverted cells in Z3 moved in a ventral to dorsal direction in the NT, but some cells exited before reaching the dorsal midline (Fig. 5C,D). Some photoconverted cells also crossed over to the contralateral side of the NT (Fig. 5C-F). Lastly, the NT cells in slice culture traveled faster in the dorsal direction compared with our 3D static data predictions of cell movements (Fig. 5D-F). However, we observed that some cells photoconverted in Z3 did not have enough time to exit the NT over the course of a typical time-lapse session (Fig. 5D-F; >13 hours).

In vivo time-lapse imaging provides new insights into NC cell precursor behaviors

To examine the *in vivo* cell dynamics in the dorsal NT, we adapted a whole chick embryo culture technique to allow for upright confocal time-lapse microscopy (Fig. 6; supplementary material Movies 5, 6). By fluorescently labeling the nuclei of dorsal NT cells on one side of the embryo and co-labeling cells with a lipophilic dye (DiI), we could follow individual cells and judge the boundaries of the NT (Fig. 6A,B; supplementary material Movies 5, 6). Time-lapse imaging revealed three major features of cell dynamics in the dorsal NT. First, we found that the majority of NC cells tracked (75%) exited the NT within one to two cell diameters of the dorsal midline (Fig. 6B; supplementary material Movie 5). After emerging from the NT, NC cells roughly maintained a spatial order of emigration into the surrounding microenvironment (Fig. 6B; supplementary material Movie 5). Observations of NC cell behaviors from a transverse view (XZ-plane) confirmed the spatiotemporal manner of NC cell exit from the dorsal NT midline (Fig. 6C; supplementary material Movie 5). Of the 25% of cells

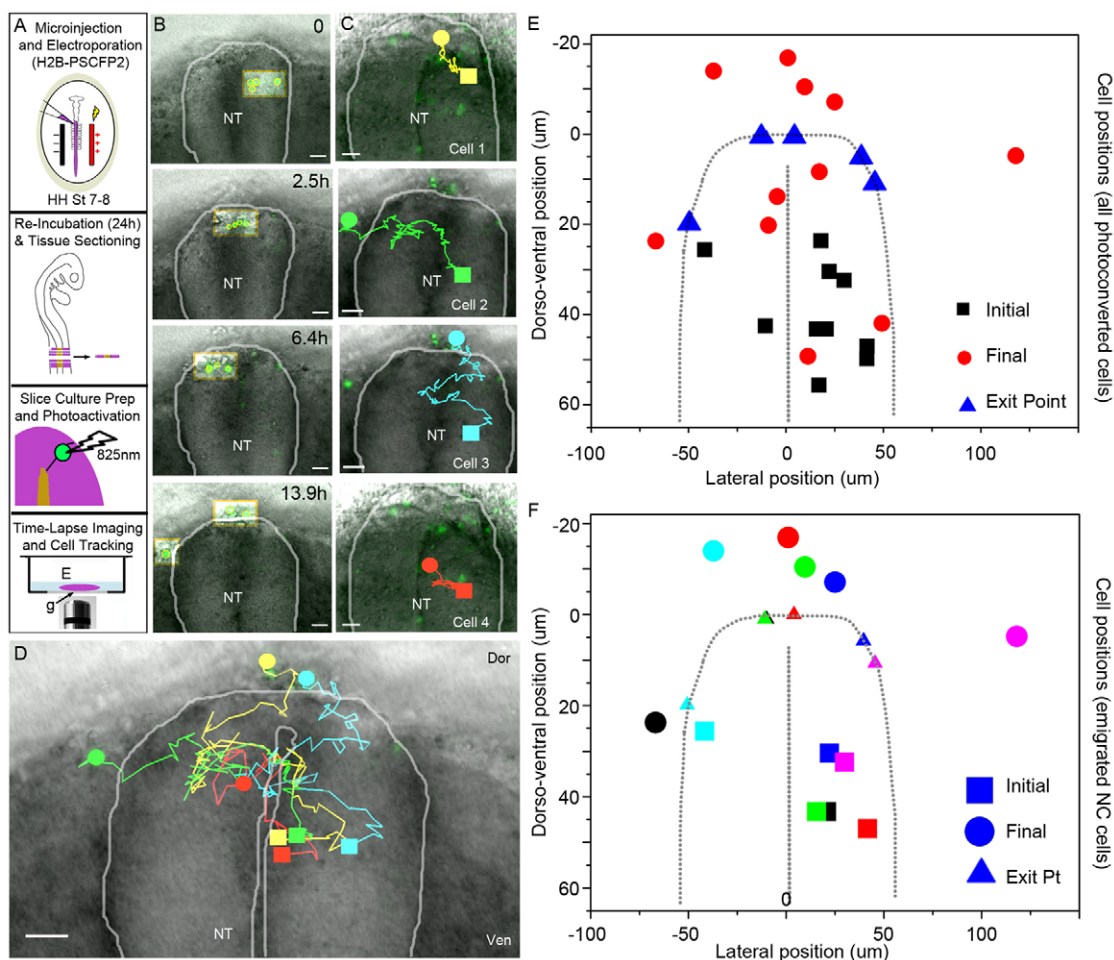


Fig. 5. Slice culture time-lapse imaging of trunk NC precursor cell dynamics and NT exit. (A) Experimental schematic. (B) Selected frames highlighting four photoconverted cells during their progression through the NT. (C) Four trajectories of different photoconverted cells from two different time-lapses overlaid on the final frames. (D) Initial (black square) and final (red circle) positions of photoconverted cells from a single time-lapse, overlaid. (E) Initial, final and NT exit positions of cells from two time-lapse imaging sessions mapped onto a template NT. (F) Initial (square) and Final (circle) positions of only cells that exited the NT. Each cell has a unique color. Scale bars: 20 μm . B, beginning, E, end.

that exited further away from the midline, all of these cells emigrated to the ipsilateral side of the embryo. NC cells that exited near the dorsal midline emigrated to both ipsilateral and contralateral sides of the embryo (data not shown).

