Dbx1-Expressing Cells Are Necessary for the Survival of the Mammalian Anterior Neural and Craniofacial Structures

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Abstract
Development of the vertebrate forebrain and craniofacial structures are intimately linked processes, the coordinated growth of these tissues being required to ensure normal head formation. In this study, we identify five small subsets of progenitors expressing the transcription factor dbx1 in the cephalic region of developing mouse embryos at E8.5. Using genetic tracing we show that dbx1-expressing cells and their progeny have a modest contribution to the forebrain and face tissues. However, their genetic ablation triggers extensive and non cell-autonomous apoptosis as well as a decrease in proliferation in surrounding tissues, resulting in the progressive loss of most of the forebrain and frontonasal structures. Targeted ablation of the different subsets reveals that the very first dbx1-expressing progenitors are critically required for the survival of anterior neural tissues, the production and/or migration of cephalic neural crest cells and, ultimately, forebrain formation. In addition, we find that the other subsets, generated at slightly later stages, each play a specific function during head development and that their coordinated activity is required for accurate craniofacial morphogenesis. Our results demonstrate that dbx1-expressing cells have a unique function during head development, notably by controlling cell survival in a non cell-autonomous manner.


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Introduction
Building the vertebrate head relies on complex developmental programs involving molecular cross-talks between multiple cell types belonging to the three germ layers [1]. Within the developing brain, the acquisition of positional identity relies on the formation of morphogen gradients, whose combination leads to the nested expression of transcription factors, allowing sharp boundaries between compartments and morphological subdivisions of the developing brain to be formed [2]. Craniofacial structures develop in close proximity with the brain, and the same signalling molecules, namely Fgfs, Wnts, BMPs and Shh, are involved in both brain and face morphogenesis [3,4]. However, patterning information is not exclusively mediated by morphogens passively diffusing from focal sources. For example, increasing evidence supports the hypothesis of alternative mechanisms involving the migration of signalling cells over long distances during cerebral cortex development [5].

Cephalic neural crest cells (CNCCs) are key players in vertebrate head development. They originate from the boundary between the surface ectoderm and neural plate and undergo epithelial-mesenchymal transformation to acquire a migratory phenotype. CNCCs generated at mesencephalic and diencephalic levels invade mainly the facial mesencephyme that will later give rise to the frontonasal structures, whereas CNCCs of rhombencephalic origin populate the branchial arches [6]. Genetic tracing experiments in mice [7] and chick-qual chimera [8] demonstrated that most of the craniofacial tissues, including the entire facial skeleton, derive from CNCCs. In addition to their direct contribution, CNCCs were proposed to convey patterning information: in avian embryos, interspecies graft experiments demonstrated that CNCCs-derived signals regulate beak morphogenesis [9]. CNCCs were also shown to control brain patterning in chick embryos. Notably, ablation and electroporation experiments revealed that CNCCs migration into the facial mesenchyme and local secretion of BMP-antagonists is required to maintain fgf8 expression in the forebrain [10,11]. On top of their function in patterning neural tissues, CNCCs were proposed to control cell survival [12] as well as neural tube closure [13].

In mice, by contrast with chicks, little is known about the possible interactions between the cranial mesencephyme and the forebrain during development. In addition, evidence indicating
that CNCCs are a source of signals involved in forebrain development is presently lacking.

**Dbx1** is a homeodomain transcription factor previously shown to be expressed from E9.5 by restricted pools of progenitors in the central nervous system [14,15]. Work performed in our laboratory indicated that, in the forebrain, cells deriving from these progenitors, born from E10.5 on, often share a migratory behaviour and a signalling function [16–10]. In this study, we identify five discrete subsets of **dbx1**-expressing cells in cephalic regions at early stages (E8.25–E8.75) of mouse development. We use a genetic ablation strategy to test the function of each of these subsets and reveal their relative involvement in forebrain and face development. We show by genetic tracing that the earliest **dbx1**-expressing cells generate a subpopulation of CNCCs which play a crucial role in head development by controlling proliferation and survival in neural and non-neural head tissues.

**Methods**

**Animals**

All animals used in this study were handled according to national regulations (approval #5096 from the French Ministry of Research on the use of genetically modified animals) and approved by the Veterinary Services of Paris (authorization to perform experimentation on vertebrate animals #75-1545). Genetic ablation of **dbx1**-expressing cells was achieved by conditional expression of the diphtheria toxin A fragment (DTA), crossing **Dbx1**ΔRES-loxP-Stop-loxP-DTA mice [16] (hereafter referred to as **Dbx1**ΔDTA) with various Cre recombinase expressing strains. **P6.5**Cre animals [19] express Cre in the germ line, therefore allowing complete ablation of all **dbx1**-expressing cells. Targeted ablations of the different **dbx1**-expressing populations were achieved using the FosglCre [20], Wnt1Cre [21], Nkx2.1Cre [22] and NestinCre [23] lines. In order to perform genetic tracing of **dbx1**-expressing cells, we crossed **Dbx1**ΔRES-Cre [16] (referred to as **Dbx1**ΔCre) and **ROSA26**loxP-STOP-loxP-YFP [24] (referred to as **R26YFP**) mice. In **Dbx1**ΔCre:**R26YFP** embryos, Cre-mediated recombination occurs at the **ROSA26** locus in every cell expressing **dbx1**, leading to a permanent and irreversible expression of YFP.

Animals and embryos were genotyped by PCR using the appropriate specific primers and DNA extracted from the tail or yolk sack (for E8.5 and E9.5 embryos) as a template. At least three embryos of each genotype were analysed for each embryonic day.

**Embryo processing**

For staging of the embryos, midday of the vaginal plug was considered as embryonic day 0.5 (E0.5). To stage E8.5 and E9.5 embryos more precisely, the somites were counted. Subtle variations in the size and morphology of embryos displaying the same number of somites were observed. Thus, we reasonably estimate that counting the somite for staging is accurate with a margin of error of 1ss. All embryos were collected and dissected in cold PBS and immediately fixed by immersion in 4% paraformaldehyde in 0.12 M phosphate buffer (PB) pH 7.2 at 4°C. Fixation duration was determined depending on the subsequent use of the embryos (see below). The neural tubes of E9.5 and E12.5 embryos were opened at the level of the rhombencephalon.

E8.5, E9.5 and E12.5 embryos subjected to immunostaining were fixed 4–8 h at 4°C. For storage, E8.5 and E9.5 specimens were dehydrated and stored in methanol whereas E12.5 heads were embedded in gelatine/sucrose.

**Immunostaining**

The following primary antibodies were used: chick anti-GFP (AvesLab; 1:2000), rabbit anti-AP2α (Santa Cruz; H-79; 1:100), rabbit anti-PH3 (Upstate; 1:500) and rabbit anti-activated Caspase-3 (Cell Signalling; 5A1; 1:800). For fluorescent staining, secondary antibodies were purchased from Jackson Immunoresearch and slides were mounted in Vectashield with DAPI (Biovalley). In some cases, biotinylated secondary antibodies were used (Jackson Immunoresearch) and revealed using the VECTASTAIN ABC kit (Vector) and diaminobenzidine (Sigma) as a substrate. Slides were then mounted in Mowiol.

**Embryos used for in situ hybridisation**

Embryos for in situ hybridisation, TUNEL or Nissl staining were fixed 4–8 h at 4°C. For storage, E8.5 and E9.5 specimens were dehydrated and stored in methanol whereas E12.5 heads were embedded in gelatine/sucrose.

