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Reduced salivary gland size and increased presence of epithelial progenitor cells in DLK1-deficient mice

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Abstract DLK1 (PREF1, pG2, or FA1) is a transmembrane and secreted protein containing epidermal growth factor-like repeats. *Dlk1* expression is abundant in many tissues during embryonic and fetal development and is believed to play an important role in the regulation of tissue differentiation and fetal growth. After birth, *Dlk1* expression is abolished in most tissues but is possibly reactivated to regulate stem cell activation and responses to injury. We have recently reported that DLK1 regulates many aspects of salivary gland organogenesis. Here, we have extended our studies of the salivary gland phenotype of *Dlk1* knock-out mice. We have observed that salivary glands are smaller and weigh significantly less in both *Dlk1* knock-out males and females compared with gender and age-matched wild-type mice and regardless of the natural sexual dimorphism in rodent salivary glands. This reduced size correlates with a reduced capacity of *Dlk1*-deficient mice to secrete saliva after stimulation with pilocarpine. However, histological and ultrastructural analyses of both adult and developing salivary gland tissues have revealed no defects in *Dlk1* $^{(-/-)}$ mice, indicating that genetic compensation accounts for the relatively mild salivary phenotype in these animals. Finally, despite their lack of severe anomalies, we have found that salivary glands from *Dlk1*-deficient mice present a higher amount of CK14-positive epithelial progenitors at various developmental stages, suggesting a role for DLK1 in the regulation of salivary epithelial stem cell balance.

Keywords DLK1 · Salivary glands · Saliva ·
Progenitor cells · Mouse

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Introduction

Salivary gland development depends strongly on communication between epithelial progenitor cells and mesenchymal cells, which are the precursors of the adult salivary gland acini and tubuli, and the stroma (Tucker 2007). Salivary gland regeneration is the subject of intense research concerning tissue engineering (Patel and Hoffman 2014; Ogawa et al. 2013; Liu and Wang 2014). This has permitted the identification of important signaling cues and pathways involved in salivary gland development (Cantara et al. 2012; Lombaert et al. 2008; Lombaert et al. 2013; Knox et al. 2010), in particular, signaling pathways including Fgf (fibroblast growth factor), sonic hedgehog, wingless integration site, ectodysplasin, and NOTCH receptors and their ligands. The activity of many of these signals is regulated by salivary gland innervation and parasympathetic nerve activity (Knosp et al. 2012). Recently, we have characterized an inhibitory role of the non-canonical NOTCH ligand Delta-like homolog 1 (DLK1) protein on salivary gland development. We have shown that external application of DLK1 blocks the branching morphogenesis of the

developing submandibular salivary gland (SMG) through the inhibition of NOTCH signaling (Garcia-Gallastegui et al. 2014).

DLK1, also named preadipocyte factor 1 (PREF1), pG2, or fetal antigen 1 (FA1), is a transmembrane and secreted protein belonging to the epidermal growth factor (EGF)-like repeat-containing family, to which all NOTCH receptors and ligands belong. *Dlk1* expression is abundant in many tissues during embryonic and fetal development and also in the placenta in which it is believed to play an important role in the regulation of fetal growth (Yevtodiyenko and Schmidt 2006). *Dlk1* is a member of a cluster of imprinted genes that are only expressed from the paternally inherited chromosome (Schmidt et al. 2000; Takada et al. 2000). Genes with genomic imprinting have a crucial function in the control of fetal growth and development (Murphy and Jirtle 2003; Rand and Cedar 2003; Wilkins and Haig 2003), and *Dlk1* is no exception. In the mouse embryo, *Dlk1* expression can be detected from embryonic day 8.5 (E8.5; Smas et al. 1994). At E12.5, *Dlk1* is expressed at high levels in endocrine organs such as the developing pituitary and adrenal gland, in many mesodermally derived tissues including skeletal and adipose tissues, and, finally, in organs developing by branching morphogenesis, such as the lung, the kidney, and the salivary gland. After birth, *Dlk1* expression is abolished in most of these tissues but remains throughout adulthood in endocrine organs, most notably in hormone-secreting cells of the pituitary, the adrenal gland, pancreatic islets, and the gonads (Hedlund et al. 2003). In addition, although normally silent under physiological conditions, *Dlk1* can also be locally overexpressed in adult tissues after injury, being associated possibly with stem cell activation and tissue repair responses (Andersen et al. 2009; Samulewicz et al. 2002).

Various signaling pathways appear to be regulated by DLK1. For instance, an interaction has been described between DLK1 and insulin growth factor-1/extracellular regulated kinase (IGF-1/ERK) signaling (Ruiz-Hidalgo et al. 2002) and with the C-terminal region of the extracellular matrix protein fibronectin, which is fundamental for DLK1 action on ERK activation (Wang et al. 2010). DLK1 also interacts with the NOTCH1 receptor, through its extracellular EGF-like repeats (Baladrón et al. 2005; Sanchez-Solana et al. 2011; Nueda et al. 2007b). Unlike other canonical NOTCH ligands, DLK1 lacks the delta-serrate-LAG2 domain, which is involved in receptor activation. Thus, DLK1 is regarded as a NOTCH inhibitory non-canonical ligand (Baladrón et al. 2005; Sanchez-Solana et al. 2011; Falix et al. 2012). One possible mechanism of DLK1 inhibition of NOTCH signaling is by competition for receptor binding with canonical ligands (Dikic and Schmidt 2010).