Second, in approximately half (55%) of the tracked cells that exited the NT, we did not detect a cell division immediately before exit (Fig. 7A; see also supplementary material Movie 7). When cells divided near the dorsal NT midline and exited the NT, we found that only one progeny exited the NT (83%; Fig. 7A,A'; supplementary material Movie 7). Both cell progeny were observed to exit the NT occasionally (Fig. 7B,B'; supplementary material Movie 8). Cells that divided near the basal surface of the NT resulted in both or just one cell progeny exiting the NT (Fig. 7B,B'; supplementary material Movie 8). Third, we observed that neighboring cells within the dorsal NT exited at different times, and as much as 2 hours later (Fig. 7C,C'; supplementary material Movie 9). In addition, some NT cells were able to bypass or move around another cell at the dorsal midline and exit the NT, with the 'bypassed' cell remaining in the NT for several hours (Fig. 7D,D'; supplementary material Movie 10). A summary of the typical cell dynamics scenario is shown in Fig. 7E.

DISCUSSION

We used advanced optical imaging to study avian NC cell precursors and their contribution to peripheral trunk targets. Previous analyses have led to contradictory conclusions regarding whether NC cells exit from a common location in the dorsal NT, undergo asymmetric cell divisions, share a lineage with central nervous system cells, and/or are fate restricted before emigration. This is largely owing to the technical challenges associated with labeling and visualizing cells within the dorsal NT. Previous NT cell labeling and imaging techniques have had to 'guess' the number of labeled cells and their dorsoventral position, as iontophoretic injection and either a fluorescence widefield or dissection microscope cannot reproducibly label cells and accurately measure fluorescently labeled cells at a particular location of the dorsal NT, unless the NT midline is cut open and flattened to allow a glass needle to penetrate into the tissue (Krispin et al., 2010), or is prepared as a slice culture (Ahlfstrom and Erickson, 2009). Such physical interventions may have affected cell behaviors. Thus, there was a need to clarify the emigration of trunk NC cells using more sophisticated cell labeling and imaging.

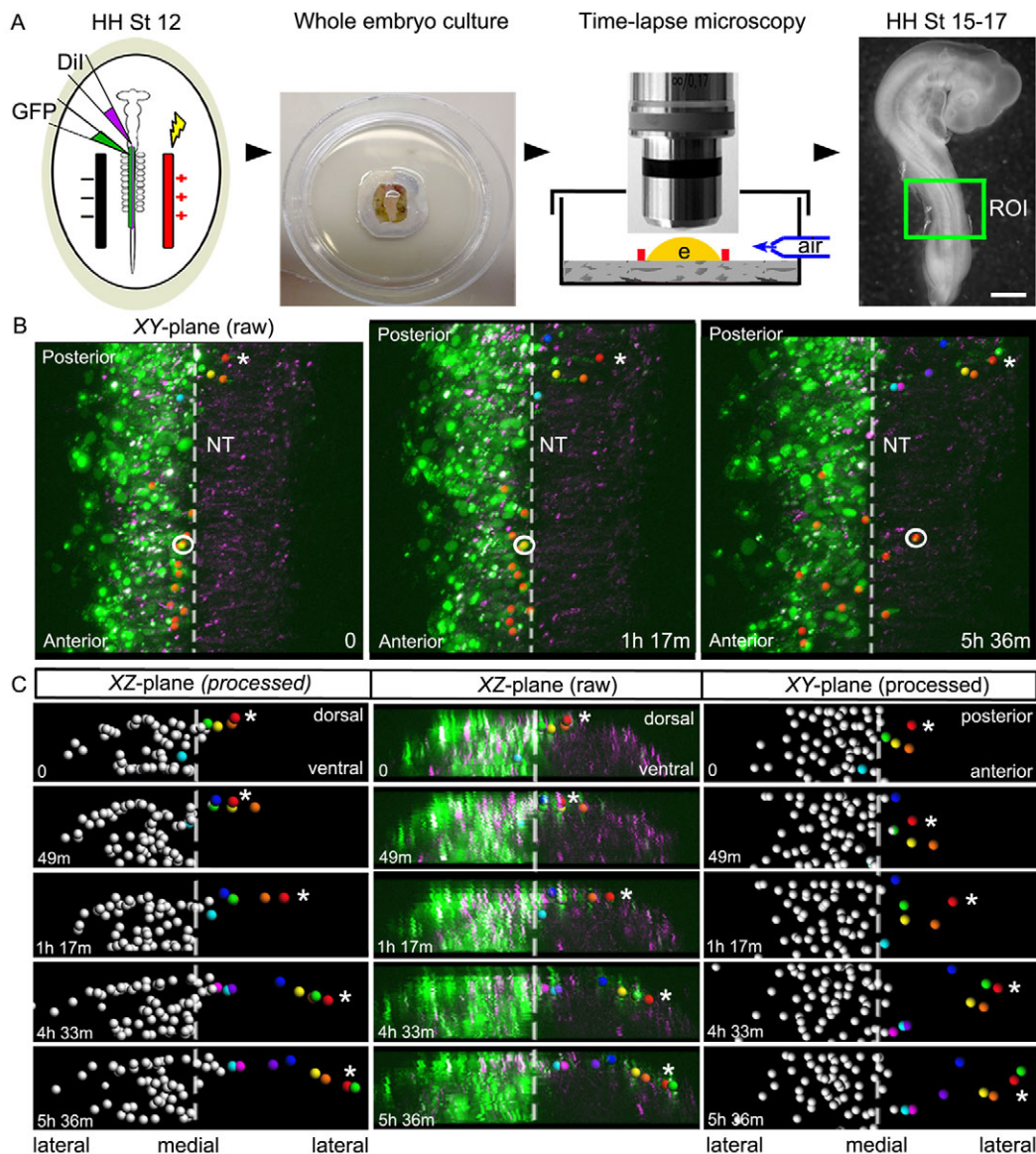


Fig. 6. *In vivo* time-lapse imaging of trunk NC precursor cell dynamics and NT exit. (A) Experimental schematic. (B) Dorsal view with Dil (magenta) and GFP (green) and tracked cells highlighted with spots. The group of spots is colored according to the order in which they emerged from the NT (red-to-magenta). The midline is marked with the dashed white line and the image has been oriented to more clearly see the cell positions. (C) *xz*- (first two columns) and *xy*- (third column) projections of a subregion through the NT (first two columns). All GFP-labeled cells (white spots) and same cells (color spots) as in B.

