

1 **Reconciling diverse mammalian pigmentation patterns with a fundamental mathematical**
2 **model**

3

4 Richard L. Mort^{1*}, Robert J. H. Ross^{2*}, Kirsten J. Hainey¹, Olivia J. Harrison¹, Margaret A.
5 Keighren¹, Gabriel Landini³, Ruth E. Baker², Kevin J. Painter⁴, Ian J. Jackson^{1,5} and Christian A.
6 Yates⁶

7

8 1. MRC Human Genetics Unit, MRC IGMM, Western General Hospital, University of Edinburgh,
9 EH4 2XU, UK

10

11 2. Centre for Mathematical Biology, University of Oxford, Andrew Wiles Building, Woodstock
12 Road, Oxford, OX2 6GG, UK

13

14 3. School of Dentistry, College of Medical and Dental Sciences, University of Birmingham, St.
15 Chad's, Queensway, Birmingham, B4 6NN, UK

16

17 4. Department of Mathematics and Maxwell Institute for Mathematical Sciences, Heriot-Watt
18 University, Edinburgh, EH14 4AS, UK

19

20 5. Roslin Institute, University of Edinburgh, Roslin, EH25 9RG, UK

21

22 6. Centre for Mathematical Biology, Department of Mathematical Sciences, University of Bath,
23 Claverton Down, Bath, BA2 7AY, UK

24

25

26 *These authors contributed equally to this work

27

28 Corresponding author Ian.Jackson@igmm.ed.ac.uk

29 **Abstract**

30 Bands of colour extending laterally from the dorsal to ventral trunk are a common feature of mouse
31 chimaeras. These stripes were originally taken as evidence of the directed dorsoventral migration of
32 melanoblasts (the embryonic precursors of melanocytes) as they colonise the developing skin. De-
33 pigmented 'belly-spots' in mice with mutations in the receptor tyrosine kinase Kit are thought to
34 represent a failure of this colonisation, either due to impaired migration or proliferation. Tracing of
35 single melanoblast clones, **however**, has revealed a diffuse distribution with high levels of axial
36 mixing - hard to reconcile with directed migration. **Here we construct** an agent based stochastic
37 model calibrated by experimental measurements to investigate the formation of diffuse clones,
38 chimaeric stripes and belly-spots. Our observations indicate that melanoblast colonisation likely
39 proceeds through a process of undirected migration, proliferation and tissue expansion and that
40 reduced proliferation is the cause of the belly-spots in Kit mutants.

41 **Introduction**

42 Melanoblasts, the embryonic precursors of the pigment producing melanocytes of skin and hair,
43 colonise the developing epidermis during development. Mice heterozygous for mutations of the
44 receptor tyrosine kinase Kit present with a de-pigmented ventral belly-spot long thought to
45 represent a failure of either melanoblast proliferation or migration¹. Melanoblasts are specified at
46 embryonic day 9 (E9) in the pre-migratory neural crest² by down regulation of the transcription
47 factors Foxd3 and Sox2 and up-regulation of the master transcription factor Mitf^{3,4}. At E10.5
48 melanoblasts de-laminate from the neural crest, up-regulate the melanoblast specific genes *Pmel*,
49 and *Dct*^{5,6} and accumulate in a region known as the migration staging area (MSA)^{1,7,8}.
50 Melanoblast survival in the skin is dependent on signalling between Kit (expressed in melanoblasts)
51 and its ligand Kitl (expressed by dermal fibroblasts and keratinocytes), however initial delamination
52 does not require Kit signalling^{1,9}. At E10.5 melanoblasts begin to leave the MSA in a Kitl and
53 endothelin 3 (Edn3) dependent manner and embark on a dorsolateral migratory pathway in the
54 dermis between the developing somites and the ectoderm^{1,7,8,10}. At E12.5 melanoblasts move from
55 the dermis to the epidermis, up-regulate E-cadherin, lose their dependence on Edn3 and continue to
56 migrate and proliferate¹¹⁻¹³. A dermal population also persists whose size remains constant (and so
57 proportionally decreases in relation to the epidermal population)¹⁴. Epidermal colonisation is
58 complete by around E15.5, after which melanoblasts down regulate E-cadherin and begin to localise
59 to the developing hair follicles^{11,15,16}. Luciani *et al.*¹⁴ measured melanoblast doubling times in
60 the dermis and epidermis and used a mathematical model to estimate the number of melanoblast
61 progenitors specified in the pre-migratory neural crest¹⁴.

62

63 Dorsolateral migration was first demonstrated by the capacity of grafted mouse skin to produce
64 melanin in chick coelom^{17,18}. Later this early work was confirmed in chimaeric mice generated by
65 the aggregation of albino and pigmented cleavage stage embryos¹⁹. In this work, Mintz described

66 a 'standard pattern' of 17 successive bands/stripes extending dorsoventrally. Although the
67 interpretation of the number of stripes was later criticised^{20,21}, the acceptance of the stripes as
68 representing directed dorsolateral migration remained. However, using a *Dct::lacZ* transgene to
69 label individual *lacZ* revertant melanoblast clones, Wilkie *et al.*²² observed a surprising degree of
70 mixing at the axial level. The patterns observed were hard to reconcile with directed migration and
71 suggested that different cell dispersal mechanisms may act in the head and trunk²².

72
73 Experimental measurements and mathematical modelling have formed the basis of a number of
74 studies on neural crest colonisation. The failure to colonise the embryonic intestine by neural crest
75 precursors in Hirschsprung's disease has been extensively studied²³⁻²⁷, as has the behaviour of
76 chick cranial neural crest populations²⁸. Both continuous partial differential equation (PDE)
77 population-level models²³ and discrete lattice-based individual-level models²⁹ have been
78 employed to model Hirschsprung's disease. Zhang *et al.*²⁹ demonstrated, using a lattice based
79 model, that colonisation of the intestine is more sensitive to changes in proliferation rate than in cell
80 migration^{23,29}. Stochastic effects, proposed to underlie incomplete penetrance in some
81 neurocristopathies³⁰, have also been investigated using individual-level modelling approaches. In
82 the present study we use experimental observations to parameterise an agent-based stochastic model
83 that allows us to explore colonisation of the trunk epidermis by migrating melanoblasts. We
84 conclude that a simple mechanism of undirected migration, proliferation and tissue expansion can
85 reconcile the patterns described above. Furthermore, Kit mutant melanoblasts migrate normally but
86 have a proliferation defect that results in a depigmented ventral belly spot.

87

88 **Results**

89 **Dermal and epidermal melanoblast migration is undirected**

90 To better understand the behaviour of melanoblasts during their colonisation of the developing

91 embryo, and to understand how this behaviour may result in the patterns described above
92 (Supplementary Fig. 1a-b), we performed *ex vivo* live imaging of whole mouse embryos at E11.5
93 and E12.5 and of mouse embryonic skin at E14.5^{31,32}. At E14.5 we used a *Tyr::Cre* transgene
94 expressing Cre-recombinase under the control of the tyrosinase promoter with *R26R-EYFP* to label
95 melanoblasts with EYFP. At earlier stages, where *Tyr::Cre* activity in other neural crest lineages
96 made imaging of melanoblasts difficult, we used a *Pmel::CreERT2* knock-in line combined with
97 *R26R-EYFP* (Methods). In all time-lapse sequences between E11.5 (Supplementary Movies 1-2)
98 and E15.5 melanoblasts migrated constantly, only pausing when they briefly pass through M-phase
99 (Supplementary Movie 3). They are polarised and move along their longest axis (or Feret's diameter
100 – See Fig. 1a and Supplementary Movie 4). The Feret's angle is therefore a good proxy for the
101 direction of instantaneous migration. The distribution of these angles in our time-lapse sequences is
102 uniform, consistent with completely random melanoblast orientation and undirected migration. To
103 confirm that this was not an artefact of the *ex vivo* system we stained *Dct::lacZ* embryos expressing
104 β -galactosidase in the melanoblast lineage⁶ and observed the same random distribution of
105 melanoblast angles at time points between E11.5 and E15.5. (Supplementary Table 1). There is thus
106 no directionality associated with melanoblast migration in developing dermis at E11.5-E12.5 or
107 epidermis at E13.5-E15.5. We therefore assume this is also the case at E10.5 when cell numbers are
108 too low to perform a rigorous analysis.

109

110 **Melanoblast dispersal is not mediated by repulsion**

111 Time-lapse sequences were sampled from E14.5 embryos in the mid-trunk region at a range of
112 dorsoventral positions resulting in a range of melanoblast densities. To investigate whether any
113 mechanism of mutual repulsion between adjacent melanoblasts influenced their position, we tested
114 whether the spacing of melanoblasts conformed to complete spatial randomness (CSR)³³. There was
115 no association between melanoblast density and the *P*-value of a Berman's test comparing each

116 pattern to CSR, and 99.5% of examples did not deviate from CSR (Fig. 1b). We reasoned that there
117 should still be an effect over very short distances due to exclusion at the scale of single cells and so
118 examined the pair-correlation function (PCF)³⁴⁻³⁶, a summary statistic that provides a measure of
119 spatial patterning on different scales (Methods). This showed a non-random spatial segregation of
120 melanoblasts below around 28 μm (Fig. 1c), consistent with volume exclusion at the scale of an
121 individual cell. Above this distance the spacing of cells conformed to CSR. The melanoblast
122 distribution at E14.5 (before hair follicle localisation) is therefore spatially random, and repulsion
123 between melanoblasts does not contribute to their dispersal.

