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HYPOTHESIS

Cyclical fate restriction: a new view of neural crest cell fate specification

Robert N. Kelsh[1,*], Karen Camargo Sosa[1], Saeed Farjami[2], Vsevolod Makeev[3,4], Jonathan H. P. Dawes[5] and Andrea Rocco[2,6]

ABSTRACT

Neural crest cells are crucial in development, not least because of their remarkable multipotency. Early findings stimulated two hypotheses for how fate specification and commitment from fully multipotent neural crest cells might occur, progressive fate restriction (PFR) and direct fate restriction, differing in whether partially restricted intermediates were involved. Initially hotly debated, they remain unreconciled, although PFR has become favoured. However, testing of a PFR hypothesis of zebrafish pigment cell development refutes this view. We propose a novel 'cyclical fate restriction' hypothesis, based upon a more dynamic view of transcriptional states, reconciling the experimental evidence underpinning the traditional hypotheses.

KEY WORDS: Fate specification, Melanocyte, Neural crest cell, Pigment cell, Zebrafish

Introduction

Neural crest cells (NCCs; see Glossary, Box 1) are vital for vertebrate development, and are a key model system for developmental biology. They are ectodermally derived, undergoing delamination (see Glossary, Box 1) and pausing in the premigratory 'staging area' near the dorsal neural tube (Marusich and Weston, 1991), before migrating extensively throughout the body. They generate diverse cell types, including most of the peripheral nervous system (PNS), all body pigment cells and skeletogenic cell types (so called 'ectomesenchymal cell fates') (Le Douarin and Kalcheim, 1999). NCCs can be divided into cranial and trunk populations, which differ in both their migration pathways and fate repertoire, with skeletogenic fates generally confined to the head. The isolation and *in vitro* characterisation of neural crest-derived stem cells, known as neural crest stem cells (NCSCs; see Glossary, Box 1), has added further interest to NCCs and has provided a controlled experimental paradigm for defining the molecular basis for fate specification and differentiation (see Glossary, Box 1).

Our interest here is in fate restriction (see Glossary, Box 1), the process whereby NCCs become committed to individual fates. Early *in vivo* labelling and transplant experiments in avian embryos demonstrated the remarkable fate potential, or potency (see Glossary, Box 1), of NCCs, with heterotopic transplantation showing that the potential of NCC populations was even greater than actually exhibited *in vivo* (Le Douarin, 1986). Early discussions debated two extreme hypotheses for the potency of premigratory NCCs: (1) NCCs are initially homogeneous, fully multipotent cells (see Glossary, Box 1); or (2) the neural crest (NC) is a heterogeneous mixture of predetermined unipotent cells. Of course, it was acknowledged that the NC might consist of a mixture of these, perhaps with some fates specified independently, whereas others are derived from (nearly fully) multipotent progenitors (Fraser and Fraser, 1991; Vogel and Weston, 1988; see also Weston and Thiery, 2015).

Closely entwined with the issue of multipotency, was the question of when and how fate choices were made: if NCCs are fully multipotent, then fate restriction was likely to depend upon instructive cues received during migration or at their destination, but if unipotent, then their migration would likely be targeted to appropriate locations (Fraser and Bronner-Fraser, 1991; Vogel and Weston, 1988). Alternatively, cells might migrate randomly, with appropriate cell types selected for survival by regional trophic factors (Le Douarin, 1986). Clonal analysis of chick and mouse NCCs in primary cultures led to the conclusion that they were multipotent (see Glossary, Box 1) (Dupin et al., 1990; Ito et al., 1993; Ito and Sieber-Blum, 1993; Sieber-Blum, 1989; Sieber-Blum and Cohen, 1980). However, this still left the question of how fully multipotent cells became committed to single fates (i.e. unipotent; see Glossary, Box 1), and in particular whether or not cells of intermediate potency were involved. Work in the 1980s resulted in two distinct hypotheses, progressive fate restriction (PFR) and direct fate restriction (DFR), for how this might work (Fig. 1A-F).

Progressive fate restriction

The PFR hypothesis was proposed independently by Weston and Le Douarin (Baroffio et al., 1988, 1991; Le Douarin, 1986; Weston, 1982, 1983, 1991). Noting the evidence for heterogeneity of marker expression in even premigratory or early migratory (see Glossary, Box 1) NCCs (e.g. Barald, 1988a,b; Barbu et al., 1986; Ciment and Weston, 1982, 1985; Henion et al., 1995; Kahane and Kalcheim, 1994; Tessarollo et al., 1993; Wehrle-Haller and Weston, 1995), Weston proposed that segregation of developmentally restricted subpopulations occurs progressively and in a specific sequence, with very early segregation of ectomesenchymal, and then primary sensory neuron, fates (Fig. 1A,C,E); at least some of these subpopulations have become distinct in the premigratory NCCs (Weston, 1991). Le Douarin's group built on the pioneering studies of Sieber-Blum and Cohen using single cell clones of NC in culture, which showed that primary NCCs were generally not unipotent, but also indicated considerable heterogeneity *in vitro* (Sieber-Blum and Cohen, 1980).

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[1]Department of Biology & Biochemistry, University of Bath, Bath, BA2 7AY, UK. [2]Department of Microbial Sciences, FHMS, University of Surrey, Guildford, GU2 7XH, UK. [3]Department of Computational Systems Biology, Vavilov Institute of General Genetics, Russian Academy of Sciences, Ul. Gubkina 3, Moscow, 119991, Russian Federation. [4]Department of Biological and Medical Physics, Moscow Institute of Physics and Technology, Dolgoprudny, Moscow Region, 141701, Russian Federation. [5]Department of Mathematical Sciences, University of Bath, Bath, BA2 7AY, UK. [6]Department of Physics, FEPS, University of Surrey, Guildford, GU2 7XH, UK.

*Author for correspondence (bssrnk@bath.ac.uk)

R.N.K., 0000-0002-9381-0066; K.C.S., 0000-0002-9803-6372; V.M., 0000-0001-9405-9748; J.H.P.D., 0000-0002-4347-9985; A.R., 0000-0002-0974-5522

Box 1. Glossary

Delamination. The process of NCCs undergoing epithelial-mesenchymal transition, exiting the neural epithelium to become mesenchymal.

Differentiation. The process of acquiring the specific morphological and transcriptional markers characteristic of an individual cell type (fate). We envisage differentiation as a continuum, a dynamic process in which a cell activates (or maintains, likely at elevated levels) expression of fate-specific transcription factors, gradually activating the transcriptional programme that results in adoption of the differentiated phenotype.

Fate commitment. The process whereby a cell stabilises ('locks in') its fate choice, i.e. the terminal stage of fate restriction. This is generally considered to involve loss of multipotency, resulting from epigenetic modification of the genome to 'fix' a specific transcriptional programme (ensuring unipotency). It is generally considered the terminal state of differentiation, but the realisation that some stem cells (e.g. neural stem cells in the central nervous system) adopt a distinctive differentiated phenotype means that caution needs to be exercised – a differentiated ('specialised') phenotype does not necessarily imply fate commitment or unipotency. Traditionally, demonstration of fate commitment requires a technically challenging transplantation experiment, but we consider that it could probably be assessed by examination of the transcriptome of a cell (provided that sequencing is sufficiently deep).

Fate potential (potency). The capacity of a progenitor cell to generate a defined differentiated cell type. In theory, this could be revealed in clonal cell culture, if we assume that culture conditions are suitable for all potential cell types and that all can be simultaneously distinguished by markers. In reality, clone size and other stochastic factors may vary the combination of cell types generated. Traditionally considered impossible to assess definitively *in vivo*, single-cell RNA sequencing may allow a glimpse into cell fate potential (see 'Fate specification' below).

Fate restriction. The process whereby a multipotent progenitor cell adopts (i.e. becomes restricted to) a specific fate. Cells may be described as partially fate restricted, when they can adopt one of a subset of fates, but are unable to adopt (i.e. restricted from adopting) others. However, confusingly, in a clonal study, a cell is considered a fate-restricted precursor when all its progeny adopt a single fate, but it should be noted that, although consistent with fate commitment (i.e. unipotency), it does not prove fate commitment, because limited environmental signals, small clone sizes and other factors can limit the clone's ability to display its full potency.

Fate specification. Multipotent cells are defined as showing fate specification as soon as fate-specific markers are detectable. It is crucial to remember that this expression is labile, and does not imply commitment. However, this term is limited by our ability to detect more than one marker simultaneously, with traditional whole-mount *in situ* hybridisation or immunofluorescence studies rarely allowing more than two or three markers to be assessed, and usually only one. Where considered, such studies may show co-expression of markers of different cell types, indicating that fate specification is clearly not the same as fate commitment (Petratou et al., 2021, 2018). New techniques, including single-cell RNA sequencing and *in situ* sequencing now allow assessment of ten to thousands of mRNA transcripts, revolutionising our ability to detect, and to distinguish, fate specification from fate commitment. We consider that expression of one or more fate-specific markers indicates at least the potential to differentiate into that cell type, and thus that at least a minimal estimate of the fate potential of a cell might be deduced from sufficiently deep transcriptional profiling.

Fully multipotent. A progenitor or stem cell is fully multipotent when it is still able to adopt any of its characteristic derivative fates. The term pluripotency was used in some of the early NC literature to distinguish cells generating all NC derivatives (i.e. pluripotent NCCs), but as the stem cell biology field has blossomed, so the meaning of this term has become more widely accepted to mean 'capable of generating all embryonic (as opposed to extra-embryonic) cells'.

Migratory. NCCs moving around the body, usually on defined migratory pathways in the trunk and tail, but more broadly in the head. Such cells are not usually visibly differentiated (e.g. melanised) in mouse or chick, but in fish and amphibians they are often partially differentiated (e.g. melanised or displaying other pigments).