To circumvent these problems, we have used fluorescent dyes and the photoconvertible fluorescent protein psCFP2 plus two-photon microscopy to provide a more precise and reproducible means to label and examine *in vivo* cell behaviors in distinct subregions of the dorsal quadrant of the NT. Two-photon marking at a single focal plane made possible selective marking and observation of single or small subgroups of cells without the need to cut open the NT or tear through cells above or below the region of interest. This allowed us to resolve discrepancies in previous studies and provided a clearer picture of the behavior of NC cell precursors and their contribution to peripheral targets.

Our *in vivo* results, first, revealed that NC cells came from all subregions of the dorsal quadrant of the NT. Second, NT cells moved from ventral-to-dorsal positions as a tight cluster that

expanded in volume. Cell divisions within the cluster appeared to change the cluster volume. Despite moving as a cluster, some labeled cells remained in the NT 48 hours after photoconversion, regardless of their initial dorsoventral position, raising the possibility that these cells remain as a ‘resident’ stem or precursor cell population in the dorsal midline. Third, we found that NC cell precursors exited the NT in a stochastic manner. Time-lapse imaging revealed complex cell dynamics during NT exit, with no strict relationship between symmetric versus asymmetric cell divisions or initial position of cell progeny in predicting exit times from the NT. Fourth, cells from each subregion of the dorsal quadrant were found on both ventral (neuronal) and dorsal (melanocytic) migratory pathways. Finally, molecular profiling showed that migrating NC cells along the SG or DRG pathways

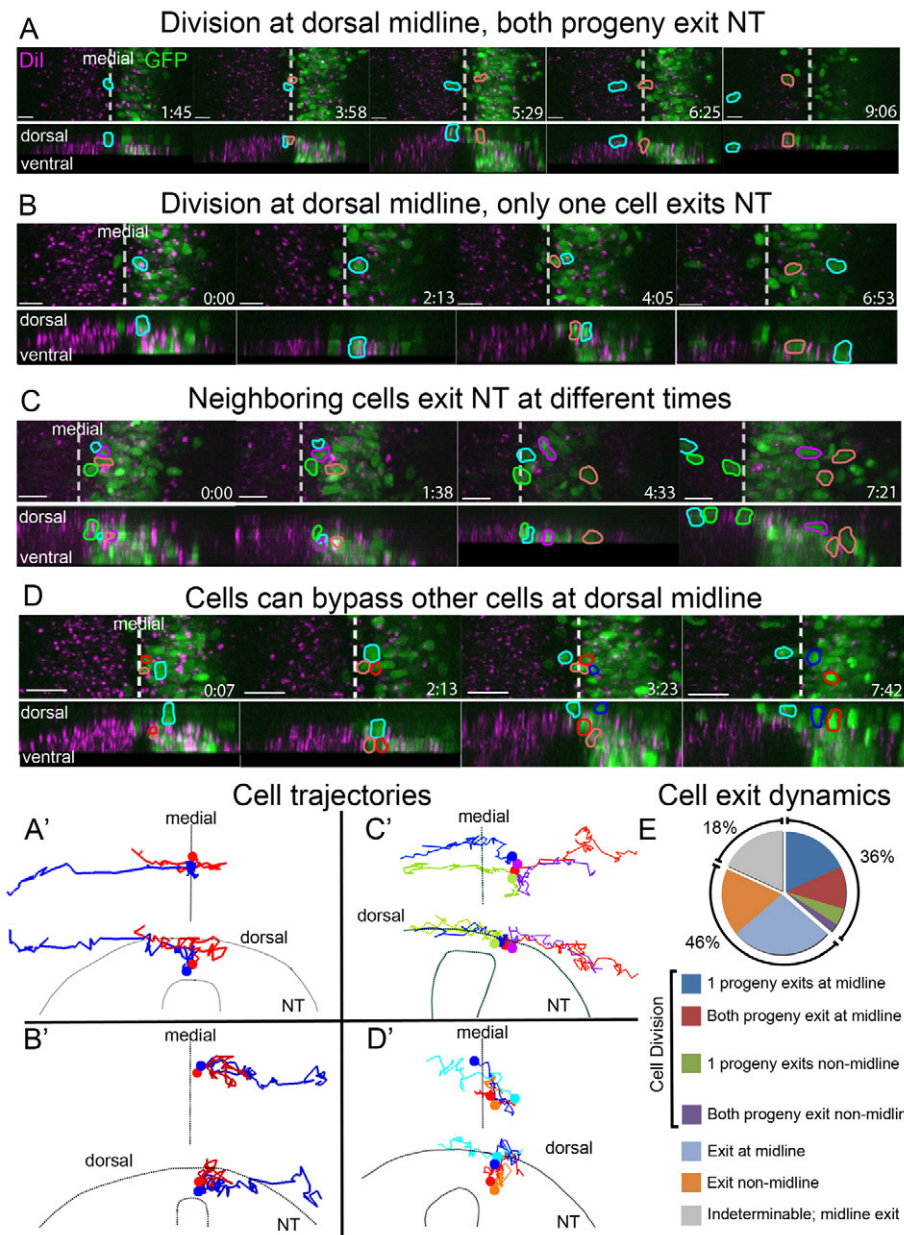


Fig. 7. *In vivo* time-lapse imaging of trunk NC precursor cell movements, cell division dynamics and NT exit. **(A)** An example of a dividing cell immediately before exit at the dorsal midline. Both progeny exit the NT. First row: xy projection. Second row: xz projection. Time is in hours:minutes. Cell nuclei are circled in cyan and red and cell trajectories (A') are shown in from both views. Initial cell positions are marked with a colored solid circle. **(B)** An example of a dividing cell at the dorsal midline, but only one progeny leaves the NT. The dividing cell nuclei are circled and the (B') cell trajectories are shown. **(C)** An example of neighboring cells that exit the NT at very different times and (C') corresponding cell trajectories. **(D)** An example of a cell bypassing another cell at the dorsal midline. (D') Trajectories of cells in D. **(E)** Distribution and description of exit dynamics for all tracked cells ($n=44$). Dil-labeled cell divisions were indeterminable (gray region). Scale bars: 20 μm .

had distinct gene expression profiles in comparison with newly emigrated cells near the dorsal NT.