**Embryos used for in situ hybridisation, TUNEL or Nissl staining**

Whole mount TUNEL was performed using the Apoptag Peroxidase in situ Apoptosis Detection kit (Millipore) according to the manufacturer’s instructions. Briefly, embryos were rehydrated and digested with 10 μg/mL proteinase K for 3 min. They were incubated with terminal deoxynucleotidyl transferase for 1 h at 37°C. The reaction was revealed using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) and NBT/BCIP (Roche) as a substrate.

**In situ Hybridisation**

Digoxigenin-labelled riboprobes for **dbx1**, **dlx1**, **emx2**, **foxl1**, **ngn2**, **otx1**, **pax6**, **srx3**, **sox10** and **wnt1** were synthesised with T3 or T7 RNA polymerases (Roche) using the Dig-RNA labelling mix (Roche) and 1 μg of linearised DNA as a template. In situ hybridisation on cryosections was performed as previously described [25]. For whole-mount staining, embryos were rehydrated, post-fixed in 4% paraformaldehyde, 0.2% glutaraldehyde in PBS, permeabilised in 1% SDS, 1%NP-40, 0.5% deoxycholate, 150 mM NaCl, 50 mM Tris pH 8, 1 mM EDTA and hybridised in a buffer containing 50% formamide, 5× SSG, 2% BBR, 2% SDS, 250 μg/mL yeast RNA, 100 μg/mL heparin and the probe (1:50 dilution).

**Images acquisition**

Embryos stained by whole-mount in situ hybridisation or TUNEL were incubated in 80% glycerol, 20% PBS and pictures acquired using a Zeiss Axiocam HRc color camera coupled to a Leica MZ FLIII stereomicroscope. Cryosections stained by Nissl, in situ hybridization or DAB immunohistochemistry were acquired using a Zeiss Axiocam HRc color camera coupled to a Zeiss Axiovert 200 microscope. Images of sections from E12.5 animals are composite that were generated by the Zeiss Axiovision software using Mosaix and Tiling functions to automatically reconstruct one image from multiple fields of the same specimen. Immunofluorescence stainings were acquired on a Leica TCS SP5 confocal microscope. All images of double-labelling correspond to a single confocal plane; in the case of sections stained with a single primary antibody, max projections of a 5–15 μm z-stack were used.

**Quantitative PCR**

RNA preparation, reverse transcription and qPCR were performed as previously described [18]. Briefly, wild-type embryos were dissected in cold PBS. For 2ss and older embryos, only tissues anterior to the prootic sulcus (which marks the boundary between
rhombomeres 2 and 3) were analysed. Several embryos of matching ages were pooled and RNA extraction was performed using the RNeasy Micro Kit (Qiagen) following the manufacturer’s instructions. 100–300 ng of total RNA was used for cDNA synthesis using the SuperScript VILO cDNA synthesis kit (Invitrogen). Samples were prepared using an epMotion 5070 (Eppendorf) automated pipetting system and qPCR was performed on a LightCycler 480 (Roche) using the LightCycler 480 SYBR green Master I (Roche) reaction mix. Dbx1 expression was calculated relative to the ribosomal protein S17 mRNA as previously described [18]. Experiments were performed at least in triplicates.

Quantifications

Counting of the percentage of cells which are PH3+ in the neural plate and mesenchyme of E8.5 embryos was performed manually, using DAPI staining to identify individual cells. Only tissues located at presumptive midbrain and forebrain levels were taken into account. At least 1000 cells were counted for n = 3 control and 4 mutant embryos. The number of pyknotic nuclei per section was manually counted following thresholding of the DAPI staining to reveal the highest levels of fluorescence. Histograms represent mean ± sem. Significance was assessed using Student’s t-test.

Counting of the percentage of Caspase-3+ cells which are YFP positive/negative was performed manually. Only Caspase-3+ cells which displayed a “normal” morphology (i.e., exhibiting a nuclear and a cytoplasmic compartment) were taken into account, thus excluding cell debris or cells that had reached advanced stages of cell death. The neuroepithelium was separated in two regions, one corresponding to the telencephalon (showing a high degree of recombination) and one corresponding to the mesencephalon and diencephalon (showing a high degree of recombination in FoxG1Cre embryos) and one corresponding to the mesencephalon and diencephalon (showing a high degree of recombination in Wnt1;Cre embryos). A total of >200 cells from two FoxG1Cre;R26YFP,Dbx1floTA embryos (at 12ss and 13ss) and three Wnt1;Cre;R26YFP,Dbx1floTA embryos (at 8ss and 10ss) were analysed.

Results

Genetic ablation of dbx1-expressing cells results in severe defects of forebrain and craniofacial structures

The Dbx1 transcription factor is expressed by restricted populations of progenitors during central nervous system development. In the forebrain, dbx1 expression is notably detected at the septum, ventral pallium and preoptic area around E10.5–E12.5 [14–16]. In order to better understand the function of the various cell populations deriving from these progenitors, we have developed a genetic strategy to perform their conditional ablation. The use of the Dbx1loxP-stop-loxP-DTA mice [16] and the appropriate Cre recombinase-expressing strain enables us to selectively eliminate specific dbx1-expressing populations. We previously used Nestin;Cre, Emx1;Cre, ddb1β2;Cre and E1-Ngn2;Cre mice to target Dbx1-derived subtypes generated between E10.5 and E12.5 [16–18]. Consistent with the limited number of dbx1-expressing cells, formation of the forebrain occurred in these mutants.

In this study, we performed the complete ablation of all dbx1-expressing cells using the PGK:Cre line [19] in which recombination occurs ubiquitously at early stages (1–2 cells stage) of development. Surprisingly, severe craniofacial and brain morphogenesis defects were observed in E12.5 PGK:Cre;Dbx1DTA (Fig. 1A, B). Consistently, a similar phenotype has been observed using another ubiquitous and early Cre-expressing line [26] to ablate all dbx1-expressing cells (not shown).

![Figure 1. Genetic ablation of dbx1-expressing cells results in severe neural and craniofacial defects. Heads of wild-type (A) and PGK:Cre;Dbx1DTA (B) E12.5 mouse embryos. Mutants are exencephalic and display craniofacial malformations. (C–H) Alternate sagittal sections of wild-type (C, D, emx2 (E, F) and foxg1 (G, H) in situ hybridisation. (I–J) Coronal sections of wild-type (I) and PGK:Cre;Dbx1DTA (J) E12.5 embryos stained by Nissl (C, E, G) and display craniofacial malformations. (C–H) Alternate sagittal sections of wild-type (C, D, Emx2 (E, F) and Foxg1 (G, H) in situ hybridisation. (I–J) Coronal sections of wild-type (I) and PGK:Cre;Dbx1DTA (J) E12.5 embryos stained by Nissl. The antero-posterior level at which coronal sections were collected is indicated by yellow dashed lines in C and D. The arrow in J points to the remaining telencephalon in ablated embryos. (K–M) High magnification of these telencephalic tissues in PGK:Cre;Dbx1DTA E12.5 embryo following emx2 (K), ngn2 (L) and dbx1 (M) in situ hybridisation. Scale bars A: 500 μm, C, I: 400 μm, K: 200 μm. doi:10.1371/journal.pone.0019367.g001](http://www.plosone.org/doi/10.1371/journal.pone.0019367.g001)