Multipotent. A progenitor or stem cell is multipotent when it is still able to adopt two or more of its characteristic derivative fates.

Neural crest cells (NCCs). Any of the numerous mesenchymal cells generated by the delamination of dorsal neural tube cells during embryonic (somitogenesis stages) development.

Neural crest stem cells (NCSCs). Originally isolated from embryonic mammals as a subset of NCCs, these are NC-derived cells that undergo extensive self-renewal and retain the potential to differentiate into one or more NC-derived cell types. Such cells can be derived from many post-migratory locations in embryos and even in adults, including skin and the PNS, reflecting the widespread maintenance of cells functioning in homeostasis (Delfino-Machin et al., 2007). Such cells in different locations normally generate only a subset of fates, and so may be named accordingly, but in at least some cases their potency has been shown to be considerably broader when environmental influences are changed by cell culture (e.g. Nishimura et al., 2002, 2010), leading to a much wider potency *in vitro* (Watanabe et al., 2016). This highlights a key inadequacy of the naming conventions within the literature. Long-term persistence of such cells, often incorporated within a stem cell definition, has been less thoroughly investigated, but the ready isolation from adult tissues implies that this feature exists here too. The term is also used more loosely for early NCCs in their fully multipotent form. Note that direct demonstration of these properties has not been performed in a zebrafish context, so that the use of the term NCSC is somewhat provisional.

Pre-delamination. NCCs still in the neural plate/dorsal neural tube.

Premigratory. NCCs sitting adjacent to the dorsal neural tube, a position designated the 'staging area' (Marusich and Weston, 1991), prior to migration.

Post-migratory. NCCs in their terminal positions. Such cells may initially be undifferentiated, but will often soon become (fully) differentiated, contributing to the physiological functions of the relevant organ or tissue.

Transdifferentiation. Transition of a cell of one differentiated morphology into the differentiated morphology characteristic of a distinct cell type, without dedifferentiation into an undifferentiated progenitor state.

Unipotent. A progenitor or stem cell is unipotent when it is stably committed to adopting one of its characteristic derivative fates (i.e. fate committed).

Le Douarin and colleagues showed that early migrating NCCs generate both clones consistent with fully multipotent cells, and a broad range of clone sizes and cell-type compositions interpreted as showing PFR during migration (Baroffio et al., 1988, 1991). They proposed that different developmental fates form by progressive restriction of fully multipotent progenitors via partially restricted cell types, publishing an early version of the now classic textbook figure (Gilbert and Barresi, 2016). Numerous studies using these 2D cultures (Calloni et al., 2007, 2009; Lahav et al., 1998, 1996; Trentin et al., 2004), but also more recent 3D cultures of NCSCs (Mohlin et al., 2019), have demonstrated the multipotency, but also the apparent heterogeneity, of many premigratory and migrating chick and mammalian NCCs (reviewed by Dupin et al., 2018).

Direct fate restriction

The DFR hypothesis was proposed based upon clonal analysis of NCC fates *in vivo* using iontophoretic labelling (whereby locally applied electrical current is used to drive a charged fluorescent dye into a single cell or small groups of cells) of chick trunk dorsal neural tube and premigratory NCCs (Bronner-Fraser and Fraser, 1989, 1988, 1991). These experiments revealed that most labelled NCCs generated heterogeneous clones with multiple derivative cell types, with some including all the fates that could be distinguished, although a significant proportion consisted of only a single cell type. These authors proposed that NCCs were homogeneous multipotent cells, and that fate choices were imposed upon them by environmental cues late in or after migration (Bronner-Fraser and Fraser, 1989, 1991) (Fig. 1B,D,F). Clonal heterogeneity in

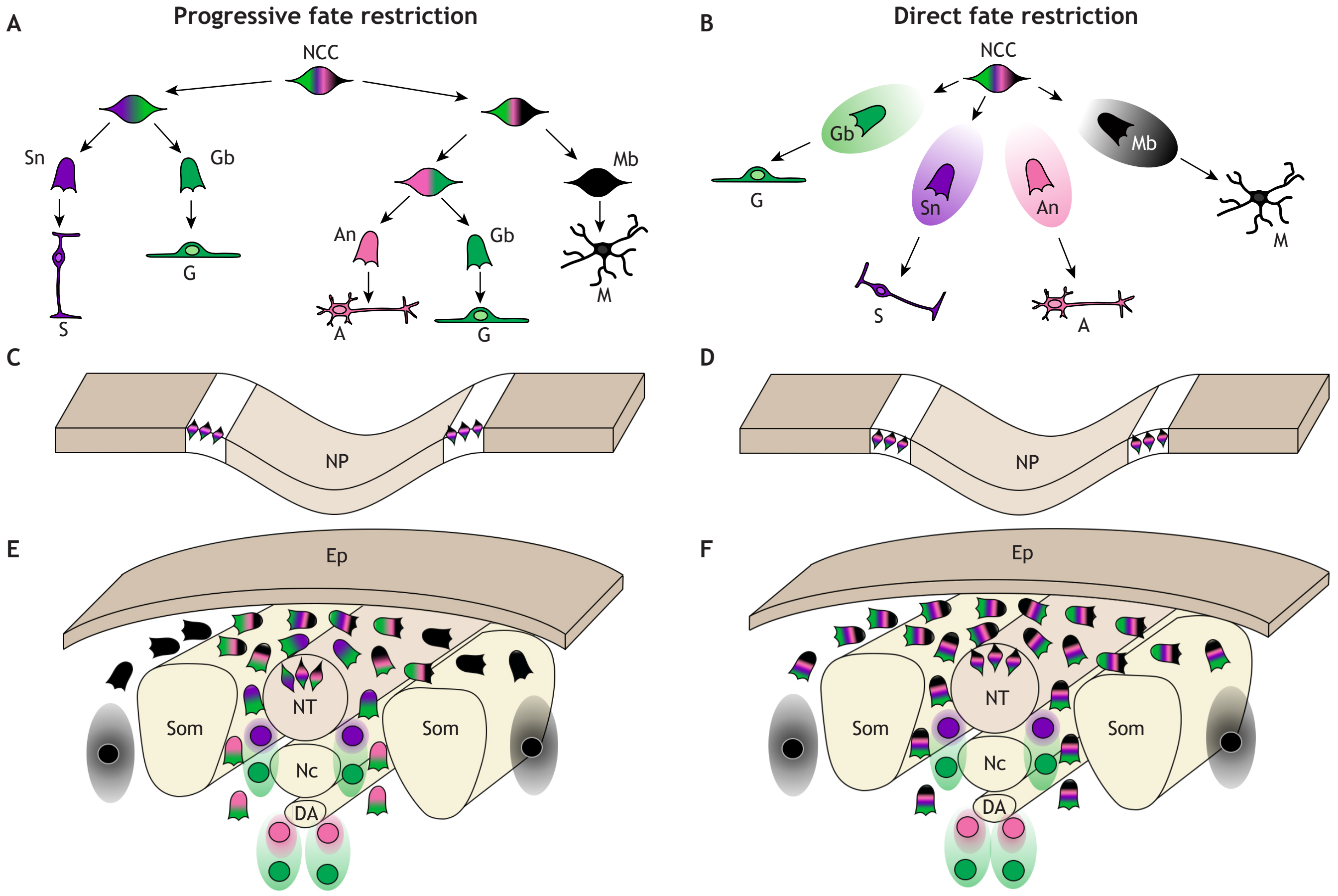

Fig. 1. Progressive and direct fate restriction hypotheses for NCC development. (A,B) Schemes show progressive (A) and direct (B) fate-restriction of NCCs, as deduced from mouse and chick studies. (A) A multipotent trunk NCC progenitor (multicoloured cell in green, purple, pink and black) produces a heterogeneous population of intermediate progenitors, here shown as bipotent sensory (purple and green) and autonomic ganglial (green and pink), but also tripotent shared autonomic ganglial and melanocyte progenitor (green, pink and black), prior to generating fate-restricted derivatives. (B) A multipotent NCC progenitor generates single-fate restricted cells: sensory neuroblast (Sn; purple); autonomic neuroblast (An; pink), glioblast (Gb; green); melanoblast (Mb; black) during or after migration. (C-F) Progressive and direct fate restriction placed in an anatomical context. (C,D) NCCs induced at the lateral border of the neural plate (NP) are considered to be fully multipotent in both hypotheses. Distinctions between the hypotheses become clear at later stages, perhaps from delamination, but especially during NCC migration. (E) Under the progressive fate restriction hypothesis, intermediates of a wide-range of partially and fully restricted potencies are rapidly segregated in premigratory NCCs [perhaps even beginning pre-delamination (see Glossary, Box 1)]. Migrating progenitors adopt routes appropriate to their potency, with melanocyte progenitors (black) in mouse and chick utilising exclusively the dorsolateral migration pathway between the epidermis and somites, and peripheral ganglial progenitors (purple and green, and pink and green) utilising the medial migration pathway between the somites and the neural tube, notochord and dorsal aorta. Ganglial progenitors accumulate in nascent ganglia, where neuronal and glial differentiation occur. Environmental cues, shown as shaded ovals, are considered to reinforce fate-restriction decisions, for example by controlling accessibility to dorsolateral migration pathway, restricting it to melanoblasts (black circle), or influencing aggregation of ganglial progenitors and the subsequent differentiation of both neuronal and glial fates (purple, pink and green circles). (F) Under the direct fate restriction hypothesis, delaminated premigratory and migrating NCCs retain full multipotency, until exposed to differentiation cues (coloured ovals) in the migratory/post-migratory environment, triggering direct differentiation into specific cell types in response to environment differentiation signals (green, purple, pink and black circles). For simplicity, the figure focuses on derivatives of trunk NCCs, although the original progressive fate restriction hypotheses also emphasised the derivation of ectomesenchymal fates (e.g. cartilage) from the cranial NC; in Weston's hypothesis this was seen as the first fate to segregate, whereas in Le Douarin's hypothesis cartilage-generating progenitors were of diverse potencies and hence likely to persist longer. Mesoderm is indicated in E and F only, and is simplified as somites (Som), without indicating segregation of sclerotome and dermomyotome; neural tube (NT), notochord (Nc), dorsal aorta (DA) and epidermis (Ep) are also indicated to delineate key dorsolateral and medial NC migration pathways. A, autonomic neuron; G, glial cell; M, melanocyte; S, sensory neuron.

cell culture and *in vivo* would then be explained by statistical effects of clone sizes and inconsistencies of environmental cues encountered.