124

125 **Melanoblast migration and proliferation are density-dependent**

126 We examined the population spread of melanoblasts in our E14.5 time-lapse sequences and
127 calculated its mean squared displacement (MSD). We found MSD to be directly proportional to
128 time, indicating that diffusion is an appropriate mathematical description of melanoblast migration
129 and consequently that the melanoblast population is not migrating with a preferred direction (Fig.
130 1d-e). Similar results were observed at E13.5 and E15.5. Due to the small number of melanoblasts
131 at E11.5 we were not able to perform a diffusion analysis but their behaviour appeared qualitatively
132 similar to E14.5 melanoblasts (compare Supplementary Movies 1 and 2 to Supplementary Movie
133 3). Diffusive motion is characterised by its diffusion coefficient (D) which is proportional to the
134 gradient of the plot in Fig. 1e. The length and width of the embryonic trunk increases linearly
135 between E10.5 and E15.5 (Fig. 2a and Supplementary Table 2), this is accompanied by ~6-fold
136 increase in the number of melanoblasts between E10.5 and E11.5 (as identified by *Dct::lacZ*
137 staining, Fig 2b-c) and an increase in the mean melanoblast density in the mid trunk region from
138 ~200 to ~700 cells mm^{-2} between E12.5 and E15.5 (Fig. 2d). We examined the relationship between
139 cell density and D and found a strong negative correlation (Fig. 2e); melanoblasts diffuse faster
140 when they are less densely packed. We also examined melanoblast proliferation by measuring the

141 frequency and length of mitotic events (Supplementary **Movie 4**). The mean melanoblast M-phase
142 length (T_m) was independent of cell density, but the proportion of mitotic cells (P_{mc}) was strongly
143 negatively correlated with density and the cell cycle time ($T_c = T_m/P_{mc}$)³⁷⁻³⁹ was consequently
144 strongly positively correlated with increasing density (Fig. 2f and Supplementary Fig. 1d-e).

145

146 **Stochastic modelling of melanoblast colonisation**

147 We hypothesised that **undirected** melanoblast movement and proliferation, in tandem with tissue
148 growth (Fig. 2a and Supplementary Table 2) are sufficient for melanoblast colonisation and that this
149 simple mechanism can explain the patterns observed in chimaeras, individually labelled clones and
150 Kit mutants. We used our observations to parameterise a stochastic model of melanoblast
151 colonisation of the trunk (**Methods**). **Our modelling framework only considers the growth of the**
152 **trunk region and its colonisation by the migrating melanoblast population. The domain is limited**
153 **axially to the region between and not including the limb buds and encompasses the complete**
154 **dorsoventral length (Fig 2a and Methods).** We assume that no new melanoblasts are specified after
155 E10.5 and therefore that the growing melanoblast population is produced solely by the proliferation
156 of this founder population. Melanoblasts migrate first within the developing dermis between E10.5
157 and E12.5 and subsequently within the epidermis and dermis between E12.5 and E15.5. We
158 collectively refer to the dermal and epidermal layers that can support melanoblast survival as the
159 dorsoventral integument (DVI – **Methods**, Supplementary Fig. 2) and assume that melanoblast
160 behaviour in these compartments is equivalent. We describe above through analysis of cell
161 orientations in *Dct::lacZ* embryos and time-lapse experiments that there is no directed migration in
162 either compartment. In our simulations we employed an agent-based, discrete-space random-walk
163 model on a growing two-dimensional lattice employing volume exclusion, whereby at most one
164 agent (melanoblast) occupies each square lattice site, and melanoblasts cannot migrate or proliferate
165 into occupied sites⁴⁰. **The stochastic** events are simulated using the Gillespie algorithm (**Methods**)⁴¹.

166

167 Using the parameters generated from our experimental observations (Methods and Supplementary
168 Table 3) our model was able to replicate the relationships between cell density, the diffusion
169 coefficient and cell cycle time described above (red lines in Fig. 2e-f). It predicts colonisation of the
170 growing domain. That is, the averaged cell density in the model at all stages of embryonic
171 development closely fits our experimental data (Fig. 3a). Examples of domain colonisation are
172 provided in Supplementary Movie 6 and Supplementary Fig 4a.

173

174 **A common mechanism can explain chimaeric and mosaic patterns**

175 To explore the relationship between mixing, tissue growth and proliferation we simulated rare
176 melanoblast clones (by mimicking *in silico* the experiments of Wilkie *et al.*²² – Methods), and
177 chimaeric patterns (two coloured and multi coloured, Fig. 3b-f and Methods). Our analysis of
178 simulation data suggests that the prominence of stripe-like patterns (an emergent property of the
179 model - Fig. 3b-c, Fig. 5 column a-g) is influenced by the number of clonal subtypes at the start of a
180 simulation and the degree to which they are mixed. We find that stripes are most apparent when we
181 initialise with only two clonal subtypes where a single, low frequency corresponding to the two-
182 striped pattern is clearly identifiable in the discrete fast Fourier transform (DFFT) of the clonal
183 signal (Fig. 4C and Methods). This corresponds to most experimental patterns in mice consisting of
184 only two colours. (Fig. 3d, Methods and Fig. 4). Our analysis suggests these stripes are composed
185 of multiple sub-clones of like genotype, formed by the stochastic coalescence of like clones and
186 that, contrary to received wisdom, stripe formation does not proceed through the directed migration
187 of coherent or descendent clones⁴² but rather stripes form from a favourable arrangement of like
188 clones in the initial conditions that are elongated dorsoventrally by the bias in growth in that
189 direction (Supplementary Table 2). Increasing the number of differently labelled clonal populations
190 removes the stripe-like pattern in the chimaeras (Fig. 4D and Fig.5). For example, compare the

191 panels in Fig 3c and Fig. 5a-h, showing two-colour and multicolour depictions of single
192 simulations. The dominant patches of colour in the first panel (Fig. 3c – top and Fig. 5 column a-g)
193 are composed of multiple sub-clones in the second (Fig. 3c – bottom and Fig. 5 column b-h).
194 Clones originating from what was previously the same labelled sub-population (but not progeny of
195 the same initial cell) now appear distinct from each other. If sub-populations are not well-segregated
196 initially then the formation of dorsoventral stripes is also less likely. Examples of well segregated
197 and poorly segregated initial sub-populations and the resulting patterns are shown in Fig. 4e and f,
198 respectively. When rare melanoblast clones (Fig. 3e-f) are simulated (Methods) and plotted
199 individually, they appear qualitatively similar to the patterns previously described in *Dct::laacZ*
200 embryos²², and are therefore reconciled with stripe formation in our model.

201

202 Stochastic evolution of dominant lineages in the model

203 Using our discrete model we observed a weak selection bias towards a small number of dominant
204 lineages. Typically the two most dominant lineages (of the 21 initially specified) in a given
205 simulation accounted for around 25% of the total number of melanoblasts at the end of the
206 simulation (Supplementary Fig. 3b). To explore this effect further we initialised our discrete model
207 with the movement and proliferation parameters employed in this study but with the same initial
208 agent distribution and domain size described by Cheeseman *et al.*^{26,27} - a two-dimensional square
209 lattice of size $L_x(t) = 50$ by $L_y(t) = 50$ in which the 10 left-most columns are fully occupied. Using
210 these conditions we observed a strong selection bias towards a small proportion of clonal subtypes.
211 On average, two agents from the original 500 contributed over 25% of the final agent density
212 (Supplementary Fig. 3c-d). This suggests that selection bias is heavily influenced by the initial
213 conditions. The relatively sparsely packed distribution of melanoblasts at E10.5 would likely result
214 in a weaker selection bias than in the developing intestine where pre-enteric neural crest cells are
215 more numerous and tightly packed in the foregut before they embark on their colonisation⁴³.

216 **Belly spots arise from reduced proliferation in *Kit* mutants**

217 Belly spot formation has long been proposed a product of either altered proliferation or migration or
218 both. To compare these effects in the model we performed a parameter sweep comparing the extent
219 of colonisation at different values of D and T_c . This revealed that our stochastic model is markedly
220 more sensitive to changes in proliferation rate than in the diffusion coefficient (Fig. 6a). *Nf1* is a
221 GTPase activating protein that negatively regulates Ras activity downstream of Kit signalling.
222 Ablation of *Nf1* results in constitutive Ras activity and increased proliferation⁴⁴. To investigate the
223 role of *Kit* signalling in melanoblast colonisation we performed live-imaging experiments at E14.5,
224 labelling melanoblasts on a *Kit*^{W-v/+} background⁴⁵ or ablating *Nf1* (Methods) to generate melanoblast
225 specific *Nf1*^{-/-} or *Nf1*^{+/-} genotypes. We found a lower density of melanoblasts in the E14.5 trunk of
226 *Kit*^{W-v/+} mice whilst the density was increased in *Nf1*^{-/-} mice implying a reduction and an increase in
227 melanoblast proliferation, respectively (Fig. 6b). However contrary to expectations we observed a
228 significantly higher rate of diffusion in *Kit*^{W-v/+} mutants. Diffusion was reduced in *Nf1*^{-/-} animals, but
229 the change was not significant (Fig. 6c). A plot of density against D (Fig. 6d) indicates that, for their
230 given densities, *Kit*^{W-v/+} and *Nf1*^{-/-} melanoblasts behave in a similar manner to wildtype (the negative
231 association with density observed in wildtype is preserved when the data are combined, Fig. 6d)
232 suggesting that the change in diffusion rates are a consequence of changes in cell density.