Nissl staining performed on coronal and sagittal sections of E12.5 PGK:Cre;Dbx1DTA embryos indicated that the neural tube was opened at presumptive midbrain levels and aberrantly folded in rostral regions (Fig. 1C, D, I, J). Frontonasal structures were reduced in size and the nasal cavity was absent. The eyes, though shifted medially and incorrectly oriented, were present and their shifted medially and incorrectly oriented, were present and their gross morphology conserved in ablated mutants (Fig. 1H, J). We performed in situ hybridisation with emx2 and foxg1 riboprobes to label telencephalic structures and found that these territories were severely affected in the mutants, with only a small anterior territory being emx2 and foxg1 positive (Fig. 1E–H). The absence of olfactory epithelium, which normally expresses emx2 and foxg1, was also confirmed in ablated mutants. The remaining telencephalic
tissue observed in PGK:Cre;Dbx1\textsuperscript{DTA} embryos appeared as a small vesicle (arrow in Fig. 1J) suggesting that neural tube closure did occur at the most anterior levels. Indeed, we observed that the \textit{dbx1\textsuperscript{m}} domain is relatively segregated from the \emx2\textsuperscript{m} and \ngn2\textsuperscript{m} domains (Fig. 1K–M), indicating that dorso-ventral patterning was maintained (ventral/dbx1\textsuperscript{m} domain is found dorsal because of the folding of the remaining anterior nervous system; see Fig. 1D).

Taken together, these results indicate that the ablation of all \textit{dbx1}-expressing cells results in an absence of dorsal neural tube closure at the midbrain level, a severe reduction in the size of the forebrain, as well as strong defects of craniofacial structures.

**Early \textit{dbx1}-expressing cells are essential for forebrain and facial development**

In a previous study, we performed the ablation of cells expressing the \textit{dbx1} gene in the central nervous system starting from E10.5 by crossing \textit{Nestin:Cre} and \textit{Dbx1\textsuperscript{flk-EGFP-flk-EGFP}\textsuperscript{DTA}} animals [16]. E12.5 embryos resulting from such crosses displayed a much milder phenotype than PGK:Cre;Dbx1\textsuperscript{DTA} mutants, consisting in an obvious reduction in the size of the mesencephalon and diencephalon (Fig. S1A, B). Importantly, despite the strong reduction in the thickness of the cerebral ganglionic eminence, the diencephalon of Nestin:Cre;Dbx1\textsuperscript{DTA} appeared normal in size [16] (Fig. S1C, D) and mutants were undistinguishable from wild-type littersmates with respect to craniofacial morphology (Fig. S1A, B). The major difference of phenotype between Nestin:Cre and PGK:Cre ablated mutants suggested that \textit{dbx1}-expressing cells generated before E10.5 and/or outside of the central nervous system are essential for midbrain, forebrain and craniofacial development.

We thus analysed earlier stages of development and found that PGK:Cre;Dbx1\textsuperscript{DTA} embryos were already severely affected at E9.5. Dorsal midline closure failed to occur at midbrain and forebrain levels and \textit{foxg1} expression was markedly reduced in ablated mutants, indicating that the telencephalic primordium was strongly reduced (Fig. 2A–D). Dorsal mesencephalic and posterior diencephalic territories, identified by \textit{wnt1}\textsuperscript{m} expression, appeared missing whereas no obvious defects were observed in the rhombencephalon (Fig. 2E, F). At 10–12ss (E8.75) mutant embryos were already distinguished from wild-type littersmates due to the failure in neural tube closure. Around 4–6ss (E8.3), the mutants could also be identified based on their morphology: measurements of the length of the headfold between the pre-otic sulcus and the anterior limit of the forebrain revealed a significant decrease in ablated mutants compared to wild-type embryos (21% reduction; Fig. S2B). We also measured a 14% reduction in the width of the neural plate at the mesencephalic level of 2–4ss (E8.25) embryos (Fig. S2A).

We thus find that morphological defects in ablated mutants are first seen at early somitic stages.

**Early \textit{dbx1} expression pattern identifies several progenitor subsets**

Our observation that ablated mutants display measurable defects from E8.25 were unexpected considering the initial report that \textit{dbx1} expression only starts at E9.5 [15]. We therefore decided to carefully re-examine the \textit{dbx1} expression pattern before E9.5 using \textit{in situ} hybridisation.

With this technique, the earliest expression of \textit{dbx1} mRNA we could detect was observed in 2ss embryos, in patches of cells located in the lateral/dorsal neural plate at the midbrain level (Fig. 3A–D, M). \textit{Dbx1} expression at this location (subsequently referred to as EM, for “early midbrain”) persisted at 3ss and 4ss but was then downregulated by 6ss (Fig. 3E–F). At this stage \textit{dbx1} expression started in the ventral diencephalon (vDi), arrow in Fig. 3E) and persisted later on (Fig. 3G–J). From 8ss (Fig. 3G–J), \textit{dbx1} expression was found in the dorso-lateral diencephalon and mesencephalon (dDM, arrow in Fig. 3G) with this domain persisting and extending posteriorly at later stages (Fig. 3I). Of note, the dDM expression domain overlaps with the midbrain area in which \textit{dbx1} is expressed at 2–4ss. Between 10ss and 13ss, \textit{dbx1} expression was transiently found in the anterior neural ridge (ANR, arrowhead in Fig. 3I), the anterior limit of the forebrain, and in cells of the facial ectoderm (FE, arrow in Fig. 3I). Due to their small size, these two domains were best visualised on coronal sections (Fig. 3K, L). These results are summarised in Fig. 3Y.

We performed quantitative PCR (qPCR) experiments using RNA extracted from embryos at stages ranging from E7.0 to 10ss (E8.75). Consistent with \textit{in situ} hybridisation experiments, we failed to detect \textit{dbx1} mRNAs at E7.0 and E7.5 (Fig. 3O). \textit{Dbx1} expression was detected at low levels in E8.0 extracts and markedly increased (by \textasciitilde 5 fold) at 2ss (E8.25). Expression then subsequently increased \textasciitilde 2 fold between 2ss and 8ss and \textasciitilde 4 fold between 8ss and 10ss. These results therefore confirm the data obtained by \textit{in situ} hybridisation.