The NOTCH pathway is a highly important regulator of cell differentiation and organogenesis (Bolos et al. 2007; Chiba 2006). As a NOTCH regulator, DLK1 is itself unsurprisingly similarly involved in cell differentiation processes during development (Falix et al. 2012), including neuroendocrine differentiation (Floridon et al. 2000), differentiation of hepatocytes and biliary epithelial cells (Tanimizu et al. 2003), differentiation of neurons and glial cells (Müller et al. 2014; Ferrón et al. 2011; Imayoshi et al. 2010), hematopoiesis (Moore et al. 1997; Mirshekar-Syahkal et al. 2013), osteogenesis (Abdallah et al. 2004), skeletal muscle differentiation (Waddell et al. 2010; Andersen et al. 2013), chondrogenic differentiation (Chen et al. 2011), and adipogenesis (Smas and Sul 1997; Baladrón et al. 2005; Nueda et al. 2007b). In addition, DLK1 expression is also a feature of stem cell niches in many adult tissues, such as the skeletal muscle, bone marrow, and the central nervous system, in which DLK1 regulates the processes of skeletal muscle regeneration (Andersen et al. 2009), hematopoiesis (Li et al. 2005), and adult neurogenesis (Ferrón et al. 2011).

Given that *Dlk1* is still expressed in many endocrine cells in the adult body, one of its main functions is endocrine and metabolic regulation at multiple levels, with associated pleiotropic effects (Da Rocha et al. 2009; Chacon et al. 2008; Charalambous et al. 2014a, b). Indeed, ample evidence is available that DLK1 is a regulator of the somatotrophic axis (Abdallah et al. 2007; Puertas-Avendaño et al. 2011). Finally, other data show that the aberrant overexpression of *Dlk1* in adult tissues might also be involved in the promotion of cell growth and cancer, which might further be associated with perturbations of either IGF-1 or NOTCH signaling (Kim et al. 2009; Yanai et al. 2010; Lopez-Terrada et al. 2009; Dezso et al. 2008; Van Limpt et al. 2003; Nueda et al. 2014).

Genetically modified mice are an extremely valuable tool for the study of DLK1 functions. For instance, mice expressing a double dose of *Dlk1* mimic the condition of loss of imprinting on this locus. Interestingly, these mice are larger than wild-type mice at birth but present a high perinatal mortality and do not thrive in adult life, in spite of the presumptive advantage of enhanced fetal growth. Moreover, mice with a triple dose of *Dlk1* present a lethal phenotype with numerous developmental abnormalities, suggesting that the correct dosage of this gene is critical for embryonic development (Da Rocha et al. 2009). Another opposite genetic modification is the global or conditional elimination of *Dlk1*, thus generating the corresponding *Dlk1* [(−/−)] or knock-out mouse. Initial studies of the phenotype of global *Dlk1*-null mice revealed growth retardation, skeletal abnormalities, and accelerated adiposity in these animals (Moon et al. 2002). Indeed, mice lacking *Dlk1* show reduced weight at birth and during the first weeks of life. Strains of mice generated on a BALB/CJ genetic background are eventually able to recover and gain a normal weight compared with wild-type littermates (Moon et al. 2002), whereas those on either a 129/SvJ or C57BL/6 background remain smaller throughout adult life (Cheung et al. 2013; Raghunandan et al. 2008).

To date, no one has examined in detail the salivary gland phenotype of $Dlk1^{(-/-)}$ mice. In the isolated SMG, the addition of sDLK1 causes many developmental anomalies, such as the disruption of branching morphogenesis and SMG innervation and the impaired maintenance of salivary epithelial progenitor cells (Garcia-Gallastegui et al. 2014). Given that DLK1 regulates multiple aspects of SMG organogenesis, our aim has been to assess whether salivary gland development and function are compromised in $Dlk1^{(-/-)}$ mice.

Materials and methods

$Dlk1^{(-/-)}$ mice $Dlk1^{(-/-)}$ mice were generated by backcrossing 129XI/SVJ and C57Bl/6 strains in order to generate $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ animals on a mixed genetic background. These mice were kindly provided by the Animal House facility of the University of Castilla La Mancha (UCLM). Knock-out of the $Dlk1$ gene was made by targeting a construct that was assembled and subsequently electroporated into SVJ129 embryonic stem cells. The construct had a neomycin-resistance cassette replacing 3.8 kb of the endogenous allele, including the promoter and the first three $Dlk1$ exons. Chimeric animals were bred to establish a $Dlk1^{(-/-)}$ mouse line (Raghunandan et al. 2008; Puertas-Avendaño et al. 2011). Genotype analysis was carried out by polymerase chain reaction (PCR) amplification with the following primers for $Dlk1$, Neo, and the constitutive ribosomal protein, $Rpl13$:

> $Dlk1$, F5′TGTGACCCCGAATATGGATTCTG
> CGAGG3′,
> R5′CATGGTTCCTTGCAGACTCCATTGACAC
> ACAGC3′;
> Neo, F5′TTGAACAAGATGGATTGCACGCAGG3′,
> R5′GGCTGGCGCGAGCCCCTGATGCTCT3′;
> $Rpl13$, F5′CCGAAGAAGGGAGACAGTTCTGCTG3′,
> R5′GGAGACTGGCAAAAGCCTTAAAGT3′.