Strong support for the DFR hypothesis resulted from the isolation of rat NCSCs by Anderson's group (Stemple and Anderson, 1992). An elegant series of studies defined the key extracellular signals driving NCSC fate specification and differentiation, and demonstrated that these acted instructively, rather than simply selecting out a subset of cells pre-specified to individual fates (Kim et al., 2003; Lo et al., 1997; Morrison et al., 2000; Perez et al., 1999; Shah et al., 1996, 1994). Although these studies were limited in the fates assessed, they reinforced a key idea of the DFR hypothesis – that single cells choose directly between multiple fates, with environmental signals instructing the fate adopted.

The PFR and DFR hypotheses are distinguished by whether (PFR) or not (DFR) they transition through cells of reduced potency before adopting individual fates. A second distinction concerns when, and especially where, fate choices begin to be made: late in migration (DFR), or beginning in or adjacent to the neural tube (PFR). It will be apparent already that the PFR hypothesis matches the way we view development in general, readily integrating with Waddington's influential epigenetic landscape model (Waddington, 1940), but therein lies the importance and the excitement of this field: is it possible that NCCs differentiate in a different way, perhaps associated with their remarkable potential (Buitrago-Delgado et al., 2015)? These contrasting hypotheses were hotly debated throughout the 1990s. The debate was then largely forgotten, primarily because PFR became the accepted ('textbook') hypothesis, but also, in part, because the meaning of the term 'multipotency', originally used to mean 'full multipotency', has tended to drift towards a more generic 'at least bipotent'. This is unfortunate, because it loses the essence of the debate – even in a PFR hypothesis most cells are at least bipotent! In recent years, certain key studies using single-cell resolution *in vivo* have reopened the discussion, but still most work has assumed a PFR interpretation.

Neural crest fate choice in recent years

This century, the PFR hypothesis has become dominant. Even the initial studies acknowledged that the data underpinning the DFR hypothesis did not rule out a PFR hypothesis (Anderson, 1989; Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991). In a later review of peripheral neuron development, Anderson concluded that segregation of sensory and autonomic lineages probably occurred prior to delamination (Anderson, 2000).

Delaminating chick NCCs were already fate restricted, but also emerged in a reproducible manner, filling more ventral locations (sympathetic ganglia) first, and then progressively occupying more dorsal ones [e.g. ventral root, dorsal root ganglia (DRG) and the skin] (Erickson et al., 1992; Kitamura et al., 1992; Reedy et al., 1998; Serbedzija et al., 1989). This was shown particularly convincingly by studies controlling carefully for time of delamination and for labelling of single cells (Krispin et al., 2010b; Nitzan et al., 2013a). Similarly, iontophoretic labelling of single premigratory NCCs in zebrafish showed that most were apparently already fate restricted (Dutton et al., 2001; Raible and Eisen, 1994; Schilling and Kimmel, 1994). Given the extensive evidence for variably multipotent (full to bipotent) migrating NCCs from primary chick culture noted before, the fate restriction demonstrated in chick *in vivo* is somewhat unexpected, but may reflect the combined impact of clone sizes and anatomical confinement of migration *in vivo*. Early fate restriction does not prove absence of multipotency (e.g. if migration is highly constrained), and thus does not strictly distinguish between the PFR and DFR hypotheses, but was consistent with early fate specification and fate-specific migration behaviour to target the appropriate locations.

This idea of fate restriction occurring prior to NCC migration was tested in mouse using the *R26R-Confetti* system to label a large sample of NCC clones with clonally distinguishable combinations of different coloured fluorescent proteins (Baggiolini et al., 2015). The authors combined clonal analysis of NCCs labelled genetically prior to delamination and in premigratory stages, with sophisticated statistical modelling to take account of proliferation rates and the relative size of the target site, and concluded that mouse NCCs at these stages show strong evidence for retained multipotency, in contrast to chick. However, in the context of our discussion, Baggiolini and colleagues defined 'multipotent' as 'fated to form at least two cell types', so that their data could be interpreted within a classic PFR hypothesis. The apparent contrasts between model systems is striking, and could be taken to indicate that there are species-specific differences in the timing of fate determination and/or regularity of migration.

Studies of fate specification have been highly limited by the number of markers that can be assessed simultaneously, making it impossible to make any authoritative statement of potency, even where fate specification was apparent, and reinforcing the difficulty of interpreting *in vivo* clonal studies showing apparent fate restriction (Box 1). However, with single-cell RNA-sequencing (scRNA-seq) offering a near-complete transcriptome, we would expect the potency of a cell to be reflected in the range of fate-specific markers that are expressed. In this context, a tour de force scRNA-seq study of mouse NC development apparently strongly reinforces the PFR hypothesis (Soldatov et al., 2019). Characterising NCCs expressing a fluorescent marker prior to delamination, NC fate specification towards skeletal and neural fates displayed an apparent pattern of sequential binary fate decisions during migration. Consistent with the conclusions of early segregation of sensory and autonomic lineages proposed previously (Anderson, 2000; Greenwood et al., 1999; Henion and Weston, 1997; Le Douarin, 1986; Perez et al., 1999; Sieber-Blum and Cohen, 1980; White et al., 2001; Ziller et al., 1983, 1987; Zirlinger et al., 2002), Soldatov and colleagues identified early segregation of sensory neuron precursors, followed by segregation of autonomic and mesenchymal progenitors. Mesenchymal fate segregation appeared not to be the primary decision, in contrast to early proposals (Weston, 1991), but in strong agreement with the apparent diversity of clones generating skeletal fates in clonal cell 2D and 3D cultures of chick NCCs (Calloni et al., 2007, 2009; Mohlin et al., 2019). This study clearly proposes a scheme highly consistent with the PFR hypothesis. However, it should be noted that (1) single-cell isolation methods usually focus (appropriately) on preserving the changing fate-specification signatures induced by *in vivo* environmental signals, rather than necessarily trying to assess the cells' fate potential, and (2) significant challenges remain in the reconstruction of developmental trajectories, even for state-of-the-art bioinformatics algorithms, as is evidenced by the necessary reliance on implicit notions, such as pseudotime, and the abundance of different algorithms available that attempt to make optimal choices of the trees describing bifurcation events, further complicated by the recent demonstration of non-binary fate choices and the dual origin of some cell types (Farrell et al., 2018).

The specific case of pigment cell fate specification: chromatoblasts and bipotent progenitors, melanocyte stem cells and NCSCs

A definitive test of the fate restriction mechanisms has been difficult to achieve. The classic textbook figure of PFR (Gilbert and Barresi, 2016) includes numerous intermediate cell types with various degrees of multipotency (see above), whereas the best-characterised examples *in vivo* are usually merely bipotent, with experimental support for well-defined progenitors with intermediate potency *in vivo* being limited (see above). Consequently, opportunities to challenge the PFR model experimentally have been lacking. One exception is the pigment cell system in fish, for which multiple bipotent progenitors have been suggested *in vivo*, but a multipotent intermediate has also been hypothesised (Bagnara et al., 1979).

The chromatoblast and bipotent pigment cell progenitors

Mammals only have a single pigment cell type (melanocyte), but most vertebrates (including zebrafish and medaka) have two or more pigment cell types (collectively known as chromatophores), including melanocytes (black), xanthophores (yellow), iridophores (iridescent, usually blue or silver), leucophores (white or cream) and others (Fujii, 1993; Schartl et al., 2016). The genetic accessibility of these cells has ensured that pigment cells are a well-studied 'model-within-a-model' for NC development (reviewed by Hashimoto et al., 2021). Here, we confine our attention to the proposed cell intermediates within pigment cell development in fish, which together formulate a widely accepted PFR model of pigment cell development.

Bagnara and colleagues proposed a common 'chromatophore stem cell' that gives rise to all (NC-derived) pigment cell types, but not to other NC derivatives (Bagnara et al., 1979). For the purposes of this Hypothesis article, we call this partially restricted progenitor a 'chromatoblast'. Although the chromatoblast idea was rather speculative, being based, in part, on the transdifferentiation (see Glossary, Box 1) of pigment cell types in prolonged cell culture (e.g. Ide, 1986; Ide and Hama, 1976), the recent demonstration that pigment cell transdifferentiation contributes to normal metamorphic development in zebrafish (Lewis et al., 2019) strengthens the concept.

However, evidence of a chromatoblast has been hard to come by. Single-cell fate mapping of premigratory zebrafish NCCs has been inconclusive; most cells are apparently fate restricted, but the very small clone sizes characteristic of this species make interpretation of this result difficult (Dutton et al., 2001; Raible and Eisen, 1994; Schilling and Kimmel, 1994). All three zebrafish pigment cells are absent in *colourless* (*sox10*) mutants, but the peripheral nervous system is severely affected too, making the phenotype more consistent with the idea of a 'non-ectomesenchymal progenitor' rather than a chromatoblast (Dutton et al., 2001; Kelsh, 2006; Kelsh and Eisen, 2000); Sox10 has a similar role in medaka (Nagao et al., 2014). However, careful assessment of the zebrafish *sox10* mutant phenotype has identified a subset of NCCs that are trapped in a premigratory position and co-express key pigment cell fate specification factors (including *sox10*, *ltk*, *tfec*); these cells were hypothesised to be the elusive chromatoblasts (Lopes et al., 2008; Petratou et al., 2018, 2021). scRNA-seq profiling, focused on adult and larval zebrafish, identified a 'pigment cell precursor' expressing *sox10*, *mitfa* and *tfec*, consistent with a putative chromatoblast (Howard et al., 2021; Saunders et al., 2019).