We thus conclude that \textit{dbx1} expression starts concomitantly to the formation of the first somites and is highly dynamic. In addition, we show for the first time that \textit{dbx1} is expressed outside of the central nervous system.
Figure 3. Dbx1 expression and tracing. (A–J) Dbx1 expression analysed by whole-mount in situ hybridisation. B and D are dorsal views of the embryos presented in A and C respectively, rostral is up. F, H and J are frontal views of the embryos shown in E, G and I respectively. Antero-posterior subdivisions of the neural plate/tube are indicated. Mb: midbrain, Fb: Forebrain, Mes: mesencephalon, Di: diencephalon, Tel: telencephalon. Arrows in E, G and I point to the vDi, dDM and FE subsets respectively; the arrowhead in I shows the ANR subset. (K, L) Coronal sections through the forebrain of a 12ss embryo showing dbx1 expression in the ANR (K) and facial ectoderm (L). (M) Coronal section collected at the midbrain level of a 3ss embryo illustrating dbx1 mRNA expression; the neural plate is delineated by dashed lines. (N) Coronal section collected at the level of the midbrain of a 6ss Dbx1<sup>Cre</sup>;R26<sup>YFP</sup> embryo and stained for YFP (green) and DAPI (blue). (O) Quantification of dbx1 expression by qPCR. Asterisks indicate stages at which dbx1 could not be amplified. The relative expression values (normalised to 1 at 2ss and expressed in arbitrary units) are indicated on top of each bar as mean ± s.e.m. (P, Q) Coronal sections of a 20ss (E9.5) Dbx1<sup>Cre</sup>;R26<sup>YFP</sup> embryo at the level of the telencephalon (P) and di/mesencephalon (Q), stained for YFP (green) and DAPI (blue). YFP is detected in regions that express dbx1 at earlier stages: ANR (red arrowhead in P), FE (white arrowheads in P), vDi and dDM (red and white arrowheads in P respectively) but also in cells located in the head mesenchyme (arrows in P). (R–W) Examples of YFP<sup>+</sup> cells found in the head mesenchyme of Dbx1<sup>Cre</sup>;R26<sup>YFP</sup> embryos at 8ss (R–T) and 11ss (U–W) revealed by DAB immunostaining. Sections in R, S and U were collected at telencephalic levels, T, V and W at diencephalic levels. Neural tissues, surface ectoderm and endoderm are surrounded by black, blue and green dash lines respectively. (X) Coronal sections of a 8ss Dbx1<sup>Cre</sup>;R26<sup>YFP</sup> embryo immunostained for YFP (green) and the neural crest cells marker AP2α (red); cell nuclei are stained by DAPI (blue). Some of the YFP<sup>+</sup> cells found in the neural plate are AP2α<sup>−</sup> (arrowhead in X1) whereas
Tracing of early dbx1-expressing populations

Although telencephalic and frontonasal tissues are severely affected upon genetic ablation of all dbx1-expressing cells, we only found little expression of dbx1 in these structures. In order to test whether tissues that form the telencephalon and face derive from dbx1-expressing progenitors but no longer express dbx1 mRNA, we used permanent tracing to follow the progeny of dbx1-expressing cells. We analysed Dbx1<sup>Cre</sup>;R26<sup>YFP</sup> embryos between E8.0 and E9.5 using YFP immunostaining to visualise cells deriving from dbx1-expressing progenitors. Consistent with expression data, we never observed YFP<sup>+</sup> cells at E8.0. YFP expression was first traced in the mesencephalon at 6ss (Fig. 3N) with a pattern reminiscent of ablated embryos. We analysed the expression of the neural tissue (in mesenchyme (arrows in Fig. 3P and Fig. 3R–W). Importantly, we found that the onset of YFP expression occurred ~3–4ss later than that of the dbx1 gene, indicating that the delay between mRNA expression and recombination and subsequent accumulation of the reporter protein spans around ~3–6 hours (at this stage, one somite is formed every ~90 minutes [27]).

One noticeable exception to the similarity between YFP and dbx1 expression patterns was the presence of YFP<sup>+</sup> cells in the head mesenchyme (arrows in Fig. 3P and Fig. 3R–W). Importantly, we never observed Dbx1 mRNA, protein, or LacZ expression in this tissue (in Dbx1<sup>Cre</sup>;R26<sup>lacZ</sup> embryos, in which the lacZ gene has been inserted at the first ATG of the dbx1 gene and allows to trace all Dbx1-derived cells without delay due to the recombination [25]). Mesenchymal Drox1-derived cells were first found around 6–8ss, suggesting that they derive from the EM population (at this stage, only EM-derived cells are YFP<sup>+</sup>). At later stages, EM and dDM progenitor domains partially overlap, making it difficult to determine whether the dDM subset also contribute to these mesenchymal cells. Cephalic neural crest cells (CNCCs) are known to be generated at early somitic stages at the interface between the meso-diencephalic neural plate and surface ectoderm and to subsequently delaminate to invade the facial mesenchyme. We therefore tested the possibility that mesenchymal cells deriving from dbx1-expressing progenitors are CNCCs by performing double immunostaining for YFP and the transcription factor AP2α (a marker of NCCs [28]) on cryosections of Dbx1<sup>Cre</sup>;R26<sup>YFP</sup> embryos. We found that all YFP<sup>+</sup> cells in the head mesenchyme, but also some in the lateral neural plate, were AP2α<sup>+</sup> (Fig. 3X), showing that EM (and possibly dDM) dbx1-expressing progenitors generate CNCCs.

We therefore conclude that Dbx1-derived cells do not massively contribute to some of the territories that are most affected in ablated mutants, namely the forebrain and face, suggesting that the defects observed are non cell-autonomous.

Genetic ablation of dbx1-expressing cells does not affect antero-posterior patterning of the neural plate but results in proliferation defects

In order to determine the mechanisms that were responsible for the defects observed in PGK<sup>Cre</sup>;Dbx1<sup>DTA</sup> embryos, we first tested whether the specification of forebrain tissues occurs correctly in ablated embryos. We analysed the expression of the neural regionalisation markers emx2, otx1, pax6 and six3 in PGK<sup>Cre</sup>;Dbx1<sup>DTA</sup> and control embryos using in situ hybridisation. Analyses were done at 5–6ss, when the initial regionalisation of the neural plate in a hindbrain, midbrain and forebrain is achieved and when the ablated phenotype is already detectable. At this stage, in wild-type embryos, six3 is expressed in the most anterior aspect of the neuroectoderm (Fig. 4A), emx2 identifies the dorsal telencephalic anlage (Fig. 4B), pax6 expression is found in the forebrain and hindbrain (Fig. 4C), and otx1 is expressed in the midbrain and forebrain (Fig. 4D). An identical expression pattern was observed in PGK<sup>Cre</sup>;Dbx1<sup>DTA</sup> embryos, although the antero-posterior extent of the various domains appeared reduced compared to wild-types (Fig. 4E–H), consistent with the smaller

Figure 4. Antero-posterior regionalisation and proliferation in ablated mutants. (A–H) Whole-mount in situ hybridisation of 5 to 6ss control (A–D) and PGK<sup>Cre</sup>;Dbx1<sup>DTA</sup> (E–H) embryos using probes against six3 (A, E), emx2 (B, F), pax6 (C, G) and otx1 (D, H). (I–J) Coronal sections collected at the level of the midbrain of 5ss control (I) and PGK<sup>Cre</sup>;Dbx1<sup>DTA</sup> (J) embryos immunostained for phosphorylated histone-3 (PH3, green) and DAPI (blue). (K–L) Higher magnification of DAPI staining, pyknotic nuclei are highly fluorescent. Quantification of PH3-positive cells control head formation.
size of mutants at this stage (see Fig. S2A, B). We therefore concluded that anterior neural structures are correctly induced and patterned in ablated mutants.

We also tested possible changes in the regulation of \( \text{dbx1} \) expression upon ablation of \( \text{dbx1} \)-expressing cells. At all stages analysed, we only detected very weak \( \text{dbx1} \) expression, which, most importantly, was systematically found in regions corresponding to \( \text{dbx1} \)-expressing areas in controls (Fig. S2C, S3D and data not shown), ruling out the possibility that the defects observed in ablated mutant result from ectopic upregulation of \( \text{dbx1} \) (and hence, ectopic toxin production).