Our *in vivo* data suggest that trunk NC cells are likely to exit from a common location in the dorsal midline, consistent with a model inferred by Kalchauer and colleagues (Krispin et al., 2010). They showed that by cutting open the dorsal NT and accessing more ventral regions for cell labeling, that cells moved in a ventral-to-dorsal manner and exited from the midline (Krispin et al., 2010). However, in the absence of time-lapse imaging, they could not confirm whether cells traveled all the way to the dorsal midline to exit or exited before this time. By contrast, Erickson and colleagues showed that NC cells exited from multiple levels of the dorsal NT, rather than at the dorsal midline (Ahlstrom and Erickson, 2009). Using an improved trunk slice culture technique, we confirmed that NC cells exited from precocious dorsal NT locations and also moved across to exit on the contralateral side of the NT (Fig. 5). Although our time-lapse results are similar to those of Ahlstrom and Erickson, we could not rule out the

possibility that these cell behaviors were due to the *in vitro* slice culture assay rather than being representative of how cells behave *in vivo*. *In vivo*, our data showed that most NC cells (~75%) undergo EMT close to the dorsal midline with only a smaller number (~25%) exiting from precocious dorsal NT locations (Fig. 6).

Cells from all subregions of the dorsal quadrant of the NT populated the presumptive neuronal and melanocyte migratory pathways (Fig. 3). This suggested that emigrating NC cells from many subregions of the dorsal NT were equipotent in their ability to populate a particular trunk NC target. Reincubation of embryos for 48 hours after photoconversion of NT cells showed that cells from both Z1 and Z3 regions migrated along ventral and dorsal migratory pathways, suggesting that the choice of NC cell migratory pathway does not correlate with position of origin in the NT.

After undergoing EMT, NC cells altered their gene expression profiles depending on whether cells selected the presumptive SG

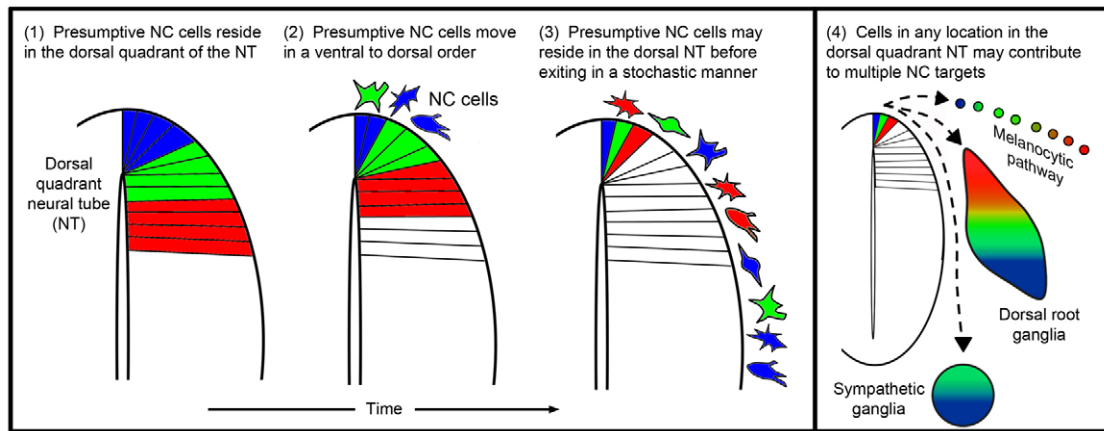


Fig. 8. Unequal replenishment model of NC cell emigration. (1) The dorsal quadrant of the neuroepithelium is divided into three zones: Z1, Z2 and Z3. (2) More ventrally located cells move dorsally and those cells in the vicinity of the dorsal midline undergo EMT and exit the NT. (3) Cells from Zones 1-3 intermingle at the dorsal midline such that a random assortment of cells from any zone may exit the NT. (4) Multiple zones of the dorsal NT can contribute to NC cells that follow distinct migratory pathways.

or DRG migratory pathways, suggesting that NC cell guidance instructions were not hardwired by signals within the NT. Because microenvironments through which NC cells travel may contain distinct guidance cues, we expected the gene expression patterns of cells traveling along the presumptive SG or DRG pathways to be different. This was indeed the case. Gene profiling showed that subpopulations of NC cells had gene expression patterns distinct from each other and from the subpopulation of NC cells, immediately after emigration from the NT (Fig. 4). NC cells traveling along the presumptive SG pathway had significantly downregulated a set of genes typical of NC cells en route to the DRG (Fig. 4).

The very dynamic and stochastic manner of NC cell exit from the trunk dorsal NT suggests an ‘unequal replenishment’ model of NC cell emigration (Fig. 8). In this model, the number of exiting NC cells does not equal the number of cells that reach the dorsal midline. Importantly, there is not an order to their emigration (Fig. 8), in that more ventral cells are able to leapfrog dorsally positioned neighbors to exit the NT (Fig. 7). This model is based on 3D static and time-lapse imaging evidence revealing that some cells that reach the dorsal midline remain resident quite some time before exiting (Figs 2, 3, 7). Although the majority of NC *in vivo* exit at the dorsal midline, a small number of cells (~25%) emigrate from other dorsal NT locations, further supporting an unordered cell emigration event.

To our knowledge, these data represent the first report of *in vivo* cell labeling and time-lapse imaging of cell movements within the dorsal NT, allowing us to mark and visualize emigrating NC cells with unprecedented accuracy. These data support our proposed ‘unequal replenishment’ model, as an alternative to the previously proposed idea that trunk NC cell precursors are specified into different lineages based on position within the NT. The latter would require NC cells to emigrate in a highly ordered fashion (Krispin et al., 2010), a finding that our imaging data fail to support. Future studies will need to address what mechanisms regulate this more complex, unordered emigration of trunk NC cells and how some cells escape EMT and remain in the NT. The approach shown here is an exciting step forward in allowing direct observation and measurement of complex cell behaviors during embryogenesis.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.083725/-/DC1>