233

234 Plotting T_c against density for *Kit*^{W-v/+}, *Nf1*^{+/-}, *Nf1*^{-/-} mutants and wildtype we find that for a given
235 density *Kit*^{W-v/+} melanoblasts proliferate more slowly than would be expected and *Nf1*^{-/-} more quickly
236 (negating the positive wildtype association with density when the data are combined, Fig. 6e). We
237 calculated a corrected cell cycle time (as T_c / Density multiplied by 200 cells mm⁻²) this revealed a
238 longer cell cycle time in *Kit*^{W-v/+} melanoblasts (Supplementary Fig. 1g), and is the likely causal
239 factor in belly spot formation. We used our model to test this conclusion with the assumption that as
240 melanoblasts are specified from the neural crest in a *Kit*/*Kitl* independent manner¹ there would be

241 equal numbers in wildtype and *Kit*^{W-v/+} melanoblasts in the MSA at E10.5. Accordingly, increasing
242 cell cycle time in the model results in the failure of melanoblasts to fully populate the domain and
243 produces a white belly spot that mimics that seen *in vivo* (Fig. 6f , Supplementary Fig. 4 and
244 Supplementary **Movie 7**). As the cell cycle time is increased, belly spots are increasingly observed,
245 followed, at longer times, by dorsal spots along with larger belly spots. This is consistent with
246 existing mutant phenotypes, small spots are usually confined to the belly, whilst larger belly spots
247 are often accompanied by dorsal spotting, such as in mutants of *Rac1*⁴⁶ or *Magoh*⁴⁷. Furthermore,
248 despite there being no change in cell motility on the *Nf1*^{-/-} background, loss of Nf1 rescues the belly
249 spot in *Kit*^{W-v/+} individuals (**Fig. 7a-d**).

250

251

252 Discussion

253 In summary we show experimentally that migrating melanoblasts do not have preferred
254 directionality, but rather diffuse and proliferate throughout the developing skin in a density-
255 dependent manner. Furthermore, repulsive events between adjacent melanoblasts do not seem to
256 contribute to their dispersal and the well-characterised 'follow my leader' behaviour observed in
257 cranial neural crest populations²⁸ was not observed. The spatial distribution of melanoblasts appears
258 random above a distance of ~28um. Consistent with this, occasional polygonal regions of exclusion
259 are observed (Supplementary **Movie** 4), reflecting the underlying packing of the epidermal
260 keratinocytes. We demonstrate experimentally that melanoblasts carrying the *Kit*^{W-v} mutation do not
261 migrate more slowly, but instead diffuse in a density-dependent manner similar to wildtype
262 melanoblasts. The mutant cells, however, proliferate more slowly than expected, and our modelling
263 suggests that this is the likely cause of the white belly spot. Many mutations result in white belly
264 spots⁴⁸, often in genes that would not be expected to affect cell motility such as the translation
265 initiation factor, *Eif3c*⁴⁹, the ribosomal protein *S7*⁵⁰, and the chromatin modifying enzyme,
266 *Mysm1*⁵¹. We suggest, that in many cases the white spotting is due to defects in melanoblast
267 proliferation rather than motility.

268

269 Using stochastic individual-level modelling we have examined the importance of density-dependent
270 diffusion and proliferation for colonisation of the DVI and conclude that colonisation is most
271 sensitive to changes in proliferation. This is in agreement with Zhang *et al.*²⁹ who explored the
272 interaction between neural crest migration and proliferation using an on-lattice model for the
273 colonisation of the gut by enteric ganglia progenitors²⁹. One weakness of our model is that it
274 assumes that melanoblast behaviour is equivalent in the relatively sparsely packed 3-dimensional
275 dermal environment between E10.5-E12.5 and in the more tightly packed 2-dimensional epidermal
276 environment between E12.5-E15.5. Experimentally, we demonstrate that this is qualitatively the

277 case but there will certainly be minor differences. The on-lattice approach we use is more
278 appropriate for the latter of these scenarios. However, to represent these two environments
279 separately would require a computationally intensive hybrid model and a number of new, and
280 potentially inaccessible parameters, which would complicate the model and hamper the
281 investigation of the patterning questions we chose to address. Our model assumes that all
282 melanoblasts arise by proliferation of the **differentiated melanoblasts present at E10.5**. This may not
283 be the case as further cells fated to be melanoblasts may **differentiate** after E10.5. Another source of
284 melanoblasts may be from **Schwann** cell precursors (SCPs) emanating from the dorsal ramus from
285 E12.5 onwards as has been proposed by Adameyko *et al.*⁵². However, as the lineage tracing
286 approach that identified these cells has been questioned^{53,54} and we have no access to the key
287 parameters of their possible behaviour, incorporating SCP derived melanoblasts into the present
288 model is not feasible.

289
290 Cheeseman *et al.*^{26,27} investigated the dominance of sub-lineages in a lattice based discrete model.
291 They found that, in many cases, the progeny of two cells (of the 500 they initialised) could
292 contribute in the order of 25% of the cells in the final population. This effect was mediated by a
293 process of sequential isolation of individual lineages deprived of space to proliferate into^{26,27}. This
294 stochastic drift in clone size has been demonstrated experimentally and explored mathematically in
295 the mouse intestine⁵⁵⁻⁵⁷. Selection of dominant lineages is relatively weak in our simulations of
296 melanoblast domain colonisation owing to the more diffuse initial conditions. More cells are able to
297 establish a significant lineage because they have the required space to proliferate initially and
298 consequently fewer lineages become spatially isolated. This implies that the stripes seen in our
299 model are predominantly formed by the coalescence of **multiple like coloured sub-clones**, and not
300 by the presence of dominant lineages. Furthermore, in our model, the domain grows in both the
301 dorsoventral and axial direction, whereas in Cheeseman *et al.*^{26,27} domain growth is only in the

302 dorsoventral direction or is absent. The two-dimensional growth in our model further reduces the
303 role of dominant lineages since cells which may previously have been isolated can gain space into
304 which they may proliferate through domain growth events. Our modelling shows that the generation
305 of rare clones²² and chimaeric patterns¹⁹ can proceed through a common mechanism employing
306 tissue expansion and density-dependent movement and proliferation. Further experimental clonal
307 analyses, using stochastic labelling methods such as brainbow/confetti^{57,58} are required to explore
308 whether our predictions of the behaviour of melanoblast subclones are accurate.

309

310 In conclusion, belly spot formation, chimaeric patterns and diffuse clonal patterns are all explained
311 by a simple model incorporating random melanoblast migration with proliferation, in conjunction
312 with domain growth, during the course of colonisation. Importantly, colonisation and the observed
313 phenotypes, are produced without the need for more complex cell-cell interactions or extracellular
314 signals and this has broader implications for cell behaviour in other NCSC lineages and their
315 associated neurocristopathies.

316 **Methods**

317 **Animal models**

318 All animal work was approved by a University of Edinburgh internal ethics committee and was
319 performed in accordance with institutional guidelines under license by the UK Home Office (PPL
320 60/4424 and PPL 60/3785). Mice were maintained in the animal facilities of the University of
321 Edinburgh. Mouse lines containing the transgenes or modified alleles; *Dct::lacZ* (generated in-
322 house)⁶ □, *R26R-YFP* (kindly provided by Prof L. Smith, The University of Edinburgh)⁵⁹ □,
323 *Tyr::CreA* and *Tyr::CreB* (kindly provided by Prof L. Larue, Institute Curie, Paris)⁶⁰ □, *Nf1^{flox}*
324 (obtained from the National Cancer Institute, Mouse Repository, Frederick, USA)⁶¹ □, and *Kit^{W-v}*
325 (obtained from the Medical Research Council, Harwell, UK)⁴⁵ □ were genotyped according to
326 published methods. *Pmel^{CreERT2}* mice (unpublished, generated in-house) were genotyped using the
327 PCR primers: *Pmel_For* (5-GGGTAAAGAAGAGGGGAGAGG-3), *Pmel_Rev* (5-
328 GGGATGTTCCATCACCTTCA-3) and *CreERT2_Rev* (5-AGGCAAATTTTGGTGTACGG-3) to
329 distinguish between targeted and wildtype alleles. Animals used to investigate adult belly spots
330 were male progeny from a cross between *Nf1^{+flox}*, *Kit^{W-v/+}* males and a *Tyr::CreA^{Tg/Tg}*; *Nf1^{+flox}*;
331 *Kit^{W-v/+}* females on a mixed genetic background. Only male animals were considered as the
332 *Tyr::CreA* transgene is X-linked. *Tyr::CreA^{+ve}*; *Nf1^{flox/flox}* animals were smaller than their litter
333 mates. For live imaging of embryonic skin on a *Kit^{W-v/+}* background E14.5 progeny from a cross
334 between *Tyr::CreB^{Tg/Tg}*; *Kit^{W-v/+}* and *R26YFPR^{Tg/Tg}*; *Kit^{W-v/+}* individuals or between *Tyr::CreB^{Tg/Tg}*;
335 *Kit^{+/+}* and *R26YFPR^{Tg/Tg}*; *Kit^{W-v/+}* individuals were used on a mixed genetic background. No
336 melanoblasts were observed in the back skin of E14.5 *Tyr::CreB^{+ve}*; *R26YFPR^{Tg+ve}*; *Kit^{W-v/W-v}*
337 individuals. For live imaging of embryonic skin on a *Nf1^{flox/+}* and *Nf1^{flox/flox}* background E14.5
338 progeny from a cross between *Tyr::CreB^{Tg/Tg}*; *Nf1^{flox/+}* and *R26YFPR^{Tg/Tg}*; *Nf1^{flox/+}* individuals or
339 *Tyr::CreB^{Tg/Tg}*; *Nf1^{flox/+}* and *R26YFPR^{Tg/Tg}*; *Nf1^{flox/flox}* individuals were used on a mixed genetic
340 background. E14.5 *Tyr::CreB^{+ve}*; *Nf1^{flox/flox}* individuals were viable and morphologically