Several bipotent pigment cell progenitors have been inferred from the study of mutant phenotypes. Melanocytes are completely absent in *nacre* (*mitfa*) mutant zebrafish (Lister et al., 1999), consistent with the known role of Mitf in mammals as a master regulator of melanocyte development (Steingrimsson et al., 1994; Tassabehji et al., 1994). Alongside the absence of melanocytes, these zebrafish mutants display a substantial increase in iridophore numbers, leading to the proposal that both cell types derive from a bipotent 'melanoiridoblast'. Lineage tracing of *mitfa*-expressing cells at 24 h post-fertilisation (hpf) indicated that these cells are post-mitotic and many develop as melanocytes or iridophores (Curran et al., 2010). Less is known about xanthophore fate specification, but studies of mutants for *pax3*, *pax7* and *sox5* in zebrafish and/or medaka have provided tantalising evidence for potential bipotent 'melanoxanthoblasts' and, especially, 'xantholeucoblasts' (Kimura et al., 2014; Minchin and Hughes, 2008; Nagao et al., 2014, 2018, 2016).

These studies lead, naturally, to a hypothesis of zebrafish pigment cell development that is explicitly a PFR hypothesis (Fig. 2). However, a study of zebrafish using sensitive NanoString detection of mRNA expression in individual NC-derived cells from several embryonic/early larval stages failed to detect cells showing signatures characteristic of a chromatoblast (*mitfa*, *tfec* and *pax7*, but not *phox2b*) or of bipotent pigment cell progenitors (e.g. *mitfa* and *tfec*, but not *pax7*, for melanoiridoblast) (Nikaido et al., 2021 preprint). Instead, progenitor cells that expressed the proposed chromatoblast fate-specification genes (e.g. *mitfa*, *tfec*, *pax7*, etc.) also expressed known fate-specification genes for neural fates (e.g. *phox2b* and *sox10*), leading to their interpretation as broadly multipotent intermediates (Nikaido et al., 2021 preprint).

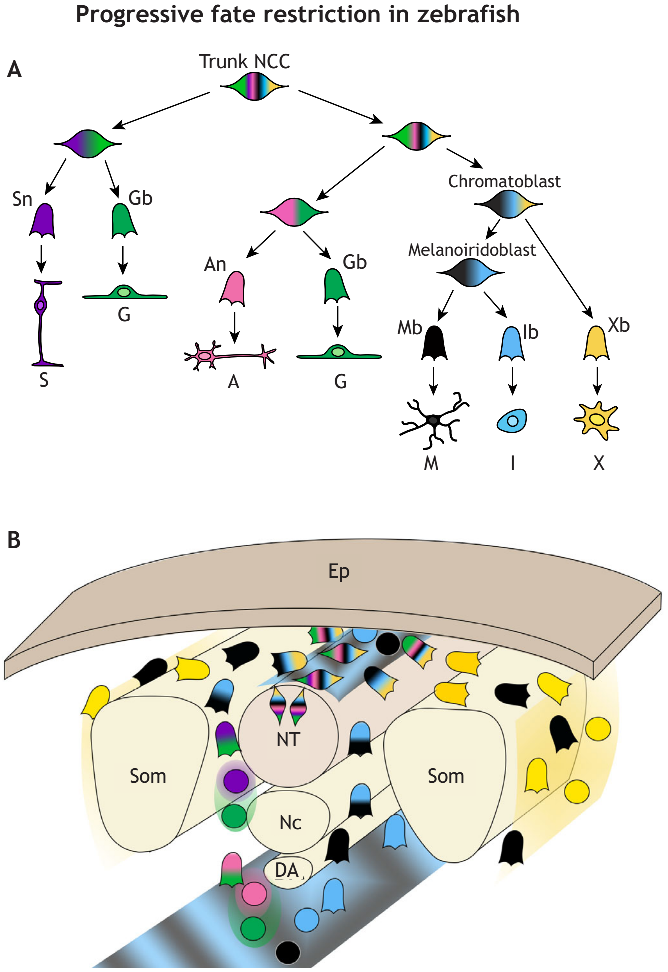

Fig. 2. A PFR hypothesis of zebrafish pigment cell development. The current working hypothesis of how zebrafish trunk NCCs generate the three distinct pigment cell types, shown as an adaptation of the general PFR hypothesis shown in Fig. 1. (A) It is assumed that the initial NC is fully multipotent, producing sensory and autonomic neurons, glia, and melanocytes, iridophores and xanthophores. A multipotent, but partially restricted progenitor of all the pigment cells (chromatoblast) has been proposed as an intermediate stage, as has a bipotent melanoiridoblast. A, autonomic neuron; An, autonomic neuroblast; G, glial cell; Gb, glioblast; I, iridophore; Ib, iridoblast; M, melanocyte; Mb, melanoblast; S, sensory neuron; Sn, sensory neuroblast; X, xanthophore; Xb, xanthoblast. (B) In an anatomical context, migrating pigment progenitors on the dorsolateral migration pathway are considered to be fate-specified melanoblasts and xanthoblasts, whereas cells on the medial pathway include both bipotent neural progenitors (indicated on left migration pathway) and pigment cell progenitors (right migration pathway), from which individual cell types emerge. Initially, migrating pigment progenitors show overlapping expression of marker genes consistent with melanoiridoblast status (Petratou et al., 2021, 2018). Note that the status of progenitors with respect to xanthophore fate has been less well-explored and is ignored here for the sake of simplicity. DA, dorsal aorta; Ep, epidermis; Nc, notochord; NT, neural tube; Som, somite.

In attempts to identify putative chromatoblasts in *sox10* mutants, expression of *leukocyte tyrosine kinase* (*ltk*), which encodes a receptor crucial for iridophore fate specification, has been noted as characteristic of cells trapped in a putative pigment cell progenitor state (Lopes et al., 2008). In a direct test of the chromatoblast hypothesis, genetic fate mapping of these *ltk*-expressing cells showed that they generate all pigment cell types, but also peripheral glial and neuronal fates (Nikaido et al., 2021 preprint). Taking into account the single-cell NanoString data, it was proposed that these *ltk*-expressing cells were not chromatoblasts, but instead NC-derived highly multipotent progenitors (NC-HMPs), conflicting with the PFR hypothesis for pigment cell development (Nikaido et al., 2021 preprint) (Fig. 3). In the NanoString data, some cells from the NC-HMP cluster were, as expected, derived from earlier stages when premigratory NCCs are prominent (Nikaido et al., 2021 preprint); however, these multipotent cell clusters included many cells from later stages (early larval zebrafish; 3-5 days post-fertilisation), when most NCCs are considered to have differentiated. Consequently, these clusters were interpreted as including both widely multipotent NCCs from the earlier stages and also likely glial cells with retained multipotency from the later stages; such an interpretation was based in part on analogy to neural stem cells (Alvarez-Buylla et al., 2001; Obernier and Alvarez-Buylla, 2019; Than-Trong and Bally-Cuif, 2015), but also on studies of so-called adult 'melanocyte stem cells' (MSCs) in the zebrafish (Box 2).

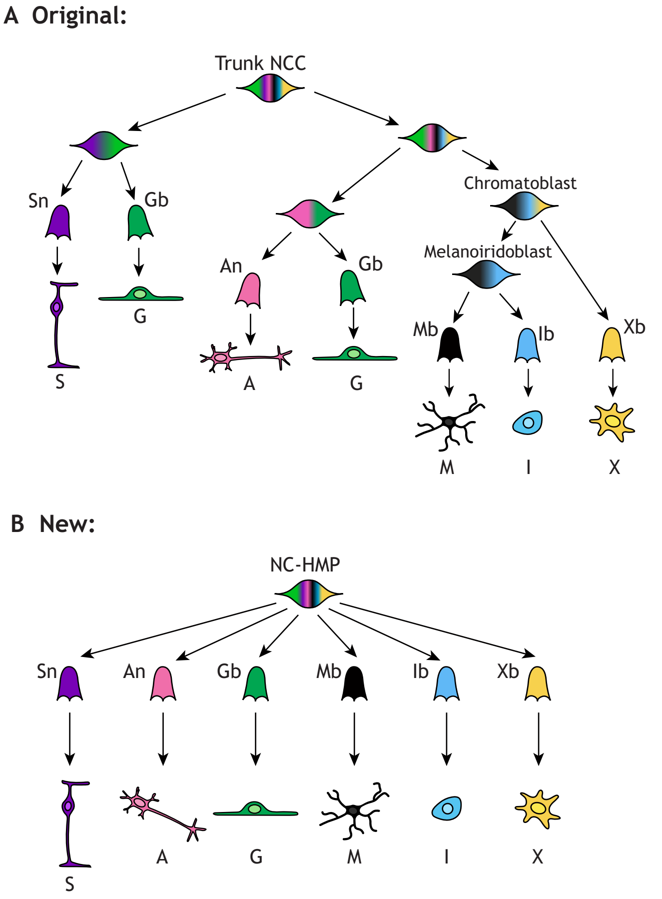

Fig. 3. Experimental test of the PFR hypothesis of zebrafish pigment cell development. (A) The original working hypothesis of pigment cell development, as shown in Fig. 2, with fully multipotent initial NC generating sensory and autonomic neurons, glia, and melanocytes, iridophores and xanthophores via multipotent, but partially restricted progenitors of all the pigment cells (chromatoblast) and a bipotent melanoiridoblast. (B) Revised hypothesis, based on findings of Nikaido et al. (2021 preprint). Owing to its unexpectedly broad multipotency, we propose the name 'NC-derived highly multipotent progenitors' (NC-HMPs) for the trunk NCCs; cells of intermediate potency were not detected. A, autonomic neuron; An, autonomic neuroblast; G, glial cell; Gb, glioblast; I, iridophore; Ib, iridoblast; M, melanocyte; Mb, melanoblast; S, sensory neuron; Sn, sensory neuroblast; X, xanthophore; Xb, xanthoblast.