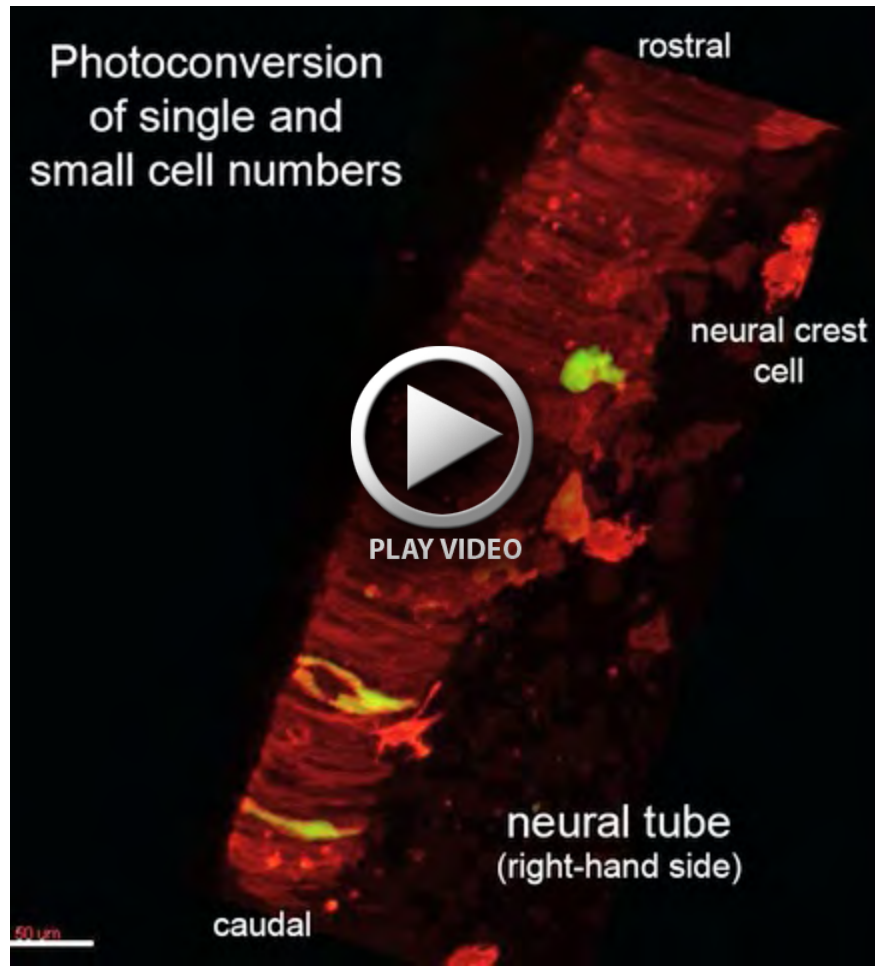
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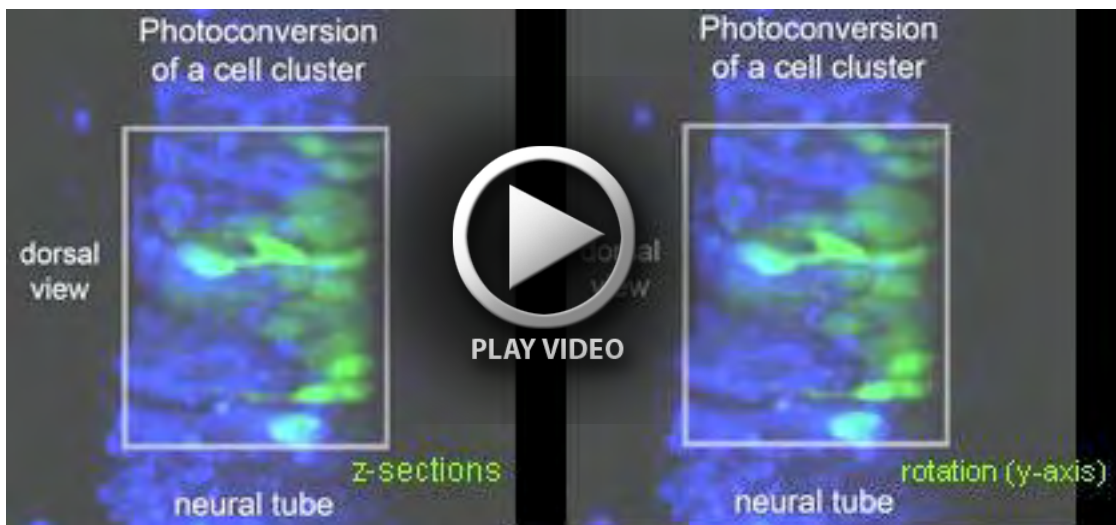
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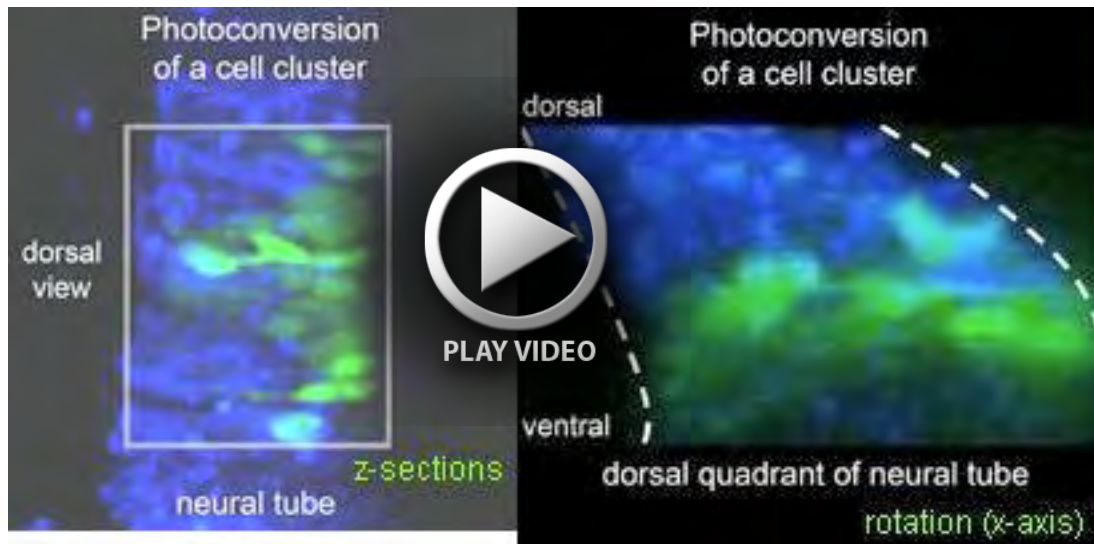
Fig. S1. In situ hybridization. (A) *FOXD3*, (B) *SNAIL2* and (C) *SOX9* expression in the chick trunk at the wing bud level (between somites 14 and 18) of HH stage 13-17 embryos, comparing neural crest specifier gene expression with previously presented images of similarly staged photoconverted embryos with approximate locations of the three zones marked.