We then analysed proliferation using phospho-histone 3 (PH3) immunostaining on 4 to 8ss embryos. Quantifications revealed a mild (27%) but significant decrease in the percentage of PH3\(^+\) (i.e. mitotic) cells in the neural plate of ablated mutants compared to controls (Fig. 4I, J, M). By contrast, we found no significant differences in the percentage of PH3\(^+\) cells in the head mesenchyme. It therefore appears that proliferation in the neural tube is reduced upon ablation of \( \text{dbx1} \)-expressing cells.

Interestingly, careful examination of DAPI staining on these specimens revealed a ~10 fold increase in the number of pyknotic nuclei compared to control littermates (Fig. 4K, L, N), suggesting enhanced cell death occurred in ablated embryos.

**Genetic ablation of \( \text{dbx1} \)-expressing cells triggers extensive apoptosis**

To test whether tissues were undergoing apoptosis in ablated mutants, we performed whole-mount TUNEL staining. At E8.0, consistent with the fact that \( \text{dbx1} \) expression is just starting (not yet detected by in situ hybridisation but found at low levels by qPCR), both ablated and wild-type littermates displayed low levels of apoptosis (Fig. S2D). At 2ss, increased apoptosis was found throughout the neural plate (Fig. 5A–D), in the forebrain (Fig. 5E, F) and midbrain (Fig. 5G, H), but not at spinal cord levels (Fig. S2E). Of note, TUNEL staining was typically more pronounced in the lateral aspect of the neural plate and at the junction with surface ectoderm. At later stages (11–13ss), increased TUNEL-staining was also observed in ablated mutants compared to wild-types (Fig. 5I–L). Reminiscent of earlier stages, apoptotic cells were mainly located in the dorsal and anterior regions of the neural tube as well as in the surface ectoderm, especially at the level of the forebrain (Fig. 5M, N).

Importantly, we found striking differences between the pattern of \( \text{dbx1} \) expression in wild type embryos and the distribution of apoptotic cells in ablated animals. At 2–4ss, when \( \text{dbx1} \) is expressed in a patch of cells in the midbrain (EM subset), ablated mutants of the same stage display apoptosis throughout the neural plate, including the most anterior and lateral regions (compare Fig. 3D and 5D). At 10–12ss, when four distinct \( \text{dbx1} \)-expressing populations are identified in control embryos (vDi, dDM, ANR and FE), \( \text{PGK:Cre;Dbx1}^{\text{DTA}} \) littermates exhibit a continuous stripe of TUNEL\(^+\) cells in the dorsal midbrain and forebrain (compare Fig. 3I and 5J). Furthermore, the number of apoptotic cells observed in the telencephalon of \( \text{PGK:Cre;Dbx1}^{\text{DTA}} \) at 11–18ss (Fig. 5J, L and data not shown) by far exceeded the one of \( \text{Dbx1} \)-derived cells as indicated by genetic tracing in 20ss \( \text{Dbx1}^{\text{Cre;R26}} \) embryos (Fig. 3P).

**Figure 5. Increased apoptosis in ablated mutants.** Whole-mount TUNEL staining of 2ss (A–D) and 11–13ss (I–L) control (A, C, I, K) and \( \text{PGK:Cre;Dbx1}^{\text{DTA}} \) (B, D, J, L) embryos. C and D are dorsal views of the embryos shown in A and B respectively. (E–H) are cryosections of the embryos shown in A and B at the level of the forebrain (E, F) and midbrain (G, H) as indicated by dashed lines in C and D. K and L are front views of the embryos shown in I and J respectively. M and N are cryosections collected at the level of the diencephalon of these embryos as indicated by dashed lines in I and J. Neural tissues are surrounded by a dashed line for better visualisation. Scale bars: A, I: 200 μm; E, M: 100 μm.

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Together, these results indicate that the apoptosis observed in ablated mutants starts almost concomitantly to the onset of dbx1 expression and, in a large extent, occurs in regions that neither express dbx1 nor derive from Dbx1-expressing progenitors. They also suggest that Dbx1-expressing cells present at 2–4ss promote the survival of neighbouring tissues in a non cell-autonomous manner.

Specific requirements of the dbx1-expressing subpopulations for forebrain and head development

In order to directly test the function of the different dbx1-expressing subsets in forebrain and head development, we attempted to perform their targeted ablation. To this end, we used Nkx2.1:Cre [22], Wnt1:Cre [21] and Foxg1 [20] mouse lines that we crossed with DboxloxP-loxp-loxp-DTA mice.

We first analysed the pattern of recombination by crossing these Cre-expressing mice with ROSA26CreERT2 reporter and compared it to the distribution of Dbx1-derived cells at E9.5. The Nkx2.1:Cre line allowed recombination in a domain encompassing the vDi dbx1-expressing population (Fig. S3A, B). We confirmed effective ablation of this subset in Nkx2.1:Cre;Dbx1DTA embryos by analysing dbx1 expression at E9.5 (19ss; Fig. S3C, D). With the Wnt1:Cre line, recombination occurred in the dorsal mesencephalon and diencephalon, overlapping with the dDM dbx1-expressing population (Fig. S3E, F). Dbx1 expression at E9.5 (19ss) in Wnt1:Cre;Dbx1DTA embryos indicated a complete ablation of the dDM subset (Fig. S3G, H). The Foxg1Cre line drove recombination in the most anterior part of the neural tube as well as the surface ectoderm at stages preceding the onset of dbx1 expression in the ANR and FE (Fig. S3I). Dbx1 in situ hybridisation on Foxg1Cre;Dbx1DTA 14ss embryos confirmed the efficient ablation of both the ANR and FE subsets (Fig. S3O–T). In addition, the Foxg1Cre line allowed a salt-and-pepper recombination in the neural tube and mesencephalon all along the antero-posterior axis (Fig. S3J), suggesting that at least some dbx1-expressing cells from the vDi and dDM subsets are also targeted. However, the extent of such an ectopic recombination did not appear to significantly affect these populations as revealed by dbx1 in situ hybridisation on Foxg1Cre;Dbx1DTA E9.5 (20ss) embryos (Fig. S3K, L).

At last, we found that in both the Foxg1Cre and Wnt1:Cre lines, some recombination occurs at early somitic stages in the area of dbx1 expression in the midbrain (Fig. S3M, N; compare with Fig. 3N), suggesting that the EM subset is partially targeted by these two lines. It is also worth noting that CNCs in the head mesencephalon were targeted by the Wnt1:Cre line (Fig. S3E, N; as previously described [21]).

In summary, the Nkx2.1:Cre line targets the vDi subset, the Foxg1Cre line partially targets the EM, vDi and dDM subsets and completely targets the ANR, and FE subsets, and the Wnt1:Cre line partially targets the EM subsets and completely targets the dDM subset (Table 1).