341 indistinguishable from their litter mates. To image melanoblast behaviour in whole embryos at
342 E11.5 we examined the progeny of a cross between $Pmel^{CreERT2/CreERT2}; R26R-YFP^{Tg/Tg}$ individuals
343 on a mixed background. The pregnant mothers were given 8mg of 4-hydroxytamoxifen (4OHT) per
344 40g body weight by at E10.5. To investigate melanoblast numbers in fixed tissues $Dct::lacZ^{Tg/Tg}$
345 and $Dct::lacZ^{Tg/+}$ embryos were used resulting from crosses between combinations of $Dct::lacZ^{Tg/+}$,
346 $Dct::lacZ^{Tg/Tg}$ and $Dct::lacZ^{+/+}$ parents on CD1 background. Embryos used for optical projection
347 tomography were F1 hybrid's from a cross between the mouse strains C57Bl6 and CBA (obtained
348 from Charles River Laboratories, UK).

349

350

351 **Embryonic skin culture and imaging of whole embryos**

352 Embryonic skin culture was performed as described in Mort *et al.*³¹. Briefly up to six cultures were
353 imaged in parallel per experiment. Skin was sampled from the flank of E13.5, 14.5 and 15.5 mouse
354 embryos. The dorsoventral position varied but was never taken at the ventral extreme. The skin
355 samples were mounted on a clip filled with 1% w/v agarose (in phosphate buffered saline - PBS)
356 and secured with suture thread. The clip was then inserted into a custom designed 6-well chamber
357 so that the skin was sandwiched against a lummox gas permeable membrane (Greiner). The wells
358 were filled with DMEM (no phenol red) supplemented with 1x Glutamax (Gibco), 1% v/v
359 Penicillin/Streptomycin and 10% v/v fetal calf serum. Whole E11.5 embryos were embedded in 1%
360 w/v agarose (in PBS) in a large custom made imaging clip so that the dorsal region of the flank was
361 just protruding above the surface of the agarose. The clip was then inserted into a custom designed
362 6-well chamber so that the protruding region of the embryo was pressed against a lummox gas
363 permeable membrane (Greiner). The wells were filled with DMEM (no phenol red) supplemented
364 with 1x Glutamax (Gibco), 1% v/v Penicillin/Streptomycin and 10% v/v fetal calf serum.

365

366 **X-Gal staining of embryos**

367 X-Gal staining of *Dct::lacZ* embryos was performed as previously described⁶. Briefly embryos
368 were fixed in 4% w/v paraformaldehyde for varying times depending on developmental stage. They
369 were then permeabilised in detergent wash solution (2 mM MgCl₂, 0.05% w/v BSA, 0.1% w/v
370 sodium deoxycholate, 0.02% v/v Igepal in 0.1 M sodium phosphate buffer, pH 7.3) before being
371 stained overnight in X-Gal stain solution (5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 0.085% w/v
372 NaCl with 0.1% w/v X-gal in detergent wash). Embryos were then subjected to further washes in
373 detergent wash solution and then PBS before being post-fixed in 4% w/v paraformaldehyde (in
374 PBS).

375

376 **Image acquisition**

377 Time-lapse sequences of migrating melanoblasts in embryonic skin culture and whole embryos
378 were captured on a Nikon A1R inverted confocal microscope using a 20x objective, images were
379 captured at two minute (for skin) or five minute (for embryos) intervals over the course of the
380 experiment. A stage top environmental chamber was used providing 5% CO₂ in air and **maintaining**
381 a constant temperature of 37 °C. Images of X-Gal stained *Dct::lacZ* embryos were captured on a
382 Nikon macroscope using a ring light for illumination and a 2x Nikon objective with 2x optical
383 zoom.

384

385 **Optical projection tomography**

386 OPT was performed on Bouin's fixed mouse embryos at 1-day stages between E10.5 and E15.5.
387 Samples were mounted in 1% low melting point agarose, dehydrated in methanol and then cleared
388 overnight in BABB (1 part benzene alcohol: 2 parts benzene benzoate). Samples were scanned
389 using a Bioptonics OPT Scanner 3001 (Bioptonics, Edinburgh, UK) using a variety of fluorescent
390 wavelengths to visualise tissue autofluorescence (excitation 425/60 nm/emission 480nm and

391 480/40nm emission 510nm emission). Resultant scans were then reconstructed using proprietary
392 software (nRecon/Skyscan, Belgium).

393

394 **Image analysis and cell tracking**

395 All image analysis tasks were performed using custom written macros for the Fiji distribution of
396 ImageJ⁶². All morphological and tracking procedures were carried out on segmented images using
397 standard ImageJ routines. To automatically track melanoblasts in the time-lapse sequences a
398 modified version of the wrMTrck plugin (<http://www.phage.dk/plugins/wrmtrck.html>) was used on
399 segmented TIFF stacks, the script used relied on Gabriel Landini's morphology collection
400 (<http://www.mecourse.com/landinig/software/software.html>). The tracking and morphology data
401 generated by the procedure was recorded in a text file and used for the down-stream analyses. The
402 mean squared displacement of the melanoblast population was calculated from this data using the
403 time ensemble averaging approach⁶³, in a custom macro written for Fiji. Feret's angles from time-
404 lapse sequences (E14.5) were calculated from the shape of the **cell** body after image segmentation.
405 The angle of migration in X-Gal stained samples (E11.5, 12.5, 13.5, 15.5) was measured manually
406 by drawing a line along the longest axis of each cell in ImageJ. To calculate cell densities for stages
407 E10.5 and E11.5 (Fig. 3a) the total number of cells was divided by the area of the trunk calculated
408 from our OPT data. For all other stages the mid-domain density was measured.

409

410 To analyse the cell cycle, only the first 4 hours of each time lapse was considered to minimise laser
411 exposure. The mean M-phase length (**T_m**) per time-lapse was calculated from the length of five
412 mitotic events. The mean proportion of cells morphologically in M-phase (**P_{mc}**) per time-lapse was
413 calculated from 5 frames spaced 60 minutes apart over the first 4 hours of each time-lapse. The cell
414 cycle time (**T_c**) for a given time-lapse was then calculated as **$T_c = T_m/P_{mc}$** ³⁷⁻³⁹.

415

416 The pair-correlation function (PCF) is a summary statistic that provides a quantitative measure of
417 spatial patterning. The function is derived by normalising the counts of the distances between pairs
418 of agents³⁴⁻³⁶. It is therefore able to capture patterning and the length scale of individual objects. We
419 applied the PCF with non-periodic pairwise distance counting to multiple microscopy images of
420 melanoblasts in the developing epidermis at E14.5 using a custom Matlab script. To avoid issues
421 associated with the image boundary (where cells had been lost due to image processing) we used
422 only a $256\ \mu\text{m} \times 256\ \mu\text{m}$ central portion of each image (we find similar results for alternative
423 window sizes if the central portion is positioned sufficiently far away from the boundary).

424

425 **Measurement of domain expansion**

426 To measure the expansion of the dorsoventral and axial domains of the trunk, optical projection
427 tomography (OPT) models were analysed using ImageJ/Fiji. Two measurements of the trunk
428 circumference were made at the levels of the fore and hind limbs and averaged. For E10.5 where
429 the umbilical hernia encompasses most of the axial width of the domain the region comprising the
430 peritoneal membrane was excluded from the measurement as there is no dermal tissue at this level
431 for melanoblasts to colonise. The dorsoventral length was defined as half the mean trunk
432 circumference. Axial width was defined as the length between the hind and fore limb junctions
433 incorporating the curve of the domain at early stages (E10.5-E12.5).