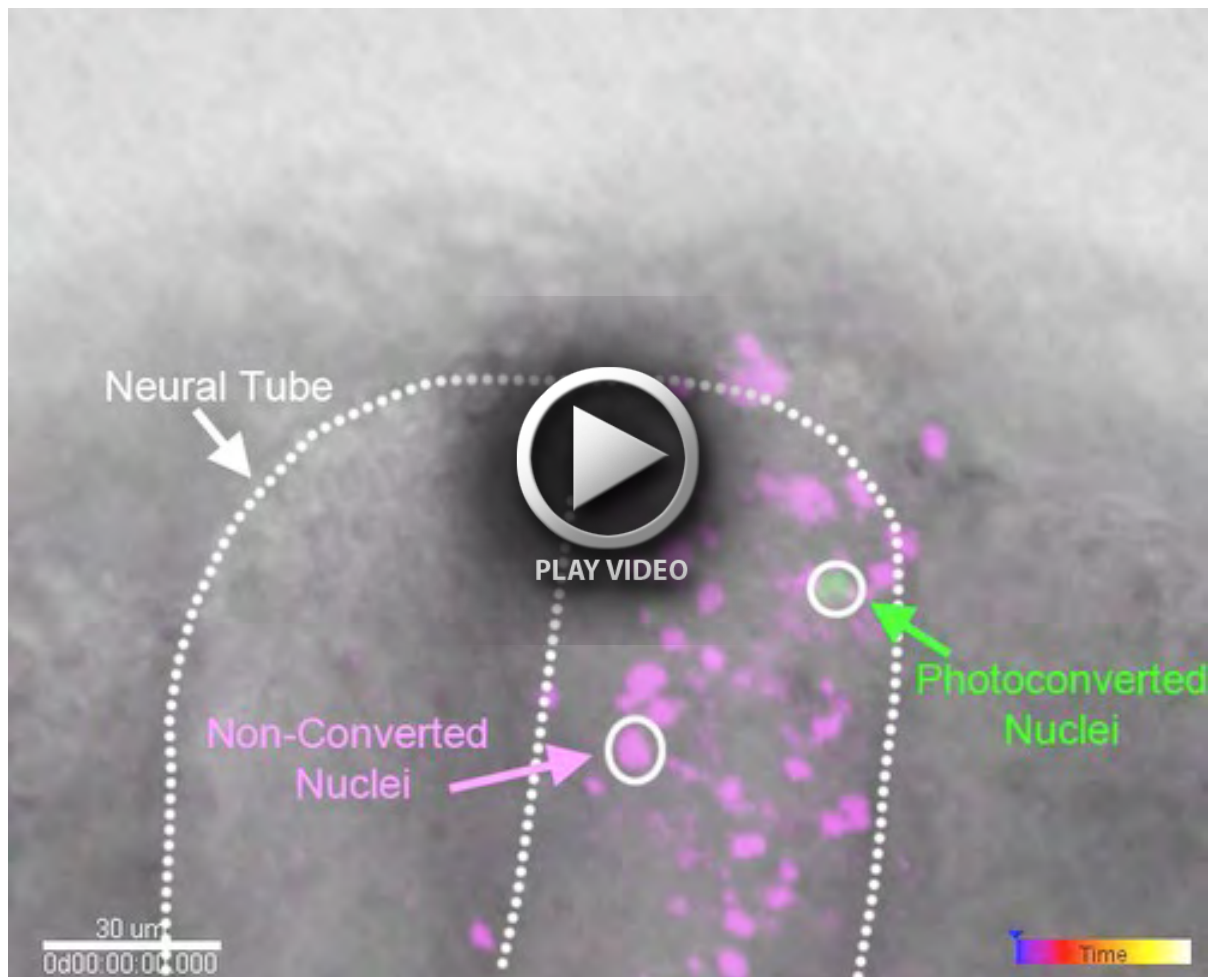
Movie 1. Two-photon photoconversion of single and small numbers of cells *in ovo* labeled with psCFP2 and Gap43-TagRFP. Confocal z-stack rotated with various channels displayed.



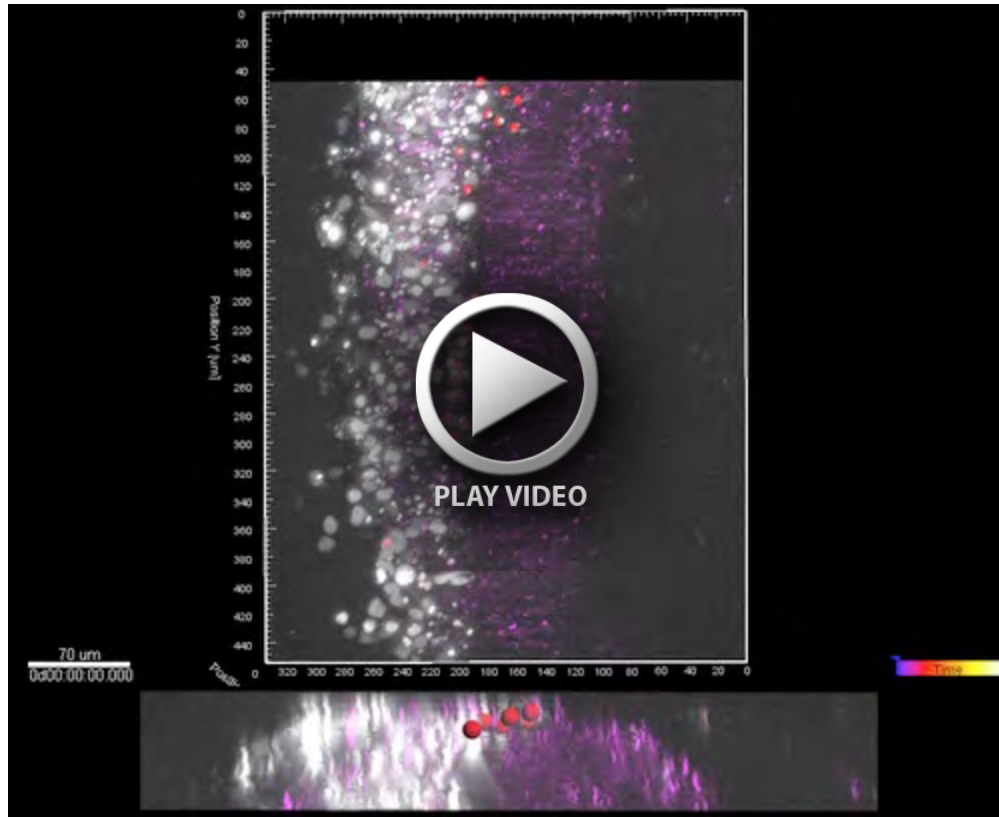
Movie 2. Two-photon photoconversion of a cluster of cells in a single zone of the dorsal quadrant of the neural tube. Confocal z-stack slice by slice as well as rotation around y-axis.