A unifying view: cyclical fate restriction

We propose a novel, dynamic view to reconcile the observations underlying the PFR and DFR hypotheses, i.e. early apparent fate specification and *in vivo* clonal restriction, but late retention of multipotency. In testing the PFR hypothesis for pigment cell development, we have identified a group of premigratory NCCs that co-express key factors involved in cell fate specification of all pigment cell types, including *ltk*, *tfec*, *mitfa*, *pax7* and *sox10* (Nikaido et al., 2021 preprint). In normal development, these markers are transient, being strongly downregulated in the majority of cells as they adopt specific fates; this observation highlights repression of key fate-specification genes, including fate-specific transcription factors and receptors, as an important, but largely overlooked, mechanism underpinning fate specification (Petratou et al., 2021, 2018). Formation of individual cell types is blocked in *sox10* mutants, leading to cells becoming trapped in an NC-HMP-like progenitor state (Dutton et al., 2001; Elworthy et al., 2003; Greenhill et al., 2011; Nikaido et al., 2021 preprint; Petratou et al., 2021, 2018)

We propose a 'cyclical fate restriction' (CFR) hypothesis in which NC-HMP progenitors are highly dynamic, cycling asynchronously through a series of sub-states, each of which is biased to adopt a single fate (Fig. 4). Here, we define a 'sub-state' as one in which the cell, which is not itself in equilibrium, is transiently biased (i.e. primed) to adopt a specific fate, such as a melanocyte, before moving into a state in which it is biased to a different fate, and so on. We use the term 'cyclical' because we envisage a process in which the cell repeatedly visits and transits through all these sub-states, until such time as it becomes committed to a single fate. It is important to note that this 'fate-cycling' process reflects changes in the transcriptome/proteome of the cell, and is considered to be independent of the cell cycle. Whether or not the process is strictly periodic, or more broadly simply involves the cell recurrently accessing these sub-states, is not our key concern here; experimental investigation to test for the recurrence of transcriptional profiles characteristic of the sub-states is highly demanding and remains to be investigated.

A molecular model for CFR

Although various underlying molecular mechanisms biasing progenitor fate can be envisaged, we consider that an attractive one is focused on the expression levels of receptors for fate-specification factors (Fig. 5) (Kelsh, 2006; Weston, 1991). The biased sub-states would then be characterised by higher level expression of one or more fate-specification receptors, thus making them more sensitive to specific environmental fate-specification signals. For example, one sub-state might have higher expression of the Ltk receptor, and this sub-state would be primed to interact with environmental cues, such as ALKAL proteins (Fadeev et al., 2018), leading to differentiation into an iridophore (Lopes et al., 2008). Indeed, the heterogeneous expression of *ltk* is striking in both whole-mount *in situ* hybridisation and single cell NanoString profiling studies (Lopes et al., 2008).

The switch to a new sub-state would involve downregulation of that fate-specification receptor, and upregulation of another, such as a Frizzled receptor, which would prime the cell for a melanocyte fate. Importantly, we propose that the shift to the next sub-state depends upon activity of an appropriate, key, fate-specific transcription factor. We emphasise transcription factor activity, rather than expression levels, to make clear that regulation need not be at the level of transcription; such a view has been eloquently expounded by Goding and colleagues (Goding and Meyskens, 2006; Goding and Arnheiter, 2019). Cyclical changes in activity of the key fate-specification transcription factors would result in cyclical changes in the fate-specification receptors, and hence in bias of the sub-states. For example, for a sub-state to express high levels of Ltk, and thus be biased to become an iridophore, the cell would first need an increase in Tfec activity, because *ltk* expression in premigratory NCCs depends upon Tfec (Petratou et al., 2021). In the absence of that key transcription factor, however, cells are unable to enter the specific sub-state.

As the cell fate-cycles through the sub-states, its final fate depends partly upon how long the cell remains in that sub-state; longer duration increases the opportunity for receiving the appropriate fate-specification signal. In addition, cell fate depends on whether fate-specification signals (i.e. ligands) are present in sufficient quantities and for a sufficient length of time to drive fate specification, as shown in the ventral neural tube, for example (Sagner and Briscoe, 2019).

The CFR model is consistent with key biological observations of NCCs

Our hypothesis is consistent with heterogeneities in gene expression in premigratory NCCs, and apparent fate specification; as the NC-HMP fate-cycles through the various sub-states, it displays varying expression profiles, appearing to be specified when seen in the static snapshot view characteristic of almost all studies, but retaining multipotency. The CFR hypothesis helps to explain the embryonic origin of NCSCs (Hultman et al., 2009). We suggest that as NC-HMPs in these PNS locations become differentiated as neurons and glia, some satellite glia in the DRG [and likely also Schwann cell precursors (SCPs) in some or all of the PNS; Adameyko et al., 2009, 2012; Dooley et al., 2013; Kelsh and Barsh, 2011; Nitzan et al., 2013b; Parichy and Spiewak, 2015; Singh et al., 2016] retain their multipotency and are thus cryptic NCSCs; their entry into a quiescent state is driven by their exposure to the niche within the PNS. It is only after some process of re-activation (e.g. at metamorphosis) that they begin to generate pigment cells; re-activation will probably involve local removal of the quiescence-maintenance signals in the niche and re-entry into the sub-state fate-cycling mode, with the local niche signals controlling the cell types formed by biasing the time spent in each poised sub-state. We note that mouse MSCs *in vivo* appear to be lineally restricted to generate melanocytes (Nishimura et al., 2002, 2010), yet *in vitro* culture studies reveal a much wider potency (Watanabe et al., 2016), consistent with them also being intrinsically highly multipotent, but with their niche restricting the fates their progeny actually adopt *in vivo*.

Importantly, the CFR hypothesis provides a natural explanation for two paradoxes. First, it explains why NCCs adopt different fates even in a crowded premigratory position. Under the DFR hypothesis, where fate specification signals were received late in migration, it is easy to see how fates adopted would be locally appropriate. However, if fate specification occurs very early – prior to migration – then how cells make different decisions needs to be explained. In fish, NCCs in the premigratory position are likely exposed to high levels of Wnt and ALKAL signals, but only a subset become each of melanocytes and iridophores. In the chick, such heterogeneity of fate choice is attributed to differences in timing of delamination from the dorsal neural tube (Krispin et al., 2010a,b; Nitzan et al., 2013a), and this is plausible in the zebrafish too. However, the CFR hypothesis offers another intriguing explanation – that cells make different choices because they are only transiently in a receptive sub-state for each of the relevant fate-specification signals.

Box 2. Adult NCSCs and MSCs

So-called MSCs, derived from the NC and set aside during embryonic development (Dooley et al., 2013), are the origin of numerous pigment cells in the adult. Fish show a prominent metamorphosis whereby body structure, including skin pigment pattern, is modified to generate the adult form. In zebrafish, *de novo* generated melanocytes, iridophores and some xanthophores, replace the embryonically derived early larval pattern, although many adult xanthophores seem to be generated through a process of dedifferentiation, proliferation and differentiation of embryonic xanthophores (Hultman and Johnson, 2010; Johnson et al., 1995; Mahalwar et al., 2014; McMenamin et al., 2014; Parichy et al., 1999, 2003; Quigley et al., 2004; Tryon et al., 2011; Walderich et al., 2016). These observations, plus those of regeneration of melanocytes after their chemical or physical ablation (Hultman et al., 2009; O'Reilly-Pol and Johnson, 2008; Yang and Johnson, 2006; Yang et al., 2004), suggested the presence of NC-derived stem cells, which normally remain quiescent until metamorphosis, but which can be activated for regeneration. The number, diversity and location of these cells remains poorly defined, although they are associated with the PNS, utilising the peripheral nerves to reach diverse locations in the skin (Budi et al., 2011; Dooley et al., 2013) and hypodermis (Iyengar et al., 2015). A breakthrough study identified a set of these stem cells residing in the DRG (Dooley et al., 2013), but indirect evidence indicates that they may be more widespread, associated with peripheral nerves (Camargo-Sosa et al., 2019). These stem cells are multipotent, with clones including all three pigment cell types, neurons and glia of the DRG and the PNS (Budi et al., 2011; Singh et al., 2016): originally named MSCs, a better name would therefore be adult NCSCs. Detailed studies of SCPs in birds and mammals identify them as an important source of melanocytes, as well as neurons and glia (Adameyko et al., 2009, 2012; Nitzan et al., 2013b), making it likely that these zebrafish adult NCSCs are their evolutionary equivalent.

Second, our hypothesis provides an alternative explanation for the observations at embryonic stages that in some fate-specification mutants (e.g. *mitfa*) the absence of one cell type (melanocyte) is accompanied by elevated numbers of another (iridophore), previously interpreted under PFR as evidence for a bipotent cell. Under our CFR hypothesis, the explanation results from the preferential order of progression through the sub-states, such that if transition is blocked by a mutation, the cell pauses in a specific sub-state. For example, in *mitfa* mutants (Lister et al., 1999), we propose that cells cannot progress from a pro-iridophore sub-state to a pro-melanocyte sub-state [or, more accurately, cannot readily progress; it is likely that cells in such mutants are not permanently trapped in the specific prior sub-state, but simply their 'dwell-time' in that sub-state is prolonged. We envisage that the underlying gene regulatory network (GRN) allows alternative dynamic routes for exit from the prior sub-state, in a manner bypassing the subsequent sub-state]. Consequently, they spend longer in the former sub-state, making them more sensitive to pro-iridophore specification signals; consequently, more iridophores are formed.