434

435 **Statistics**

436 All statistical tests were performed using the 'R' statistics package, an open source software
437 package based on the 'S' programming language (<http://www.R-project.org>). The Berman's⁶⁴ test
438 for a point process model was performed using the additional 'spatstat' package
439 (<http://www.spatstat.org/>). All correlations were explored by examining the Pearson's product
440 moment correlation coefficient. Comparisons between multiple groups were undertaken using a

441 one-way analysis of variance (ANOVA). Subsequent pairwise comparisons were performed using a
442 Tukey's honest significant difference test (Tukey's HSD test) which is corrected for multiple testing.
443

444 **Model framework**

445 In our modelling framework we consider only the growth of the trunk region of the developing
446 embryo between and not including the limb buds and its colonisation by the migrating melanoblast
447 population (Fig. 2a). We assume that melanoblast behaviour in the dermis (between E10.5 and
448 E12.5) and the epidermis (between E12.5 and E15.5) is equivalent. The dermal and epidermal
449 layers that can support melanoblast survival are collectively referred to as the dorsoventral
450 integument (DVI). We use an agent-based discrete random-walk model with volume-exclusion on a
451 two-dimensional square lattice of length $L_x(t)$ by $L_y(t)$ to model the DVI. $L_x(t)$ represents the
452 dorsoventral length of the domain at time, t , and $L_y(t)$ the axial length at time t . The lattice spacing
453 is denoted Δ and time evolves continuously. Each agent (melanoblast) is assigned to a lattice site,
454 from which it can move or place progeny into an adjacent site. Attempted agent movement or
455 proliferation events occur with rate P_m or P_p per unit time, respectively. That is, $P_m\delta t$ is the
456 probability of a given agent attempting to move in the next infinitesimally small time interval, δt ,
457 with events simulated as such using the Gillespie algorithm. If an agent attempts to move or
458 proliferate into a site that is already occupied, the event is aborted.

459

460 **Modelling tissue expansion**

461 To model domain growth we employ a stochastic 'pushing' growth mechanism as described in
462 Binder *et al.*⁶⁵. The insertion of new lattice sites into the domain occurs with rates P_{ga} and P_{gd} per
463 unit time, for growth in the axial and dorsoventral direction, respectively. When a 'growth event'
464 occurs in the dorsoventral direction (horizontal direction in Supplementary Fig. 2a), for each row of

465 the lattice one new site is added in a column which is selected uniformly at random. In order to
466 accommodate the new sites, in each row, the sites to the right of the added site are shifted a distance
467 Δ rightwards carrying their contents with them (i.e. cells move with their sites). Likewise, for axial
468 growth (in the vertical direction in Supplementary Fig. 2b) one new site is added to each column in
469 a row which is selected uniformly at random and the appropriate sites are shifted upwards. Growth
470 is linear in both the dorsoventral and axial directions as evidenced by experimental data
471 (Supplementary Table 2).

472

473 **Implementation of model**

474 Movement, proliferation and growth events are modelled as exponentially distributed ‘reaction
475 events’ in a Markov chain. Specifically we use the ‘Gillespie’ Monte Carlo simulation algorithm to
476 simulate realisations of our model system. Each realisation represents five days of real time from
477 E10.5 to E15.5. We implement zero-flux boundary conditions on all boundaries in our discrete
478 model. **This represents the assumption that melanoblast efflux is balanced by melanoblast influx at**
479 **the boundaries of the domain.**

480

481 **Modelling parameters from experimental data**

482 **Lattice spacing**

483 The lattice spacing is chosen to be $\Delta = 38 \mu\text{m}$. This implies that a single agent excludes a volume of
484 $1444 \mu\text{m}^2$ which is a realistic estimate for the size of a melanoblast. A completely colonised model
485 domain (that is every site in the computational domain is occupied by an agent) has a density of
486 approximately $692 \text{ cells mm}^{-2}$. Our experiments have established that the mean ($\pm 95\% \text{ CI}$) density
487 of a ‘colonised’ domain at E15.5 is $701.21 \pm 137.70 \text{ cells mm}^{-2}$ (main text Fig. 2d).

488

489 **Domain size and growth rates**

490 Linear isotropic domain growth for the axial and dorsoventral domains were defined from
491 morphological analysis of optical projection tomographs at embryonic stages between E10.5 and
492 E15.5 (Supplementary Table 2). We initialise the domain as a rectangle of length 1178 μm in the
493 dorsoventral direction and 1634 μm in the axial direction (corresponding to 31 lattice sites by 43
494 lattice sites, respectively). Although the domain grows stochastically, we employ constant growth
495 rates $P_{ga} = 0.00526 \text{ min}^{-1}$ and $P_{gd} = 0.0246 \text{ min}^{-1}$ in the axial and dorsoventral direction,
496 respectively, such that the mean-field growth in each direction is linear and matches with the
497 experimentally measured linear domain growth relationship.

498

499 **Initial number and position of cells**

500 We defined the number of progenitor melanoblasts by counting the melanoblasts in the trunk of
501 *Dct::lacZ* embryos at E10.5; a mean (\pm 95% CI) of 20.32 ± 5.95 melanoblasts (Fig 2C). In our
502 *Dct::lacZ* embryos we noted an under-representation of melanoblasts in the centre of the trunk
503 region, although not always clear at E10.5 this was most striking at E11.5 (Supplementary Fig. 1c).
504 We therefore weighted our initial distribution in a similar manner, initialising 21 agents such that on
505 average one third are between sites 12 and 32 of the axial axis, and the remaining two thirds are
506 evenly distributed between sites 1 and 11, and 33 and 43 corresponding to a slight under-
507 representation in the middle of the axial axis. These agents are distributed so that 95% are between
508 sites 8 to 17 of the dorsoventral axis. All agents are distributed between sites 8 to 19 of the
509 dorsoventral axis.

510

511 **Diffusion rate**

512 As described in the main text we determined experimentally a density-dependent relationship
513 between melanoblast diffusion and local density (main text Fig. 2e). To determine the same
514 relationship in our model we track agents moving on a $646 \mu\text{m} \times 646 \mu\text{m}$ domain (corresponding to
515 17×17 sites) with periodic boundary conditions. This domain size corresponds, approximately, to
516 the field of view of the microscope used to collect the experimental data on melanoblast movement.
517 At $t = 0$ in the simulation, a number of agents (from 1 to 289, representing all possible non-zero
518 agent densities) are initialised with positions chosen uniformly at random throughout the domain.
519 These agents are allowed to move (but not to proliferate, so as to keep the density constant) as
520 described above for a simulation duration equivalent to 400 minutes of real-time. This process is
521 repeated 100 times for each agent density in order to guarantee enough data for an accurate
522 representation of the mean squared displacement (MSD) of the population. In each simulation the
523 resulting agent tracks (excluding, for those agents that crossed a boundary, the portion of their
524 tracks after their first boundary crossing event, since the tracks of these agents would be lost in our
525 experimental system) are used to characterise the MSD as described in the main text. To determine
526 the movement rate, P_m , we compare the relationship between density and effective diffusion
527 coefficient for the experimental data to those for the model for a range of different values of P_m . We
528 chose the value of P_m that gives the best fit (smallest least squares error, l^2 norm). This value of P_m
529 is given in Supplementary Table 3.

530

531 **Proliferation rate**

532 We defined the maximum proliferation rate by counting the number of melanoblasts in the trunk of
533 *Dct::lacZ* E10.5 and E11.5 embryos. We found a mean (\pm 95% CI) of 20.32 ± 5.95 melanoblasts
534 increasing to 151.09 ± 27.95 melanoblasts in the first 24 hours (Fig. 2c). To estimate a maximum
535 doubling time for this period we used the mean cell number at E10.5 ($-$ the 95% CI = 14 cells) and
536 the mean cell number at E11.5 ($+$ the 95% CI = 179 cells) implying a mean dermal doubling time of

537 6.6 hours. We therefore chose a maximum possible population doubling time in the model of 7
538 hours.

539 **Simulation of rare clonal patterns**

540 In order to investigate rare clones, at a time point chosen uniformly at random during the
541 simulation, we chose one of the agents, from amongst all the agents that populate the domain at that
542 time, with equal probability. This agent is marked and all the agent's progeny inherit the same mark.
543 At the end of the simulation all marked agents are plotted in a different colour to the non-marked
544 agents resulting in a diffuse rare clonal pattern as seen in Supplementary Fig. 3a and in Fig. 3f.

545

546 **Identification of stripe-like patterns in the model**

547 To investigate chimaeric stripe-like patterns in our discrete model we initialised our simulations
548 with two distinctly labelled agent sub-populations and tracked the positions of their progeny over
549 time (Fig. 4a). When the simulation was complete, we assigned the value +1 (associated with light
550 grey cells) to one of the agent types and -1 (associated with black cells) to the other (while empty
551 lattice sites are assigned the value 0). We then averaged the values associated with the lattice sites
552 on each row. This provides a measure of the proportion of each agent colour in each row which we
553 call the 'clonal signal' (Fig. 4b). We repeat this process for each of the possible divisions of the 21
554 initial cells into two non-overlapping subsets, which we call a 'clonal ratio'. For instance in order to
555 investigate the patterning of a single clone we label one clone with +1s and remaining 20 clones
556 with -1s. In this way we can investigate the pattern formed by a single clone amongst 21 differently
557 labelled clones. Similarly, in order to investigate the pattern formed by approximately 11 distinctly
558 labelled clones we label two of the randomly selected clones with 1s and 19 with -1s. To investigate
559 the effect of having only two different clonal labels, we label approximately half (10 or 11) of the
560 randomly selected clones with 1s and the other half with -1s.