We then analysed apoptosis in the various selectively ablated mutants. Interestingly, Nkx2.1:Cre;Dbx1DTA embryos displayed the same low levels of apoptosis as their wild-type counterparts between 3ss and 15ss (Fig. 6A, B, E, F, I, J, M, N and data not shown). We therefore concluded that the vDi dbx1-expressing population is dispensable for the survival of anterior tissues. Both Foxg1Cre;Dbx1DTA embryos (Fig. 6C, G) and Wnt1:Cre;Dbx1DTA (Fig. 6D, H) showed increased apoptosis from 3–4ss compared to controls (Fig. 6A, E), but in an extent that did not match the levels observed in PGK:Cre;Dbx1DTA mutants (Fig. 6D, H). Such an observation not only confirms that the EM subset is essential for the survival of the neural plate cells, but also that it is partially (but not completely) targeted by the Wnt1:Cre and Foxg1Cre lines. At 8–9ss, increased apoptosis was observed in Wnt1:Cre;Dbx1DTA and Foxg1Cre;Dbx1DTA embryos (Fig. 6L, K–M, O, P). With the Foxg1Cre line apoptotic cells were typically sparse along the antero-posterior and dorso-ventral axis of the forebrain and midbrain; many were also found in the facial surface ectoderm (Fig. 6K, O). By contrast, using the Wnt1:Cre line, increased apoptosis was most prominent in the dorsal midbrain (Fig. 6L, P).

Each targeted ablation therefore yields a specific pattern of apoptosis, and none of them recapitulates the complete ablation. In order to precisely determine which cells undergo apoptosis in the different ablated embryos, we generated mice bearing both the DboxloxP-loxp-loxp-DTA and the ROSA26CreERT2 reporter and mated them with Cre-expressing lines. We performed immunostaining for activated Caspase-3 and YFP on embryos resulting from such crosses to reveal on the same specimen apoptotic and recombined cells respectively. When using the Nkx2.1:Cre line, we found Caspase-3+ cells in the vDi, with most of them also being YFP+ (arrows in Fig. 6Q), showing that these cells died because they were located in the recombination domain and expressed dbx1 (and therefore DTA). When recombination was driven using the Foxg1Cre strain, we observed increased apoptosis (similar to TUNEL staining) and, interestingly, we counted that 163 out of 185 Caspase-3+ cells found in the mesencephalon and diencephalon were YFP+ (i.e. not recombined; arrowheads in Fig. 6R and Fig. S4), ruling out the possibility that they died because of DTA expression. Control embryos that did not express Cre recombine displayed very low levels of Caspase-3 staining (Fig. 6T). Taken together, these data show that a significant part of the apoptosis observed in ablated mutants is non cell-autonomous as previously suggested from the analysis of PGK:Cre;Dbx1DTA embryos.

In addition to cell survival defects, we found that Wnt1:Cre ablated embryos display a number of forebrain patterning defects. Thus, around 8ss, we observed a dorsal and medial expansion of the foxg1 expression domain (Fig. S3A–D) associated with a posterior shift in the dorsal limit of the emx2 expression domain (Fig. S3E–H). Since the Wnt1:Cre line does not drive recombination in the forebrain, we believe that these defects are strong evidences in favour of a non cell-autonomous function of the dorsal midbrain on forebrain patterning.

Early midbrain dbx1-expressing cells play a crucial role in forebrain formation

When we examined the outcome of targeted ablations at E12.5, we found that each mutant exhibited a mild facial phenotype (Fig. 7A–D). Importantly, all three single mutants.
formed a forebrain that was near normal in size, although its patterning was affected (Fig. 7E–H and data not shown). Thus, each targeted ablation results in a specific phenotype and none of them recapitulate the complete ablation. This could either be explained by a cooperative activity of different subsets, or by the fact that the EM subset is not completely ablated in any of the single mutants.

To discriminate between these two possibilities, we generated Foxg1\textsuperscript{Cre};Nkx2.1:Cre; Wnt1:Cre\textsuperscript{ERT2} (referred to as Triple-Cre\textsuperscript{ERT2}) animals. We first analysed Triple-Cre;Dbx1\textsuperscript{DTA} embryos at E12.5 and found that these animals exhibit craniofacial abnormalities similar to PGK:Cre;Dbx1\textsuperscript{DTA} embryos (Fig. 8A): the nasal cavity was absent and the eyes were shifted medially in both mutants. However, Triple-Cre;Dbx1\textsuperscript{DTA} mutants vastly differ from PGK:Cre;Dbx1\textsuperscript{DTA} regarding brain development since the latter almost completely lacked a forebrain at this stage while the former formed a forebrain that was near normal in size (Fig. 8A, B). We therefore conclude that the cumulative contribution of dbx1-expressing cells targeted by both PGK:Cre and Triple-Cre\textsuperscript{ERT2} lines (i.e. vDi, dDM, ANR and FE subsets) is required for accurate craniofacial morphogenesis. Conversely, dbx1-expressing cells that are targeted in PGK:Cre but not in Triple-Cre ablated mutants (i.e. belonging to the ME subset) are absolutely critical for forebrain formation.

In situ hybridisation for \(dbx1\) at 4ss confirmed that the EM subset is mostly spared in Triple-Cre;Dbx1\textsuperscript{ERT2} mutants (Fig. 8E). Although \(dbx1\) staining was decreased compared to control embryos, it was much higher than in PGK:Cre;Dbx1\textsuperscript{ERT2} mutants, which only display very few \(dbx1\) cells (Fig. 8C–E). We then performed TUNEL staining and found that Triple-Cre;Dbx1\textsuperscript{ERT2} embryos display increased apoptosis at 4ss compared to controls and single ablations, but were not as severely affected as PGK:Cre;Dbx1\textsuperscript{ERT2} embryos (Fig. 8F–K; see also Fig. 6B–D). Consistently, 4ss Triple-Cre;
Dbx1 was previously reported to be expressed from E9.5 in the mouse brain [14,15]. We now show that five distinct subsets of dbx1-expressing cells can be identified at earlier stages (E8.25–E8.75), including one in the facial ectoderm. This study is the first to describe that dbx1 expression is not confined to the central nervous system, as previously reported [15]. Unlike other vertebrates [30] however, we did not find dbx1 expression in the anterior axial mesendoderm of mouse embryos.

One of our most striking findings is that the presence or absence of a limited number of cells from the Dbx1 lineage can have profound consequences on brain and face morphogenesis. Thus, PGK:Cre and Triple-Cre ablated mutants, which only differ by the presence/absence of the EM subset, exhibited very similar craniofacial defects at E12.5 but vastly differed regarding their forebrain phenotype. This allowed us, therefore, to dissociate forebrain formation and facial morphogenesis, and demonstrate that these two processes rely on distinct players, namely “early” (2–4ss) and “late” (from 5–6ss) dbx1-expressing subsets respectively. Since all targeted ablations resulted in different craniofacial phenotypes, and none of them recapitulated the defects observed in PGK:Cre or Triple-Cre ablated mutants, our results show that each dbx1-expressing population has a specific role and that their coordinated activity is required to ensure accurate craniofacial morphogenesis. Importantly, the dDM, vDi and ANR/FE dbx1-expressing subsets largely overlap with region producing Wnts/PGK:Cre;Dbx1;sox10 embryos displayed a strong reduction in sox10 staining whereas Triple-Cre;Dbx1;sox10 embryos appeared significantly less affected (Fig. 8L–N).

Similarly, at 8ss, doi:10.1371/journal.pone.0019367.g007
Scale bars: 500 µm.

Figure 7. Consequences of targeted ablations at E12.5. Heads of wild-type (A), Nkx2.1;Cre;Dbx1;sox10 (B), Foxg1;Cre;Dbx1;sox10 (C) and Wnt1;Cre;Dbx1;sox10 (D) E12.5 mouse embryos. (E–H) Nissl staining of coronal sections collected at the level of the eyes of the embryos shown in A–D. doi:10.1371/journal.pone.0019367.g007

Figure 8L–N.