Movie 3. Two-photon photoconversion of a cluster of cells in a single zone of the dorsal quadrant of the neural tube. Confocal z-stack slice by slice as well as rotation around *x*-axis.



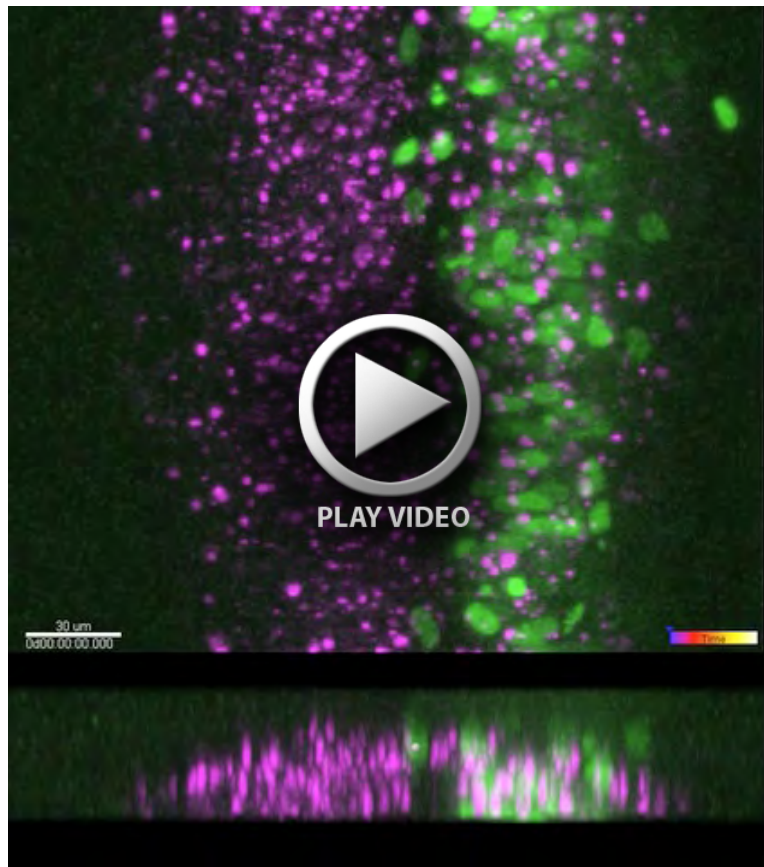
Movie 4. Time-lapse imaging of H2B-psCFP2-labeled cells in transverse slice culture. The first frame shows nonconverted and photoconverted psCFP2 cells. Subsequent images show only the photoconverted cells for clarity. Images collected every 9.6 minutes for ~13 hours.



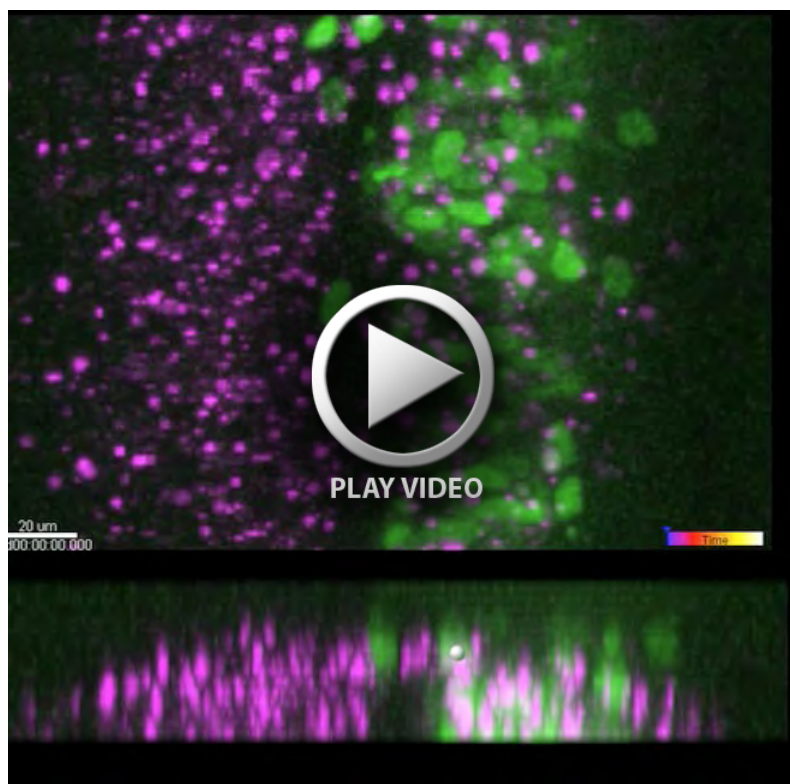
Movie 5. *In vivo* time-lapse imaging of trunk NC cells during NT exit revealing orderly emigration (related to Fig. 6) shown globally (top) and in a subregion-transverse view (bottom) with Dil (magenta)/H2B-GFP (white)-labeled cells collected in 7-minute intervals for ~6 hours.