561 To identify the presence of stripe-like patterns in our simulations we apply the discrete fast Fourier
562 transform (DFFT) to our intensity profiles. We repeat this process 100 times and generate an
563 average DFFT for each different initial clonal ratio (Fig. 4c). For simulations without stripe-like
564 patterns, (i.e. when the agents from different subpopulations are well-mixed) no dominant
565 frequency is clearly identifiable. However, in the case where different agent subpopulations are not
566 well-mixed and have formed dorsoventral stripes, a single, low frequency is identifiable that relates
567 directly to the periodicity of the stripes in the simulation. A dominant frequency (corresponding to
568 the maximum value of the averaged DFFT) can be identified for each initial clonal ratio. We call
569 this dominant frequency the 'stripe intensity' (Fig. 3d). This method allows us to systematically
570 identify the presence of stripes.

571

572 **References**

573

574 1. Wehrle-Haller, B. & Weston, J. a. Soluble and cell-bound forms of steel factor activity play
575 distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest
576 migration pathway. *Development* **121**, 731–42 (1995).

577

578 2. Henion, P. D. & Weston, J. A. Timing and pattern of cell fate restrictions in the neural crest
579 lineage. *Development* **124**, 4351–9 (1997).

580

581 3. Kos, R., Reedy, M. V, Johnson, R. L. & Erickson, C. A. The winged-helix transcription factor
582 FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in
583 avian embryos. *Development* **128**, 1467–79 (2001).

584

585 4. Nitzan, E. *et al.* A dynamic code of dorsal neural tube genes regulates the segregation
586 between neurogenic and melanogenic neural crest cells. *Development* **140**, 2269–79 (2013).

587

588 5. Baxter, L. L. & Pavan, W. J. Pmel17 expression is Mitf-dependent and reveals cranial
589 melanoblast migration during murine development. *Mech. Dev.* **3**, 703–7 (2003).

590

591 6. Mackenzie, M. A. F. A., Jordan, S. A. A., Budd, P. S. S. & Jackson, I. J. J. Activation of the
592 receptor tyrosine kinase Kit is required for the proliferation of melanoblasts in the mouse
593 embryo. *Dev. Biol.* **192**, 99–107 (1997).

594

595 7. Erickson, C. A., Duong, T. D. & Tosney, K. W. Descriptive and experimental analysis of the
596 dispersion of neural crest cells along the dorsolateral path and their entry into ectoderm in the
597 chick embryo. *Dev. Biol.* **151**, 251–72 (1992).

598

599 8. Weston, J. A. Sequential segregation and fate of developmentally restricted intermediate cell
600 populations in the neural crest lineage. *Curr. Top. Dev. Biol.* **25**, 133–53 (1991).

601

602 9. Nishikawa, S. S. I. *et al.* Distinct stages of melanocyte differentiation revealed by analysis of
603 nonuniform pigmentation patterns. *Development* **122**, 1207–14 (1996).

604

605 10. Erickson, C. A. & Goins, T. L. Avian neural crest cells can migrate in the dorsolateral path
606 only if they are specified as melanocytes. *Development* **121**, 915–24 (1995).

607

608 11. Nishikawa, S. I., Kunisada, T., Yoshida, H. & Nishimura, E. K. Regulation of E- and P-
609 cadherin expression correlated with melanocyte migration and diversification. *Dev. Biol.* **215**,
610 155–66 (1999).

611

612 12. Lee, H.-O., Levorse, J. M. & Shin, M. K. The endothelin receptor-B is required for the

- 613 migration of neural crest-derived melanocyte and enteric neuron precursors. *Dev. Biol.* **259**,
614 162–175 (2003).
615
- 616 13. Shin, M. K., Levorse, J. M., Ingram, R. S. & Tilghman, S. M. The temporal requirement for
617 endothelin receptor-B signalling during neural crest development. *Nature* **402**, 496–501
618 (1999).
619
- 620 14. Luciani, F. *et al.* Biological and mathematical modeling of melanocyte development.
621 *Development* **138**, 3943–54 (2011).
622
- 623 15. Hirobe, T. Histochemical survey of the distribution of the epidermal melanoblasts and
624 melanocytes in the mouse during fetal and postnatal periods. *Anat. Rec.* **208**, 589–94 (1984).
625
- 626 16. Geissler, E. N., Ryan, M. A. & Housman, D. E. The dominant-white spotting (W) locus of
627 the mouse encodes the c-kit proto-oncogene. *Cell* **55**, 185–92 (1988).
628
- 629 17. Rawles, M. E. The Development of Melanophores from Embryonic Mouse Tissues Grown in
630 the Coelom of Chick Embryos. *Proc. Natl. Acad. Sci. U. S. A.* **26**, 673–80 (1940).
631
- 632 18. RAWLES, M. E. Origin of pigment cells from the neural crest in the mouse embryo. *Physiol.*
633 *Zool.* **20**, 248–66 (1947).
634
- 635 19. Mintz, B. Gene control of mammalian pigmentary differentiation. I. Clonal origin of
636 melanocytes. *Proc. Natl. Acad. Sci. U. S. A.* **58**, 344–51 (1967).
637
- 638 20. West, J. D. A theoretical approach to the relation between patch size and clone size in
639 chimaeric tissue. *J. Theor. Biol.* **50**, 153–160 (1975).
640
- 641 21. Wolpert, L. & Gingell, D. Striping and the pattern of melanocyte cells in chimaeric mice. *J.*
642 *Theor. Biol.* **29**, 147–50 (1970).
643
- 644 22. Wilkie, A. L., Jordan, S. A. & Jackson, I. J. Neural crest progenitors of the melanocyte
645 lineage: coat colour patterns revisited. *Development* **129**, 3349–3357 (2002).
646
- 647 23. Landman, K. A., Simpson, M. J. & Newgreen, D. F. Mathematical and experimental insights
648 into the development of the enteric nervous system and Hirschsprung's disease. *Dev. Growth*
649 *Differ.* **49**, 277–86 (2007).
650
- 651 24. Simpson, M. J., Zhang, D. C., Mariani, M., Landman, K. A. & Newgreen, D. F. Cell
652 proliferation drives neural crest cell invasion of the intestine. *Dev. Biol.* **302**, 553–68 (2007).
653

- 654 25. Simpson, M. J., Merrifield, A., Landman, K. A. & Hughes, B. D. Simulating invasion with
655 cellular automata: connecting cell-scale and population-scale properties. *Phys. Rev. E. Stat.*
656 *Nonlin. Soft Matter Phys.* **76**, 021918 (2007).
657
- 658 26. Cheeseman, B. L., Zhang, D., Binder, B. J., Newgreen, D. F. & Landman, K. A. Cell lineage
659 tracing in the developing enteric nervous system: superstars revealed by experiment and
660 simulation. *J. R. Soc. Interface* **11**, 20130815 (2014).
661
- 662 27. Cheeseman, B. L., Newgreen, D. F. & Landman, K. A. Spatial and temporal dynamics of cell
663 generations within an invasion wave: A link to cell lineage tracing. *J. Theor. Biol.* **363**, 344–
664 56 (2014).
665
- 666 28. McLennan, R. *et al.* Multiscale mechanisms of cell migration during development: theory
667 and experiment. *Development* **139**, 2935–44 (2012).
668
- 669 29. Zhang, D., Brinas, I. M., Binder, B. J., Landman, K. A. & Newgreen, D. F. Neural crest
670 regionalisation for enteric nervous system formation: implications for Hirschsprung’s disease
671 and stem cell therapy. *Dev. Biol.* **339**, 280–94 (2010).
672
- 673 30. Binder, B. J., Landman, K. A., Newgreen, D. F. & Ross, J. V. Incomplete penetrance: The
674 role of stochasticity in developmental cell colonization. *J. Theor. Biol.* **380**, 309–314 (2015).
675
- 676 31. Mort, R. L., Keighren, M., Hay, L. & Jackson, I. J. Ex vivo Culture of Mouse Embryonic
677 Skin and Live-imaging of Melanoblast Migration. *J. Vis. Exp.* 1–6 (2014).
678
- 679 32. Mort, R. L., Hay, L. & Jackson, I. J. Ex vivo live imaging of melanoblast migration in
680 embryonic mouse skin. *Pigment Cell Melanoma Res.* **23**, 299–301 (2010).
681
- 682 33. Maimon, O. & Rokach, L. *Data Mining and Knowledge Discovery Handbook*. (Springer
683 Science & Business Media, 2010).
684
- 685 34. Agnew, D. J. G., Green, J. E. F., Brown, T. M., Simpson, M. J. & Binder, B. J. Distinguishing
686 between mechanisms of cell aggregation using pair-correlation functions. *J. Theor. Biol.* **352**,
687 16–23 (2014).
688
- 689 35. Fozard, J. A. *et al.* Techniques for analysing pattern formation in populations of stem cells
690 and their progeny. *BMC Bioinformatics* **12**, 396 (2011).
691
- 692 36. Binder, B. J. & Simpson, M. J. Quantifying spatial structure in experimental observations and
693 agent-based simulations using pair-correlation functions. *Phys. Rev. E* **88**, 022705 (2013).
694
- 695 37. Nowakowski, R. S., Lewin, S. B. & Miller, M. W. Bromodeoxyuridine immunohistochemical