Discussion

In this study, we show that Dbx1-derived cells are essential for forebrain and craniofacial development in mice. We have identified five restricted dbx1-expressing subsets in the head at early (E8.25–E8.75) developmental stages (EM, vDi, dDM, ANR and FE). Complete genetic ablation of these cells results in extensive and non cell-autonomous apoptosis as well as decreased proliferation in tissues that will later give rise to the midbrain, forebrain and face, leading to dramatic defects in head morphogenesis. Careful analysis of the dynamics of dbx1 expression in wild-types and apoptosis in ablated mutants indicated that the EM subset, which generates a small subpopulation of CNCCs, is crucial to maintain the survival of neighbouring tissues. In addition, targeted and combinatorial ablations of the different subsets, using mutants carrying multiple Cre-expressing transgenes, revealed that the vDi, dDM, ANR and FE are collectively required to ensure accurate craniofacial morphogenesis, whereas the EM is necessary for forebrain formation.

Distinct contribution of Dbx1-derived cells to head development

Since tracing experiments showed that the EM subset generates CNCCs (Fig. 3X), we analysed CNCCs production in Triple-Cre and PGK:Cre ablated mutants by in situ hybridisation for sox10, a marker of NCCs [29]. At 4ss, when CNCCs are mainly premigratory, PGK:Cre;Dbx1;sox10 embryos displayed a strong reduction in sox10 staining whereas Triple-Cre;Dbx1;sox10 embryos appeared significantly less affected (Fig. 8L–N). Similarly, at 8ss, when CNCCs have invaded the cephalic mesenchyme in wild-type counterparts than to PGK:Cre;Dbx1;sox10 embryos (Fig. 8L–N). Similarly, at 8ss, when CNCCs are mainly premigratory, PGK:Cre;Dbx1;sox10 embryos displayed a strong reduction in sox10 staining whereas Triple-Cre;Dbx1;sox10 embryos appeared significantly less affected (Fig. 8L–N). Similarly, at 8ss, when CNCCs have invaded the cephalic mesenchyme in wild-type embryos, PGK:Cre;Dbx1;sox10 counterparts were almost devoid of sox10+ cells, whereas Triple-Cre;Dbx1;sox10 embryos were only mildly affected (Fig. 8O–Q). By contrast, all three genotypes showed similar strong staining in the branchial arch, which is mainly invaded by rhombencephalic NCCs. These results show that the severity of the forebrain phenotype, but not of the craniofacial phenotype, correlate with the loss of CNCCs.

Taken together, our results suggest that a small number of early-born dbx1-expressing cells and their CNCCs progeny have a unique function during early steps of forebrain development by promoting the survival and proliferation of neighbouring tissues in a non cell-autonomous manner.
of neural tube closure, indicating that none of these processes can account for increased cell death. Conversely, the failure of neural tube closure could arise from increased apoptosis as it is observed in several exencephalic mouse mutants [35]. The specific enrichment of apoptotic cells observed at the lateral borders of the neural plate of PGK:Cre;Dbx1^{DTA} embryos could thus preclude fusion of the neural folds. However, the failure of forebrain maintenance in these mutants cannot be attributed to the neural tube closure defect since watch-Cre;Dbx1^{DTA} embryos, which also display an opened brain at E9.5 (data not shown), form a forebrain.

Apoptosis in PGK:Cre;Dbx1^{DTA} embryos was observed in regions that neither express dbx1 nor derive from dbx1-expressing cells and dbx1 expression was not upregulated upon ablation suggesting that apoptosis in non cell-autonomous. We unambiguously demonstrated that cell death is indeed non cell-autonomous using targeted ablation in the presence of the R26^{+} reporter allele, thus allowing us to show that a large number of apoptotic cells are not recombined in Foxg1^{Cre} and watch-Cre ablated mutants, and therefore cannot undergo cell death because of DTA expression. In addition to increased apoptosis, PGK:Cre;Dbx1^{DTA} embryos also displayed a reduction in the proliferation within the neural plate. However, given the respective extent of the proliferation and apoptosis defects in PGK:Cre;Dbx1^{DTA} embryos, we believe that the phenotype observed upon ablation mainly result from increased cell death.

We have recently shown that other later-born Dbx1-derived populations, generated between E10.5 and E12.5 and migrating over long distances to settle in the cerebral cortex, regulate proliferation and differentiation in a non cell-autonomous manner, but were not involved in regulating cell survival [17,18]. We now bring evidence that the earliest-born Dbx1-derived cells also share the capacity to influence territories at distance from their generation site but they have a unique role in cell survival. The ability to regulate the development of adjacent tissues therefore appears to be a common feature among Dbx1-derived cells.

Role of CNCCs during forebrain development

We have shown that the EM dbx1-expressing subset gives rise to a specific subpopulation of CNCCs and we have correlated the ablation of this subset with extensive apoptosis in surrounding tissues and failure in forebrain formation. Tracing experiments indicated that CNCCs are not the only progeny of the EM subset, as some EM-derived cells remain in the neural plate where they later intermingle with cells deriving from the dDM subset. Since tracing of the Dbx1 lineage and ablation are mutually exclusive strategies, it is not possible to precisely say which cells belonging to the EM subset are spared in Triple-Cre;Dbx1^{DTA} mutants. However, the fact that (i) the watch-Cre line drives recombination in all NCCs, (ii) apoptotic cells in 4ss Triple-Cre;Dbx1^{DTA} mutants are mostly found in lateral regions, whereas (iii) untargeted dbx1^{+} cells are more medial, suggest that Dbx1-derived CNCCs are likely to be ablated in Triple-Cre;Dbx1^{DTA} mutants. This is also supported by the small reduction in sox10 staining observed at 4ss following ablation with the Triple-Cre line, which matches the relatively small number of Dbx1-derived CNCCs found in wild-types. By contrast, the strong decrease in sox10 staining observed in PGK:Cre;Dbx1^{DTA} embryos suggests that a significant number of CNCCs which are not from the Dbx1 lineage undergo cell death in these mutants. It is thus likely that dbx1-expressing cells belonging to the EM subset control forebrain formation by regulating the production/survival of CNCCs.

To date, little is known concerning the role of CNCCs during forebrain development in mice. By contrast, this question was
previously addressed using avian models. In chicken, surgical excision of the neural folds generating facial skeletogenic CNCCs prior to their delamination (2–5ss) results in the complete absence of the telencephalon [12]. When the excision is performed at slightly later stages (5–6ss), a forebrain is formed, although its patterning is severely affected and dorsal midline closure does not occur [10,13]. Subtle variations in the timing of the ablation therefore have a profound influence on its outcome.

In our system, we mimic the consequences of a surgical removal of the mid-diencephalic to anterior-rhombencephalic neural folds, using genetic means to perform the ablation of only a very specific and molecularly characterised population of cells. In addition, we show that forebrain formation is maintained when some of the earliest dbx1-expressing cells are spared from the ablation.

Increased apoptosis in PGK:Cre;Dbx1<sup>DTA</sup> embryos is observed from early stages and is clearly the main cause to the phenotype we observe. In chicken experiments, increased apoptosis was observed in forebrain tissues, but it was reported 24 to 48 hours after surgery and only in the case of 2–5ss ablations [10–13]. The contribution of apoptosis in the avian paradigm of CNCCs depletion thus remains to be precisely assessed in order to better understand how CNCCs contribute to forebrain development.