It is worth considering carefully how our hypothesis compares with the original DFR hypothesis. Although the full transcriptional profile of the NC-HMP state remains to be defined, its potency apparently includes all pigment cell, peripheral neuron and glial fates, so our CFR hypothesis is, in terms of biological behaviour, closer to DFR (wherein migrating NCCs are homogeneous, fully multipotent progenitors), than to PFR. However, we note that the

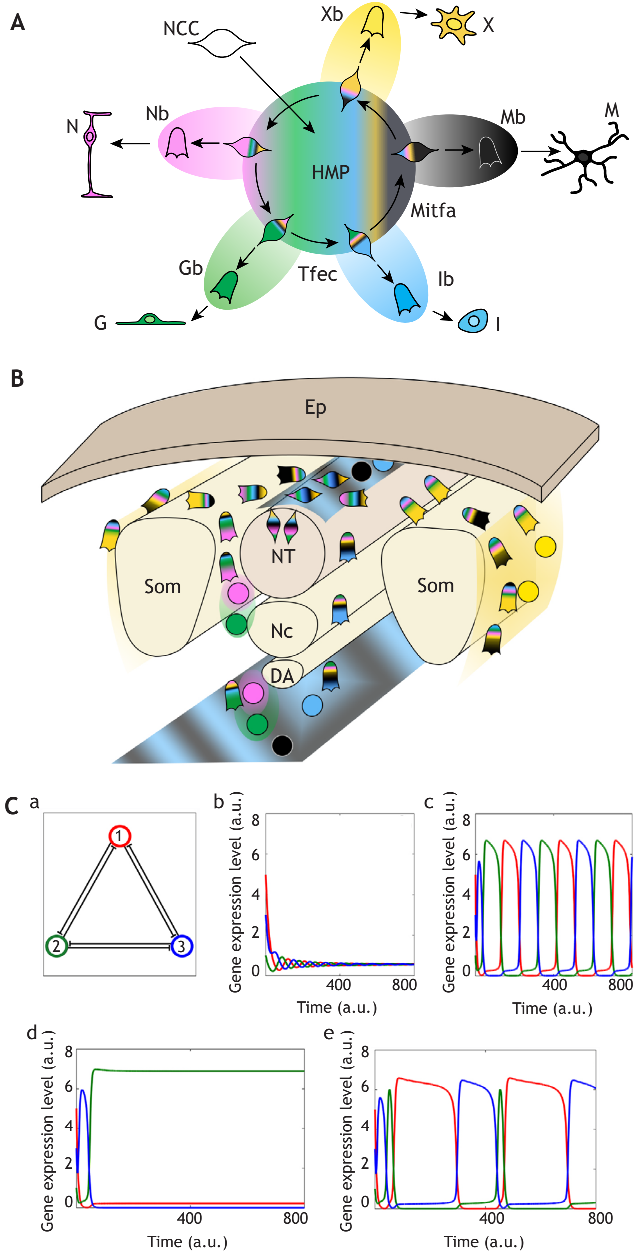

Fig. 4. CFR and CFR modelling. (A) In our new hypothesis, we propose that the fully multipotent NCC transitions to an NC-HMP (large multicoloured circle). Crucially, we envisage the HMP as fate-cycling through a series of sub-states (shown as cells spaced around the circle), each biased to adopt a single fate (indicated by expansion of one colour in the 'rainbow': neuroblast (Nb; pink), glioblast (Gb; green), iridoblast (Ib; blue), melanoblast (Mb; black), xanthoblast (Xb; yellow). Single fate specification occurs upon an NC-HMP encountering specific differentiation signals (pink, green, blue, black and yellow ovals), otherwise the multipotent progenitor continues cycling through the subsequent sub-states. Transition from one sub-state to the next is promoted by fate-specific transcription factors (TFs), including Mitfa and Tfec for pro-melanoblast and pro-iridoblast sub-states, respectively. G, glial cell; I, iridophore; M, melanocyte; N, neuron; X, xanthophore. (B) Initially (prior to delamination?), NC-HMPs show unbiased multipotency, indicated by even multicoloured shading. However, influenced by local environmental cues (specification factors; pink, green, black, blue and yellow shading) encountered before and/or during migration, cells become biased in their fate preferences, although not actually committed; such cells would appear to be fate-specified in a snap-shot view (e.g. by whole-mount *in situ* hybridisation). Depending upon the signalling environment, these biases may favour one or more fates (indicated by expansion of one or two colours in the 'rainbow' shading). In response to continuing fate specification signalling, cells exit the transcriptional fate-cycling phase and begin differentiation (unicoloured circles). DA, dorsal aorta; Ep, epidermis; Nc, notochord; NT, neural tube; Som, somite. (C) A simple mathematical model of a 'cross-repressilator' (Farjami et al., 2021) GRN, which exhibits different behaviours under different conditions. (Ca) Topology of a GRN in which each of the three TFs shown (1-3) mutually cross-represses the others; simulations show that this simple GRN readily displays behaviours matching key features of CFR. (Cb-Ce) Time courses of expression levels of TFs in the mathematical model, with Cb-Cd showing effects of increasing external (environmental) signal under conditions where it increases the production rates of all TFs equally; as the external signal intensifies, the GRN transitions from a non-cycling state with all TFs at very low levels (Cb), mimicking early NCCs, to a cycling state with each TF transiently and sequentially expressed at a comparatively high level (Cc), mimicking NC-HMPs, and, finally, to a stable state with one TF constantly expressed at a high level (Cd), mimicking the differentiated state. (Ce) TF levels in a cycling progenitor when the increasing level of external (environmental) signal increases production rates for TFs 1 and 3 (red, blue) but decreases that for TF 2 (green) compared with the simulation shown in Cc. Note that the cycling behaviour continues, but now the cell lingers sequentially in states favouring each of two ('red' and 'blue') fates, and the cell only very transiently lingers in a state favouring the third ('green') fate; this is one example of how the system can display a behaviour compatible with bias towards a subset of fates, while still retaining multipotency (cycling through all states). Note that the time courses and expression levels are illustrative, and in arbitrary units (a.u.). For simplicity, the model for an NC-HMP with just three fates is shown, but the model can be generalised to higher multipotency (Farjami et al., 2021).

progenitor state in our model is distinct from the earliest NCCs, as these do not express *sox10* initially (Lopes et al., 2008), whereas our single-cell study sorted cells using a reporter of *sox10* expression (Nikaido et al., 2021 preprint). Furthermore, even amongst these *sox10*$^+$ cells, our clustering identifies multiple clusters equivalent to NC-HMP progenitors, including ones without ('early NC-HMP') and with ('late NC-HMP') elevated *ltk* expression; conceivably, these might represent detection of distinct sub-states themselves. Careful analysis of marker expression *in vivo* by RNAscope also indicates a series of early progenitor states (Petratou et al., 2021), although their exact correspondence to the cell types defined by NanoString clustering remains to be determined. We speculate that our NC-HMPs may best correspond to a classic 'trunk NCC', and for that cell type might be similar to the original DFR. However, the key feature we are proposing, the dynamic nature of the GRN within these cells, makes our hypothesis distinctive. It also has some interesting consequences, as we will now begin to explore.

Modelling CFR – beyond bifurcations in fate specification

To formalise our conceptual model and to begin to explore its properties and feasibility, we took a mathematical modelling approach. Here, we consider a mathematical model based only on deterministic dynamics, although alternative models incorporating stochastic fluctuations as key drivers of transitions between sub-states are also attractive, having been proposed in other developmental contexts (e.g. Corson and Siggia, 2017). In these latter models, deterministic gene regulation is responsible for creating the relevant sub-states, and gene expression fluctuations allow for transitions between them. These models are attractive because they readily display features mimicking the biology, such as a 'noisy' and blurred state corresponding to a multipotent progenitor state, becoming more differentiated under the control of external signals capable of reshaping the basins of attraction of the sub-states. However, such stochastic models require a careful tuning of parameters balancing constraining deterministic dynamics with heterogeneity-inducing stochastic components, leading us to pursue