- 696 determination of the lengths of the cell cycle and the DNA-synthetic phase for an
697 anatomically defined population. *J. Neurocytol.* **18**, 311–8 (1989).
698
- 699 38. Martynoga, B., Morrison, H., Price, D. J. & Mason, J. O. Foxg1 is required for specification
700 of ventral telencephalon and region-specific regulation of dorsal telencephalic precursor
701 proliferation and apoptosis. *Dev. Biol.* **283**, 113–27 (2005).
702
- 703 39. Mort, R. L. *et al.* Fucci2a: a bicistronic cell cycle reporter that allows Cre mediated tissue
704 specific expression in mice. *Cell Cycle* **13**, 2681–96 (2014).
705
- 706 40. Liggett, T. M. *Stochastic Interacting Systems: Contact, Voter and Exclusion Processes.*
707 (Springer-Verlag, 1999).
708
- 709 41. Gillespie, D. T. Exact stochastic simulation of coupled chemical reactions. *J. Phys. Chem.* **81**,
710 2340–2361 (1977).
711
- 712 42. West, J. D. in *Development in Mammals* (ed. Johnson, M. H.) 413–460 (Elsevier, 1978).
713
- 714 43. Durbec, P., Larsson-Blomberg, L., Schuchardt, A., Costantini, F. & Pachnis, V. Common
715 origin and developmental dependence on c-ret of subsets of enteric and sympathetic
716 neuroblasts. *Development* **122**, 349–358 (1996).
717
- 718 44. Bollag, G. *et al.* Loss of NF1 results in activation of the Ras signaling pathway and leads to
719 aberrant growth in haematopoietic cells. *Nat. Genet.* **12**, 144–8 (1996).
720
- 721 45. Cable, J., Jackson, I. J. & Steel, K. P. Mutations at the W locus affect survival of neural crest-
722 derived melanocytes in the mouse. *Mech. Dev.* **50**, 139–50 (1995).
723
- 724 46. Li, A. *et al.* Rac1 Drives Melanoblast Organization during Mouse Development by
725 Orchestrating Pseudopod- Driven Motility and Cell-Cycle Progression. *Dev. Cell* **21**, 722–
726 734 (2011).
727
- 728 47. Silver, D. L., Leeds, K. E., Hwang, H.-W., Miller, E. E. & Pavan, W. J. The EJC component
729 Magoh regulates proliferation and expansion of neural crest-derived melanocytes. *Dev. Biol.*
730 **375**, 172–81 (2013).
731
- 732 48. Lamoreux, M. L., Delmas, V., Larue, L. & Bennett, D. C. *The Colors of Mice.* (Wiley-
733 Blackwell, 2010).
734
- 735 49. Gildea, D. E. *et al.* The pleiotropic mouse phenotype extra-toes spotting is caused by
736 translation initiation factor Eif3c mutations and is associated with disrupted sonic hedgehog
737 signaling. *FASEB J.* **25**, 1596–605 (2011).

- 739 50. Watkins-Chow, D. E. *et al.* Mutation of the diamond-blackfan anemia gene *Rps7* in mouse
740 results in morphological and neuroanatomical phenotypes. *PLoS Genet.* **9**, e1003094 (2013).
741
- 742 51. Liakath-Ali, K. *et al.* Novel skin phenotypes revealed by a genome-wide mouse reverse
743 genetic screen. *Nat. Commun.* **5**, 3540 (2014).
744
- 745 52. Adameyko, I. *et al.* Schwann cell precursors from nerve innervation are a cellular origin of
746 melanocytes in skin. *Cell* **139**, 366–79 (2009).
747
- 748 53. Mort, R. L., Jackson, I. J. & Patton, E. E. The melanocyte lineage in development and
749 disease. *Development* **142**, 620–632 (2015).
750
- 751 54. Hari, L. *et al.* Temporal control of neural crest lineage generation by Wnt/ β -catenin signaling.
752 *Development* **139**, 2107–2117 (2012).
753
- 754 55. Fletcher, A. G., Breward, C. J. W. & Jonathan Chapman, S. Mathematical modeling of
755 monoclonal conversion in the colonic crypt. *J. Theor. Biol.* **300**, 118–33 (2012).
756
- 757 56. Mirams, G. R., Fletcher, A. G., Maini, P. K. & Byrne, H. M. A theoretical investigation of the
758 effect of proliferation and adhesion on monoclonal conversion in the colonic crypt. *J. Theor.*
759 *Biol.* **312**, 143–56 (2012).
760
- 761 57. Snippert, H. J. *et al.* Intestinal crypt homeostasis results from neutral competition between
762 symmetrically dividing *Lgr5* stem cells. *Cell* **143**, 134–44 (2010).
763
- 764 58. Livet, J. *et al.* Transgenic strategies for combinatorial expression of fluorescent proteins in
765 the nervous system. *Nature* **450**, 56–62 (2007).
766
- 767 59. Srinivas, S. *et al.* Cre reporter strains produced by targeted insertion of EYFP and ECFP into
768 the ROSA26 locus. *BMC Dev. Biol.* **1**, 4 (2001).
769
- 770 60. Delmas, V., Martinuzzi, S., Bourgeois, Y., Holzenberger, M. & Larue, L. Cre-mediated
771 recombination in the skin melanocyte lineage. *genesis* **36**, 73–80 (2003).
772
- 773 61. Zhu, Y. *et al.* Ablation of NF1 function in neurons induces abnormal development of cerebral
774 cortex and reactive gliosis in the brain. *Genes Dev.* **15**, 859–76 (2001).
775
- 776 62. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods*
777 **9**, 676–82 (2012).
778

- 779 63. Charsooghi, M. A., Akhlaghi, E. A., Tavaddod, S. & Khalesifard, H. R. A MATLAB program
780 to calculate translational and rotational diffusion coefficients of a single particle. *Comput.*
781 *Phys. Commun.* **182**, 400–408 (2011).
782
- 783 64. Berman, M. Testing for Spatial Association Between a Point Process and Another Stochastic
784 Process. *Appl. Stat.* **35**, 54 (1986).
785
- 786 65. Binder, B. J., Landman, K. A., Simpson, M. J., Mariani, M. & Newgreen, D. F. Modeling
787 proliferative tissue growth: a general approach and an avian case study. *Phys. Rev. E. Stat.*
788 *Nonlin. Soft Matter Phys.* **78**, 031912 (2008).
789

790 **Acknowledgements**

791

792

793 **Author Contributions**

794 **RLM, RJHR, CAY, IJJ** and **KJP** conceived the work and designed the experimental and modelling
795 approach. **RLM, OJH, KJH** and **MAK** performed the wet lab work. **RJHR** and **CAY** performed the
796 mathematical modelling with help and support from **KJP** and **REB**. **RLM** and **GL** designed and
797 performed the image analysis routines. **RLM, RJHR, REB, CAY and IJJ** prepared the manuscript.

798

799 Reprints and permissions information is available at www.nature.com/reprints

800

801 **Competing financial interests**

802 The authors have no competing financial interests.

803

804 **Figure legends:**

805 **Figure 1: Melanoblast migration is undirected and not driven by repulsion. a:** A time-lapse
806 **sequence of melanoblasts migrating in *ex vivo* culture of E14.5 skin.** The Feret's diameter of each
807 cell body is indicated in cyan. The path of a single migrating cell is indicated in red. Melanoblasts
808 migrate along their Feret's diameter – the longest distance between any two points along a given
809 boundary. The distribution of the Feret's angles in the time-lapse sequences invariably conformed to
810 a uniform distribution (Kolmogorov-Smirnov Test $P > 0.05$ in all cases, $n = 414$ independent tests)
811 implying that melanoblast migration is undirected. **b:** An analysis of the spacing of melanoblasts in
812 the time-lapse sequences at E14.5. A range of cell densities was observed at the start of each time-
813 lapse ($t = 0$) between 242 cells mm^{-2} and 780 cells mm^{-2} (mean \pm 95 CI = 448.52 ± 57.46 cells mm^{-2} ,
814 $n = 20$). Independent spatial patterns were generated by sampling each time-lapse at 40 minute
815 intervals. Each spatial pattern ($n = 414$ patterns) representing the x, y position of the centre of mass
816 of each cell was tested for complete spatial randomness (CSR) using a Berman's test for a point
817 process model. In the majority of examples the pattern conforms to CSR ($n = 412$ out of 414
818 independent tests). The 0.05 significance line is indicated in red, the Berman's P value is plotted
819 against the cell density at $t = 0$. **c:** Average pair-correlation function (PCF) for melanoblast spatial
820 organisation at E14.5 **for data in panel 1b.** The red line indicates complete spatial randomness at all
821 distances. Melanoblast spacing conforms to CSR at distances over $\sim 28 \mu\text{m}$. **d:** All tracks of a time-
822 lapse sequence plotted from a zero origin showing a homogeneous population spread. **e:** Plot of the
823 mean squared displacement (MSD) of the tracks in against time. A straight line through the origin
824 (red) can be fitted to the data (black) indicating that the population **is** diffusing. The slope of the line
825 is used to derive the diffusion coefficient (D). Abbreviations: PCF; pairwise correlation function.
826 Scale-bar **in panel 1a = $50 \mu\text{m}$.**