Mechanisms of Dbx1-derived cells function

In PGK:Cre;Dbx1<sup>DTA</sup> embryos, anterior neural territories are correctly induced but fail to be maintained and undergo extensive apoptosis. One possible explanation of our observations is that Dbx1-derived cells normally provide signals (whose identity remains to be established) necessary for cell viability or cell survival in surrounding tissues. Such a function could be fulfilled by long-range diffusing molecules originating from the neural tissue itself and/or by signals locally released by migrating CNCCs. Further studies will be required in order to discriminate between these two hypotheses. Of note, although the function of the dbx1 gene is not precisely known during forebrain development, Dbx1 itself is most likely not involved in the processes we describe here as dbx1-deficient embryos form a forebrain and do not display defects in craniofacial morphogenesis (25) and unpublished results).

A large number of mouse mutants displaying aberrant head morphogenesis have been identified [36], but in most instances the defects result from improper induction or patterning rather than increased cell death as it is the case in PGK:Cre;Dbx1<sup>DTA</sup>. A specific feature of our model is the fact that the eye fields remain unaffected despite extensive apoptosis in neighbouring areas, suggesting that the signalling partners mediating the survival of presumptive forebrain tissues are different from those involved in eye development.

All thing considered, the model that best recapitulates the phenotype of PGK:Cre;Dbx1<sup>DTA</sup> mutants is probably the CNCC ablation paradigm in chick [10,12,13]. However, although a mechanism involving BMP antagonists was proposed to mediate CNCCs function [11], the fact that it relies on the control of neural tube closure, forebrain growth and patterning but not on the regulation of survival suggests significant divergence between our respective systems.

In this study, we identify novel dbx1-expressing populations at early stages of neural development and demonstrate that, despite their limited number, they exert a critical function in maintaining forebrain and craniofacial development. In gastrulating zebrafish embryos, the ablation of only a dozen of anterior ectodermal cells was shown to result in major brain patterning defects followed by widespread apoptosis [37]. In mice, early dbx1-expressing cells constitute a unique example of such few cells having a crucial function during brain development.

Supporting Information

Figure S1 Nestin:Cre driven ablation of Dbx1-derived cells at E12.5. Heads of wild-type (A) and Nestin:Cre;Dbx1<sup>DTA</sup> (B) E12.5 mouse embryos. The obvious reduction in the size of the midbrain in mutants is indicated by the yellow arrowhead in B. (C–D) Coronal sections collected at the level of the eyes of wild-type (C) and Nestin:Cre;Dbx1<sup>DTA</sup> (D) E12.5 mouse embryos. The red arrowhead in D points at the reduction in the thickness of the medial ganglionic eminence in mutants. Scale bars: A, C: 500 μm.

Figure S2 Morphological defects in E8.5 PGK:Cre;Dbx1<sup>DTA</sup> embryos. (A) Quantification of the width of the neural plate at the level of the midbrain in 2–4ss control and PGK:Cre;Dbx1<sup>DTA</sup> embryos. (B) Quantification of the length between the pre-otic sulcus and the anterior limit of the forebrain in 3 to 6ss control and PGK:Cre;Dbx1<sup>DTA</sup> embryos. * p<0.001. (C) In situ hybridisation for dbx1 on tss control and ablated mutants. (D) TUNEL staining of presomitic E8.0 embryos showing the normal levels of apoptosis in mutants. Embryos are shown from above (anterior is up); the red asterisk indicates the node. (E) Sections collected at the level of the spinal cord of 3ss control and PGK:Cre;Dbx1<sup>DTA</sup> embryos stained by TUNEL. Scale bars: C: 200 μm; Dr: 50 μm.

Figure S3 Targeted ablation of the Dbx1-expressing subsets. The Nkx2.1:Cre line drives recombination in a domain (arrow in A) overlapping with the vDi subset (arrow in B) and allows its ablation (C, D). The Wnt1:Cre line drives recombination in the dDM region (arrows in E, F) and allows the complete ablation of the dbx1-expressing cells in this area (G, H). Using the FoxG1<sup>lox<sup>5flo</sup></sup> line, recombination occurs in the facial ectoderm (arrowhead in I) and anterior forebrain (arrow in I) but also in a salt and pepper manner along the neural tube and mesencephyme (J). Ablation driven by the FoxG1<sup>lox<sup>5flo</sup></sup> line does not significantly affect the vDi and dDM subsets (K, L). At 5ss, both the FoxG1<sup>Cre</sup> (M) and Wnt1:Cre (N) lines allow recombination in the mesencephalic neural plate (arrows in M and N). The Wnt1:Cre line also drive recombination in CNCCs (arrowhead in E and N). (O-P) Whole-mount in situ hybridisation for dbx1 in control (O) and FoxG1<sup>Cre</sup>;Dbx1<sup>DTA</sup> (P) 4ss embryos. Arrows in O and P as well as sections (Q and S) indicate the effective ablation of the ANR subset in FoxG1<sup>Cre</sup>;Dbx1<sup>DTA</sup> embryos. Arrowheads in O and P as well as sections (R and T) show that the FE subset is efficiently ablated in FoxG1<sup>Cre</sup>;Dbx1<sup>DTA</sup> embryos. Scale bars: A: 100 μm; C, O: 200 μm; Q: 50 μm.

Figure S4 Quantification of cell-autonomous and non-cell-autonomous apoptosis. Counting of the number of YFP positive/negative cells among Caspase-3<sup>+</sup> cells in FoxG1<sup>Cre</sup>;R26<sup>-YFP</sup>;Dbx1<sup>DTA</sup> and Wnt1:Cre;R26<sup>-YFP</sup>;Dbx1<sup>DTA</sup> embryos. Most apoptotic cells located within the recombination domain (grey) were YFP<sup>+</sup> (29/41 in the telencephalon of FoxG1<sup>Cre</sup>;R26<sup>-YFP</sup>;Dbx1<sup>DTA</sup> and 116/169 in the mesencephalon and diencephalon of Wnt1:Cre;R26<sup>-YFP</sup>;Dbx1<sup>DTA</sup> embryos). On the contrary, most Caspase-3<sup>+</sup> cells found in the mesencephalon and diencephalon of FoxG1<sup>Cre</sup>;R26<sup>-YFP</sup>;Dbx1<sup>DTA</sup> or in the telencephalon of Wnt1:Cre;R26<sup>-YFP</sup>;Dbx1<sup>DTA</sup> embryos were YFP<sup>−</sup> (163/165 and 69/81 respectively), ruling out the possibility that these cells died because of DTA expression.
Figure S5 Patterning defects in Wnt1:Cre ablazed embryos. Side (A, C) and front (B, D) views of foxg1 expression in control (A, B) and Wnt1:Cre;Dbx1;DTA (C, D) 9ss embryos. The arrows in G and D point to the dorsal and medial expansion of the expression domain respectively. Side (E, G) and top (F, H) views of emx2 expression in control (E, F) and Wnt1:Cre;Dbx1;DTA (G, H) 9ss embryos. Ablated mutants display a posterior shift in the dorsal limit of emx2 expression (arrows). Scale bar: 200 µm. (TII).

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Author Contributions

Conceived and designed the experiments: FC ME AP. Performed the experiments: FC ME AT TLdC. Analyzed the data: FC ME AP. Contributed reagents/materials/analysis tools: NK WDR. Wrote the paper: FC AP.

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