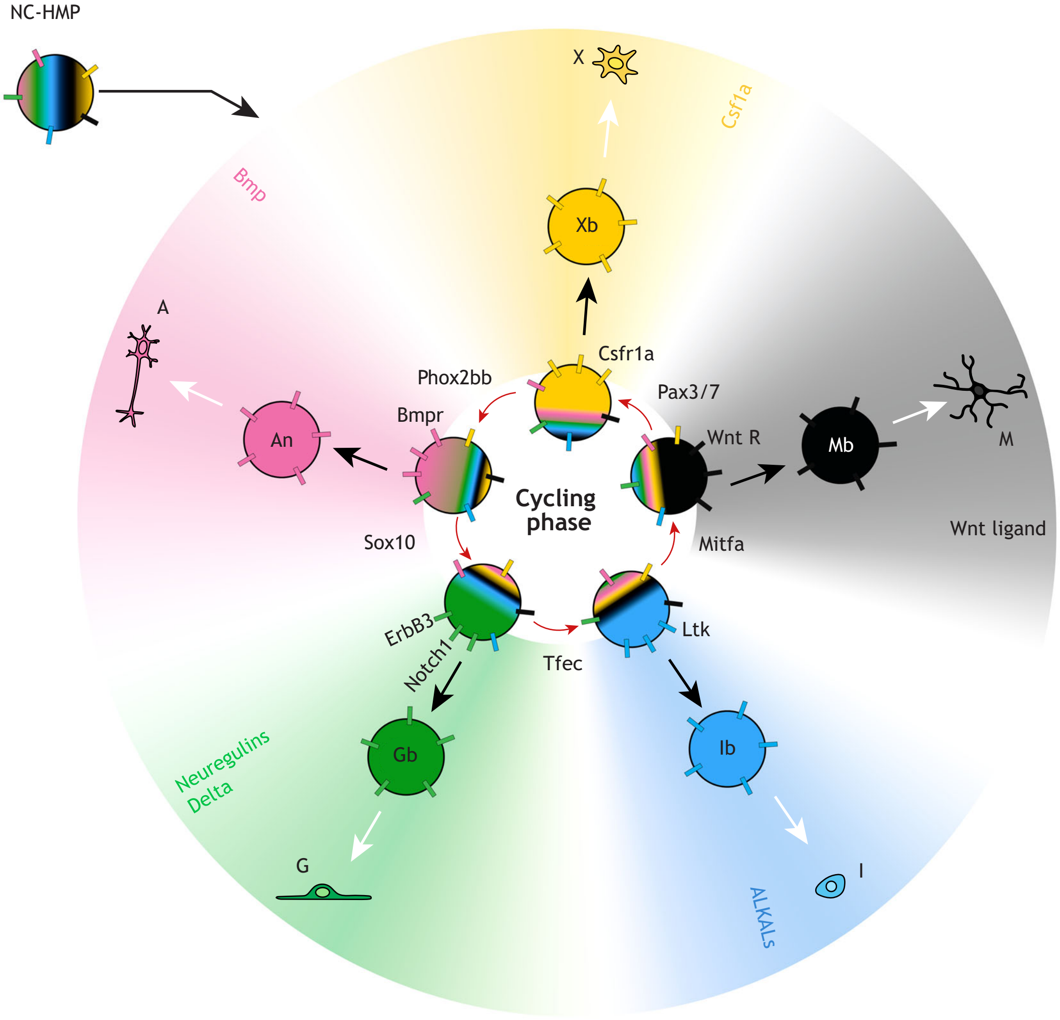

Fig. 5. Potential molecular basis for cyclical fate restriction. The CFR model expanded to show a plausible molecular mechanism underpinning the key features, although we note that other molecular interpretations would also be compatible with the concept we are proposing. NC-derived highly multipotent progenitors (NC-HMPs; multicoloured circles) express key transcription factors for different cell fate specification programmes [Phox2bb (autonomic neuron), Sox10 (glia), Tfec (iridophore), Mitfa (melanocyte) and Pax3/7 (xanthophore)] and enter fate-cycling phase under influence of environmental signals (not shown). During the cycling phase (centre), the NC-HMP cycles through a series of sub-states (multicoloured circles with a larger area coloured in pink, green, blue, black and yellow), each of them biased towards the specification of a single cell fate: autonomic neuroblast (An), glioblast (Gb), iridoblast (Ib), melanoblast (Mb) and xanthoblast (Xb), respectively. Transition to a new sub-state is promoted by increased activity of key transcription factors (hypothesised to be Phox2bb, Sox10, Tfec, Mitfa and Pax3/7) specific to each cell specification programme. Before entering the cycling phase, the NC-HMP has unbiased numbers of receptors [rectangles around cell surface coloured in pink (Bmpr), green (ErbB3 and Notch1), blue (Ltk), black (Wnt receptor; Wnt R) and yellow (Csfr1a)], each responsive to specific environmental cell fate specification signals [coloured areas: Bmp (pink), Neuregulins and Delta (green), ALK and LTK ligand ALKALs (blue), Wnt ligand (black) and Csf1a (yellow)]. Upon entering the fate-cycling phase, and as result of increased activity of transcription factors, the receptors specific to the sub-state are increased and those of other sub-states are decreased. When the cycling NC-HMP receives insufficient of the sub-state-specific environmental differentiation signal, it transitions to a new substate (red arrows); this is considered an emergent property of the GRN underlying the NC-HMP. In contrast, an NC-HMP exposed to sufficient sub-state-specific differentiation signal will activate the corresponding cell fate specification programme, and downregulate all other transcription factors and the receptors for other cell fate specification signals, thus exiting the cycling phase (black arrows), i.e. the cell has become committed to a single fate. These committed progenitors [single-coloured circles in pink (autonomic neurons), green (glioblast), blue (iridoblast), black (melanoblasts) and yellow (xanthoblast)] will differentiate (white arrows) into the respective cell type [autonomic neuron (A), glial cell (G), iridophore (I), melanocyte (M) or xanthophore (X), respectively].

the 'extreme' version of a fully deterministic system in the first instance.

Mathematical modelling of fate specification has mainly focused on mutual cross-repression between a pair of key fate-specific transcription factors, resulting in paired fate choices, e.g. macrophage versus neutrophil (Huang et al., 2007; Laslo et al., 2006). This approach may reinforce the impression that fate choice obligatorily proceeds through a series of bifurcating fate decisions, but this need not necessarily be the case. A simple expansion of the cross-repressive model to encompass multiple transcription factors driving multiple different fates reveals intrinsic cycling behaviour strikingly similar to that envisaged under, and hence providing theoretical support for, the CFR hypothesis (Fig. 4C) (Farjami et al., 2021). From a mathematical perspective, the emergence of cycling is a natural and generic consequence of negative-feedback loops imposed by cross-repression. Our modelling work indicates that the key features of the dynamics we observe appear over a wide range of model parameter values and indeed for different choices of the precise form of the mathematical equations describing the cross-repression.

The possibility of oscillatory dynamics within GRNs incorporating negative feedback was highlighted by the well-known synthetic ‘repressilator’ network constructed by Elowitz and Leibler (2000). In the context of the Notch signalling pathway, oscillations have been previously observed to result in important features that explain observed biology, such as sequential formation of somites, and the balance of neural stem cell maintenance and neuronal differentiation (Lewis, 2003; Monk, 2003; Ochi et al., 2020). We have developed a series of mathematical models of such cross-repression models (Fig. 4Ca) of cell differentiation from multipotent progenitors, exploring their outputs in simulations. Analytically, we have identified remarkable behaviours that mimic many aspects of the biology envisaged in the CFR hypothesis (Farjami et al., 2021). Particularly interesting is the effect of changing intrinsic cellular properties, such as production and degradation rates of specific regulatory transcription factors, which in turn can be viewed as a response to alterations in extrinsic fate-specification signals. Time spent in the vicinity of each of the successive stable sub-states expands, consistent with cells becoming (apparently) more specified and, in response to the correct signals, actually committed. Thus, transitions from a non-cycling, multipotent phase – analogous to the early NCC, prior to any fate specification (Fig. 4Cb) – into a dynamic phase cycling through all sub-states (Fig. 4Cc), and to eventual stable adoption of a fate (i.e. committed final fates) (Fig. 4Cd), can be displayed over wide ranges of model parameters (Farjami et al., 2021). We note that, *in vivo*, there is a considerable delay between the first expression of *sox10* as part of NC induction and the first evidence of individual fate specification in response (e.g. in zebrafish, *sox10* expression begins in the trunk at around 12 hpf, but the first downstream fate-specification response, *mitfa* transcription, is not detected until around 18 hpf; Dutton et al., 2001; Lister et al., 1999; Montero Balaguar et al., 2006); perhaps this delay reflects the time when the cells are in the non-cycling multipotent phase (Fig. 4Cb). When in the cycling phase, cells linger close to one sub-state (characterised by higher expression of relevant markers), but rapidly move on to another sub-state primed for another fate. This is consistent with the dynamicity and heterogeneity (for the appropriate markers, i.e. those undergoing cycling of expression) of the NC-HMP. Intriguingly, our modelling shows that the balance of probabilities of progenitors adopting different individual fates can be modified from equivalent (all fates equal) to highly biased [a few, or just one, fate(s) strongly favoured], by altering the relative production rates of specific transcription factors, as might happen, for example, in response to specific environmental signals (Fig. 4Ce). This recalls the evidence for strong and more subtle shifts in NCSC potency, depending upon their anatomical origin (White et al., 2001), which clearly indicates that their multipotency is ‘tweaked’ to alter the favoured fates and, indeed, that it becomes tuned by their environment. Thus, in contrast to early NCCs (most of which are considered to be NCSCs), NCSCs isolated from the sciatic nerve seem unable to contribute to sensory neurons (as opposed to sensory ganglial glia) in transplant studies. More subtly, their ability to generate sympathetic neurons decreases in favour of production of parasympathetic neurons, an effect associated with a decreased sensitivity to BMP2 (White et al., 2001).

Thus, we see from the model an indication of the type of mechanism that might underpin the behaviour proposed in the CFR hypothesis, with environmental factors influencing the apparent fate restriction of migrating and post-migratory (see Glossary, Box 1), progenitors. The effect of the environment is to change the balance of time spent in each of the sub-states, so that, for example, in cells in the skin, more time is spent in the sub-state favouring melanocyte specification. This, likely combined with local differences in the levels of fate-specification signals, would have the effect that, *in vivo*, these cells would be far more likely to differentiate into the favoured fate (e.g. melanocyte) rather than others, and thus would appear to be fate restricted. Indeed, as they migrate, local signals along the migration route will drive their cycling behaviour to biases that, by definition, become appropriate to that route (e.g. iridophores on the medial migration route). Importantly, depending upon ongoing signalling, dwell times may favour two (or possibly more) biased states. This combined with mRNA/protein perdurance may explain the ‘double specification’ observations, and the simultaneous partial activation of fate-specification programmes, seen in migrating NCCs (Petratou et al., 2021, 2018; Soldatov et al., 2019). In a snapshot view, this gives the impression of a PFR process, masking *in vivo* the underlying full multipotency that is revealed when cells are removed from those environmental signals.