827

828

829 **Figure 2: Key biological parameters. a:** For our experimental measurements we only considered
830 the trunk region between the limb buds indicated by the coloured lines on the OPT models at E10.5
831 (left) and E15.5 (right). This was defined as the axial width. **b:** We used X-Gal stained *Dct::lacZ*
832 embryos to analyse cell numbers between E10.5 and E11.5. **c:** Melanoblast numbers increase
833 around ~6-fold between E10.5 ($n = 25$) and E11.5 ($n = 11$) accompanied by an increases in axial
834 width of ~200 μm and in dorsoventral length of ~1000 μm . **d:** Melanoblast mid trunk density at
835 E12.5 ($n = 8$), E13.5 ($n = 8$), E14.5 ($n = 32$) and E15.5 ($n = 7$) accompanied by linear increases in
836 axial width of ~900 μm and dorsoventral length of ~3200 μm (between E12.5 and E15.5). **e:** The
837 diffusion coefficient (D) was defined for each time-lapse experiment ($n = 20$ E14.5 wildtype
838 samples) and the values plotted against the initial density for each time-lapse. Pearson product-
839 moment correlation indicates a significant negative correlation between diffusion (D) and density (r
840 = -0.49, degrees of freedom ($d.f.$) = 18, $P = 0.026$). The functional form of the relationship between
841 the diffusion coefficient and the cell density is recapitulated as an emergent property of the model
842 (red line). **f:** Analysis of cell cycle time (T_c) in E14.5 time-lapse sequences ($n = 19$ wildtype
843 samples). Pearson product-moment correlation indicates a significant positive association between
844 cell cycle time (T_c) and density ($r = 0.55$, $d.f. = 17$, $P = 0.016$). The functional form of the
845 relationship between cell cycle time (T_c) and cell density described is as an emergent property of the
846 stochastic model (red line). Scale bar in panel 2a = 1500 μm , scale-bar in panel 2b = 1000 μm .
847

848 **Figure 3: Random migration and proliferation are sufficient to generate chimaeric and diffuse**
849 **rare clones. a:** Comparison of the mid-domain melanoblast densities at E10.5 ($n = 25$), E11.5 ($n =$
850 11), E12.5 ($n = 8$), E13.5 ($n = 8$), E14.5 ($n = 32$) and E15.5 ($n = 7$) with results from the stochastic
851 model (model data are averaged over 100 repeats). **b:** Two examples of the final time point of
852 discrete simulations ($t = 5$ days, equivalent to E15.5) using random labelling of the initial cells in
853 two colours (black or light grey, inset) to form balanced chimaeric patterns (analogous to mouse

854 aggregation chimaeras). **c:** Comparative plots ($t = 5$ days) of the same simulation using two clonal
855 subtypes or 21 clonal subtypes. The coherent patches seen in the two-colour plots are composed of
856 multiple coalescent sub-clones. **d:** When only two clonal subtypes are present stripes are most
857 apparent. As the number of subtypes is increased ‘stripe intensity’ is reduced (where stripe intensity
858 is defined as the dominant frequency emerging from the mean discrete fast Fourier transform
859 (DFFT) of the intensity profile of 100 simulations). **e:** Two examples of rare *Dct::lacZ* revertant
860 melanoblast clones generated using the *Dct::laacZ* mouse model described in Wilkie *et al.*²²□
861 (replotted here for consistency - clones shown in black). **f:** Patterns qualitatively similar to those in
862 panel 3e generated by randomly labelling a single cell during a simulation and tracing all its
863 progeny ($t = 5$ days, clones shown in black). In panels 3b, 3c, 3e and 3f each plot represents one
864 side of the embryo extending from the dorsal most aspect on the left to the ventrum on the right.
865 Scale bars = 500 μm in all cases. Error bars in panel 3a = s.e.m.

866

867 **Figure 4: Segregation of clonal subtypes in the initial pattern determines stripe intensity. a:** A
868 stripe-like pattern from a single simulation of the discrete model initialised with agents of two
869 clonal subtypes (black and light grey) in equal proportions (analogous to a balanced mouse
870 aggregation chimaera). **b:** The axial profile of the agent intensity for the simulation depicted in
871 panel 4a reflecting the axial change in the dominant subtype (Methods). **c:** Amplitude spectrum
872 from a discrete fast Fourier transform (DFFT) of the clonal signal (shown in panel 4b). **d:** Data
873 from the simulation in panel 4a plotted as individual clones. Increasing the number of clonal
874 subtypes in the initial conditions from two to 21 removes the appearance of the stripe-like pattern
875 and reveals the extent of mixing of the individual sub-clones. **e:** A simulation in which the initial
876 pattern containing two clonal subtypes has been deliberately well segregated in the initial conditions
877 (black and light grey cells, shown in inset). Segregation of the initial sub-populations promotes
878 dorsoventral stripe formation. **f:** A simulation in which the initial pattern containing two clonal

879 subtypes has been deliberately evenly mixed in the initial conditions (black and light grey cells,
880 shown in inset). Even mixing of the initial sub-populations inhibits dorsoventral stripe formation,
881 however areas that contain a single dominant clonal subtype are still present. Each plot in panels 4a,
882 4d, 4e and 4f represents one side of the embryo extending from the dorsal most aspect on the left to
883 the ventrum on the right. Scale bars = 500 μm in all cases.

884

885 **Figure 5: Extensive mixing of individual clones in the discrete model. a-h.** Example patterns
886 from single simulations of the discrete model initialised with either agents of two clonal subtypes in
887 equal proportions (panels 5a-5g - analogous to a balanced mouse aggregation chimaera) or with 21
888 differently coloured subtypes (panels 5b-5h). The left-hand and right-hand plots are generated from
889 a single simulation. Each plot represents one side of the embryo extending from the dorsal most
890 aspect on the left to the ventrum on the right. Scale bars = 500 μm in all cases.

891

892 **Figure 6: Reduced proliferation results in belly spot formation. a:** A heat map generated from a
893 parameter sweep comparing colonisation in the model for different values of diffusion (normalised
894 with respect to D_0 , the diffusion coefficient used in our simulations) and cell cycle time (T_c). The
895 model is substantially more sensitive to changes in T_c than in diffusion as indicated by the red
896 region (blue: low probability of colonisation, red: high probability of colonisation). 100 repeats of
897 the model were performed for each combination. **b:** Mid trunk melanoblast densities of $Kit^{+/+}$;
898 $Nf1^{+/+}$ ($n = 20$), $Kit^{W-v/+}$; $Nf1^{+/+}$ ($n = 12$), $Kit^{+/+}$; $Nf1^{+/-}$ ($n = 7$) and $Kit^{+/+}$; $Nf1^{-/-}$ ($n = 14$) embryos.
899 Melanoblast density is reduced in $Kit^{W-v/+}$; $Nf1^{+/+}$ mice and is increased in $Kit^{+/+}$; $Nf1^{-/-}$ mice. One-
900 way analysis of variance (ANOVA) $P < 0.0001$, Tukey's honest significant difference test (Tukey's
901 HSD) $P < 0.001$ in both cases. **c:** Melanoblast diffusion coefficients (D) for $Kit^{+/+}$; $Nf1^{+/+}$ ($n = 21$),
902 $Kit^{W-v/+}$; $Nf1^{+/+}$ ($n = 12$), $Kit^{+/+}$; $Nf1^{+/-}$ ($n = 7$) and $Kit^{+/+}$; $Nf1^{-/-}$ ($n = 14$) embryos. Diffusion is

903 increased in $Kit^{W-v/+}; Nf1^{+/+}$ mice despite the failure of the melanoblast population to completely
904 colonise the dorsoventral domain (One-way ANOVA $P < 0.0001$, Tukey's HSD $P < 0.01$). **d:** Plot
905 of D against density for $Kit^{+/+}; Nf1^{+/+}$ ($n = 20$), $Kit^{W-v/+}; Nf1^{+/+}$ ($n = 12$), $Kit^{+/+}; Nf1^{+/-}$ ($n = 7$) and
906 $Kit^{+/+}; Nf1^{-/-}$ ($n = 14$) embryos. Pearson product-moment correlation indicates a significant negative
907 association ($r = -0.62$, $d.f. = 51$, $P < 0.0001$). **e:** Plot of T_c against cell density for $Kit^{+/+}; Nf1^{+/+}$ ($n =$
908 19), $Kit^{W-v/+}; Nf1^{+/+}$ ($n = 12$), $Kit^{+/+}; Nf1^{+/-}$ ($n = 7$) and $Kit^{+/+}; Nf1^{-/-}$ ($n = 14$) embryos. Pearson
909 product-moment correlation indicates no association ($r = 0.26$, $d.f. = 50$, $P = 0.058$) **f:** An increase
910 in T_c (from 7 to 10 hours) results in a ventral belly spot in our simulations qualitatively similar to
911 the pattern observed in $Kit^{W-v/+}$ mice ($t = 5$ days, equivalent to E15.5). Each plot represents one side
912 of the embryo extending from the dorsal most aspect on the left to the ventrum on the right. Scale
913 bars in panel 6f = 500 μm .

914

915 **Figure 7: Deletion of $Nf1$ rescues the belly spot in Kit mutants.** **a:** No pigmentation is present in
916 homozygous $Kit^{W-v/W-v}$ mutants on an $Nf1^{+/+}$ or $Nf1^{+/-}$ background (pictured) due to a complete lack
917 of melanocytes in the adult. **b:** Heterozygous $Kit^{W-v/+}$ mice on an $Nf1^{+/+}$ background exhibit a large
918 ventral belly spot due to a failure of complete melanoblast colonisation of the developing epidermis.
919 **c:** The belly spot is partially rescued in heterozygous $Kit^{W-v/+}$ mice on an $Nf1^{+/-}$ background. **d:** The
1955