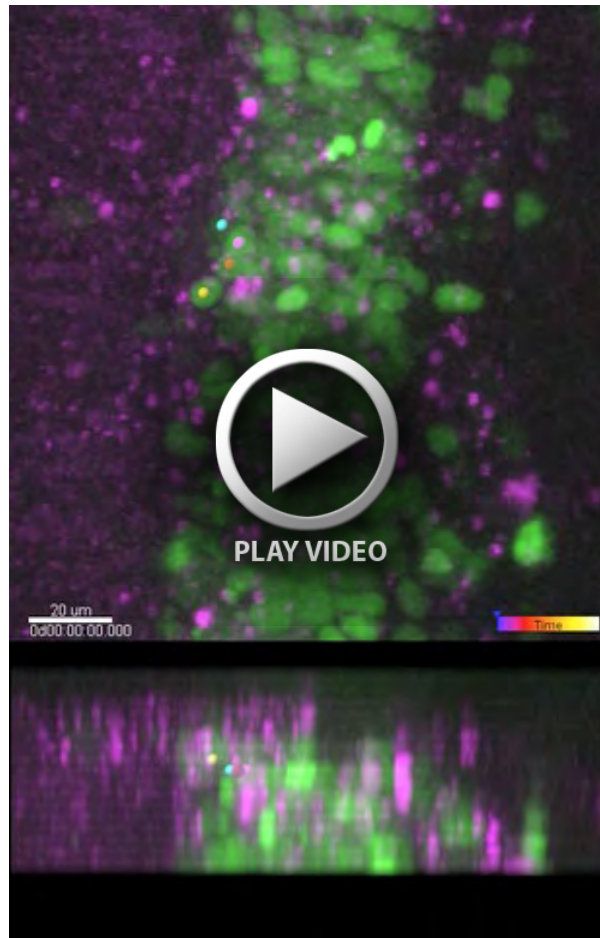
Movie 6. *In vivo* time-lapse imaging of trunk NC cells during NT exit (related to Fig. 7) with Dil (magenta)/H2B-GFP (green)-labeled cells collected in 7-minute intervals for ~7 hours.



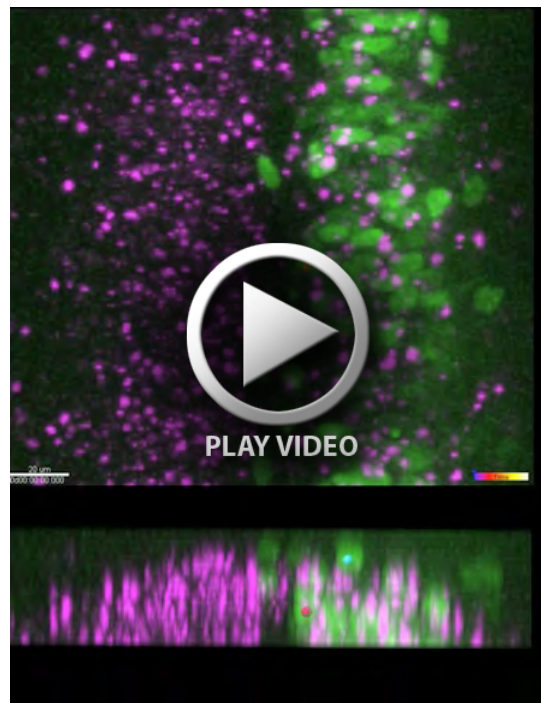
Movie 7. *In vivo* time-lapse imaging of trunk NC cells during NT exit (related to Fig. 7A; an example of a dividing cell immediately before exit at the dorsal midline; both progeny exit the NT) with DiI (magenta)/H2B-GFP (green)-labeled cells collected in 7-minute intervals for ~9 hours.



Movie 8. *In vivo* time-lapse imaging of trunk NC cells during NT exit (related to Fig. 7B; an example of a dividing cell at the dorsal midline, but only one progeny leaves the NT) with DiI (magenta)/H2B-GFP (green)-labeled cells collected in 7-minute intervals for ~9 hours.



Movie 9. *In vivo* time-lapse imaging of trunk NC cells during NT exit (related to Fig. 7C; an example of neighboring cells that exit the NT at very different times) with DiI (magenta)/H2B-GFP (green)-labeled cells collected in 7-minute intervals for ~9 hours.



Movie 10. *In vivo* time-lapse imaging of trunk NC cells during NT exit (related to Fig. 7D; an example of a cell bypassing another cell at the dorsal midline) with DiI (magenta)/H2B-GFP (green)-labeled cells collected in 7-minute intervals for ~8 hours.

Table S1. Gene expression assays

<u>Gene symbol</u>	<u>Assay ID</u>
<i>BMPR1A</i>	Gg03339693_m1
<i>BMPR1B</i>	Gg03366638_m1
<i>BMPR2</i>	Gg03345679_m1
<i>EDNRA</i>	Gg03363278_m1
<i>EFNB2</i>	Gg03338769_m1
<i>EPHA2</i>	AIMSF5V
<i>EPHA3</i>	Gg03340036_m1
<i>EPHA4</i>	Gg03371260_m1
<i>EPHB1</i>	Gg03320093_m1
<i>EPHB2</i>	Gg03349515_m1
<i>EPHB3</i>	Gg03320080_m1
<i>ERBB2</i>	Gg03360033_m1
<i>FGFR1</i>	Gg03340352_m1
<i>FGFR3</i>	Gg03340332_m1
<i>FOXD3</i>	Gg03815041_s1
<i>MITF</i>	Gg03348224_m1
<i>NEDD9</i>	Gg03310488_m1
<i>NEFM</i>	Gg03361912_m1
<i>NGN1</i>	Gg03814966_s1
<i>NRP1</i>	Gg03371276_m1
<i>NRP2</i>	Gg03364404_m1
<i>PAX3</i>	Gg03364367_m1
<i>SLUG</i>	Gg03333502_m1
<i>SNAI1</i>	Gg03366757_m1
<i>SOX10</i>	Gg03371326_m1
<i>SOX9</i>	Gg03364395_m1
<i>SPON1</i>	Gg03338910_m1
<i>TRKC</i>	Gg03366901_m1
<i>VEGFR2</i>	Gg03346169_m1