The original suggestion in the DFR hypothesis that fate specification occurs in a post-migratory location, with local signals determining the fate chosen, is now replaced in the CFR hypothesis with the idea that fate maintenance (i.e. fate commitment; see Glossary, Box 1) is strengthened and stabilised by that post-migratory environment. This view suggests the extreme hypothesis that cells locked in the differentiated state by epigenetic reinforcement of this transcriptional programme might retain latent multipotency, which could be liberated when that epigenetic lockdown is released. This is, in essence, the mechanism likely to underlie transdifferentiation (e.g. Shen et al., 2000).

Moreover, mathematical modelling of the CFR hypothesis allows us to see how NCSCs (Box 2), which we propose are retained NC-HMP cells, might be held by local environmental conditions (the niche) in a (pseudostabilised) sub-state (as a glial cell in a DRG or in peripheral nerves), but might retain potency for other fates when environmental conditions change [e.g. on liberation from the niche, such as when the DRG niche becomes activating, or upon losing contact with peripheral dendrites (Adameyko et al., 2009), or, alternatively, when single cells are isolated under conditions in which environmental signals are diluted and cells ‘relax’ from their varied specified states]. For these ‘differentiated’ cells, multipotency can be readily reactivated by changing environmental signals and cause dedifferentiation, perhaps even back to the cycling progenitor state.

Could CFR apply more widely, to include neuronal fates?

Our work has focused on pigment cell fates, but several observations suggest that CFR applies not just to chromatophores, but more widely, at least to neural fates. First, *sox10* expression is a prominent feature of the NC-HMP state that we propose is fate-cycling. Given the key roles for Sox10 in both NCC multipotency and glial fate specification and differentiation (Delfino-Machin et al., 2017; Dutton et al., 2001; Kelsh and Eisen, 2000; Kuhlbrodt et al., 1998; Liu et al., 2020; Paratore et al., 2001; Peirano and Wegner, 2000; Sonnenberg-Riethmacher et al., 2001), it seems likely that any multipotent NCC will have glial fates as an option. Second, our fate mapping of *ltk*-expressing cells, interpreted as the NC-HMPs and their progeny, showed the unexpected inclusion of peripheral neuronal and glial fates (Nikaido et al., 2021 preprint). Third, our modelling considerations, described above, and the dynamic view of differentiation as a potentially pseudo-stabilised state that results from them, readily encompasses the emerging view of glial cells as (including) NCSCs. In agreement with Dupin and colleagues (Dupin et al., 2018), we propose that multipotent NCSCs are retained in numerous locations, as observed for SCPs (Box 2). However, we take this one step further, proposing the radical view that these cells might be fully multipotent, albeit often constrained by their local stem cell niche. Fourth, various studies have shown a close link between melanocytes and the PNS, especially glial cell types (e.g. Adameyko et al., 2009; Dupin et al., 2000, 2003; Girdlestone and Weston, 1985; Kunisada et al., 2014; Motohashi et al., 2009; Real et al., 2006; Watanabe et al., 2016). Finally, scRNA-seq reveals the transcriptional similarity between glia and migratory multipotent cells (Soldatov et al., 2019).

One key aspect of the PFR hypothesis when applied to the PNS is the ‘bipotent’ ganglial progenitors (Fig. 1A); we note that although these cells are generally labelled as ‘bipotent’, they give rise to multiple types of neurons and glia and hence might equally be considered more multipotent. As noted above, there is considerable evidence for early segregation of sensory and autonomic ganglial progenitors, with the choice of neurons and glia arising only later, suggesting that cells become at least strongly biased towards these fate combinations. This is readily accommodated within the CFR hypothesis by suggesting that the nascent ganglial niche drives cells into a fate-cycling state in which the pro-neuronal and pro-glial sub-states are dominant, biasing NC-HMPs in these regions to neuronal and glial fates (Fig. 4Ce). We note that, under the CFR hypothesis, these ‘neuroglial progenitors’ are only strongly biased towards appropriate neural fates; they retain full multipotency, explaining the NanoString data at later embryonic stages noted above. In the case of these progenitors, this cryptic multipotency is rather easier to envisage because the evidence for melanocyte derivation from peripheral nerve SCPs is so strong, and given that, in zebrafish, adult melanocyte stem cells (MSCs) also generate neurons and glia (Box 2) (Adameyko et al., 2009, 2012; Dooley et al., 2013; Kelsh and Barsh, 2011; Nitzan et al., 2013b; Parichy and Spiewak, 2015; Singh et al., 2016).

How do we reconcile the detailed dissection of mouse peripheral neural development using scRNA-seq, which provided strong support for a PFR hypothesis (Soldatov et al., 2019)? In many respects, their data can be readily reconciled with the CFR hypothesis. Although the authors were unable to resolve melanocyte development, *Mitf* expression was widespread amongst delaminating and migrating NC. They show that the earliest phase of *Neurog2* expression, in premigratory NCCs, corresponds to fully multipotent cells, and not to committed sensory neuron progenitors. More broadly, their data show that these delaminated, premigratory NCCs exhibit low level expression of a wide range of markers of derived fates, including three other key neuron specification transcription factors: *Phox2b*, *Neurog1* and *Pou4f1*. We propose these cells are likely equivalent to our NC-HMPs and might be fate-cycling in the manner we propose. Detecting this would be challenging using current bioinformatics tools because cycling behaviour would result in overlapping data that would be hard to analyse in terms of a well-defined pseudotime (Mao et al., 2017). Interestingly Soldatov and colleagues characterise a gradual process of fate decisions, initiating early in migration with co-activation of differentiation programmes, then gradually biased towards genetic programmes associated with specific fates, and becoming distinct in committed cells in post-migratory locations (Soldatov et al., 2019); this seems to be compatible with the gradual emergence of a committed differentiated state from multipotent progenitors that we are proposing.

Conclusions

Our CFR hypothesis provides a novel framework for thinking about the developmental mechanisms underpinning NCC fate specification and differentiation, reconciling data supporting both traditional hypotheses, which may also be relevant in other stem cell and developmental contexts. Indeed, it has been proposed that oscillatory dynamics might underpin stemness itself (Furusawa and Kaneko, 2012). Although our CFR hypothesis is still speculative, it is certainly time to think differently in order to resolve the contest between the historically proposed and irreconcilable mechanisms. The CFR hypothesis calls into question the validity of assuming repeated bifurcations as a necessary feature of progenitor cell development. This, in turn, highlights the need for improvements in scRNA-seq analysis algorithms that may be required in order to assess differentiation trajectories in more detail, including the ability to detect increasingly complex graph structures, including cycles, robustly. Recent developments in bioinformatics algorithms, such as the use of reversed graph embedding (Qiu et al., 2017) and partition-based graph abstraction (Wolf et al., 2019), are extremely welcome.

Recent results in single-cell analysis have supplied a growing number of examples of dedifferentiation and reversible differentiation, especially in the contexts of early development (Papatsenko et al., 2015) and regeneration (Lin et al., 2021). In this context, we consider that a simple two (sub-)state model of reversible differentiation, such as that proposed by Papatsenko et al. (2015), is a primitive example of a cyclical-type model, with cells having the option to continue fate-cycling by switching to the alternative state or exiting the cycle at the current state. In this respect, the multistate CFR model suggested here can be considered as a generalisation of this two-state model for a larger number of cell types. Obviously, the concept of recurrence does not necessarily require any prescribed ordering of sub-states and the dynamics may move between sub-states that have differing sensitivities to different signals, controlling transitions to other sub-states or the exit from the fate-cycling regime. The GRN dynamics in general is likely to include additional stochastic effects; in this case, fate-cycling would describe only the most probable pathway of transitions, assuming that each transcription factor becomes active relatively often. In the case of absence of such a transition-inducing transcription factor (e.g. in a mutant context), the cell would be delayed or prevented from moving on to the next sub-state and would become (transiently) trapped in a particular sub-state. A wide range of detailed alternatives can be envisaged, including, in another extreme case, these transitions all being spontaneous. We propose that the key feature of recurrence of specific sub-states is the distinctive feature of such systems, and justifies our use of the term ‘cyclical’.

The CFR hypothesis makes a series of testable predictions, which together are distinctive; some already have at least some experimental support, but now require comprehensive assessment. These predictions include:

1. Co-expression of key fate-specification transcription factors, in early progenitor stages, reflecting their potency.
2. Consequently, the process of differentiation and fate commitment is only partly about activation of expression of key transcription factors, and is also about their maintenance

(and upregulation) while repressing those driving alternative fates.
3. Expression of fate-specification receptors in premigratory NCCs should be fate-specification transcription factor dependent.
4. Cyclical expression of some genes in NC-HMPs, likely including those encoding these fate specification signal receptors.
5. Cyclical expression underpinned by a pattern of cross-repression between fate-specific transcription factors.
6. Retained, but cryptic, multipotency in most NCCs, persisting at least until differentiation. This cryptic multipotency may underpin the setting aside of APSCs/NCSCs, which may be much more widespread within the peripheral nervous system than currently envisaged.
7. This multipotency is most clearly revealed by transcriptional profiling studies in which conditions favour both highly sensitive detection of very low level gene expression, and 'relaxation' of the fate-specified state induced by environmental signals.
8. Although many cells may undergo terminal differentiation, with that transcriptional state 'locked in' by epigenetic mechanisms (commitment), quiescent APSCs/NCSCs may retain propensity for a more dynamic, transcriptional state as a result of local factors forming their niche.
9. Activated APSCs/NCSCs will have broad, but cryptic, multipotency, with local niche factors dictating the specific cell fates they generate *in vivo*.

In summary, the CFR hypothesis provides a novel and rich framework within which to consider the diverse and conflicting data surrounding NCC fate specification and differentiation. Its testing, and the resolution of this long-standing conflict over developmental mechanisms, will continue to provide exciting challenges. We propose that it may even be more widely applicable, for example in the context of haematopoietic and neural stem cells.

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Competing interests
The authors declare no competing or financial interests.

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