Phenotype analysis was performed after three backcrosses to C57Bl/6, and then intercrosses of heterozygotes were used to generate homozygous $^{(-/-)}$, heterozygous $^{(+/-)}$, and wild-type (WT; $^{+/+}$) mice. Reverse transcription (RT)-PCR confirmed that all $Dlk1^{(-/-)}$ mice presented a specific replacement of a portion of the $Dlk1$ gene for a Neo cassette (Electronic Supplementary Material, Fig. 1). For all experiments, age-matched WT littermates were bred and used as controls (Raghunandan et al. 2008). $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mice were bred in the Animal House facilities of the Faculty of Medicine in the University of the Basque Country (UPV/EHU), under standard conditions of cleanness and a light cycle from 8 am to 8 pm, and were fed *ad libitum*. All mice were housed according to institute regulations, and procedures were carried out in compliance with Biscay Council (Spain) licenses.

Histological procedures Adult (16 weeks old; $n = 4$ of each strain) glands from male and female $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mice were prepared for hematoxylin and eosin (H/E) staining after being paraffin-embedded and sectioned. Images were captured in an OLYMPUS optical microscope BX50FO, run with CellA software. To examine the tissues at higher magnification, we prepared them for transmision electron microscopy (TEM). Adult male and female SMGs from $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mice were embedded in Epon Polarbed resin. Ultrathin sections (70 nm) were deposited onto 150 mesh copper grids, post-stained with uranyl acetate and lead citrate, and visualized in a Philips EM208S transmission electron microscope. Digital images were acquired with an Olympus SIS Morada digital camera.

Saliva measurement $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ adult male and female mice (16 weeks old; $n = 5$ of each strain) were anesthetized by 1 % isoflurane inhalation, after which saliva secretion was stimulated by intraperitoneal injection of pilocarpine (0.05 mg/100 g body weight), a non-selective muscarinic agonist. Secreted saliva was then absorbed onto previously weighed paper plugs inserted into the oral cavity. Saliva-saturated plugs were weighed and corrected for the original weight of the paper plug. The amount of secreted saliva was calculated as the increase in weight of the paper plug (in milligrams) per unit body weight (in grams).

Immunostaining E16, postnatal day 1 (P1), and adult SMGs were fixed for several hours in 4 % paraformaldehyde (depending on the size of the tissue), embedded in O.C.T. Compound (Tissue-Tek, Sakura), and frozen in dry ice. Sections (10 μm thickness) of every sample were obtained by cryostat (LEICA CM3050S) and incubated overnight with primary antibodies that recognized ductal progenitor/stem cells, rabbit anti-cytokeratin 5 (CK5, ab53121, Abcam; 1:400 dilution), epithelial or end-bud progenitor/stem cells, and rabbit anti-cytokeratin 14 (CK14, ab49747, Abcam; 1:200 dilution). The membrane-bound DLK2 protein was stained with the primary antibody previously described by Rivero et al. (2012). Axons of the parasympathetic ganglia (PSG) were stained with βIII-tubulin (ab18207, Abcam; 1:500 dilution) to give whole-mount staining for examination by extended focus acquired in XYZ to scan the whole section (around 40 μm) and then stacked in a single three-dimensional image. Nuclei of the cells were counterstained with 4,6-diamidino-2-phenylindole. Thereafter, sections were incubated with Alexa Fluor 488 conjugated to goat anti-rabbit IgG (A11029, Invitrogen). Fluorescent preparations were recorded by using

a confocal Olympus FV500 Fluoview microscope run with the software package Nikon DS-Qi1Mc.

Quantitative RT-PCR RNA extraction from embryonic glands (E13–P1) was performed with an RNA miniKiT Ambion (cat. no. AM1931). To obtain cDNA from RNA, we used the iScripts cDNA Synthesis kit (BioRad) following the manufacturer's instructions. Quantitative RT-PCR (qPCR) was carried out in an iCycler MyiQ Single-Color Real-Time PCR Detection System (BioRad) with the Power SYBR Green PCR Master Mix (Life Technologies, cat. no. 4367659). We used the following primers to detect various genes:

Ck14, F5′ACCGCCAGATCCGCACCAAG3′,
R5′TCCTAAGCCTGAGCAGCATGTAGC3′;
Ck5, F5′ TCCTGTTGAACGCCGCTGAC3′,
R5′CGGAAGGACACACTGGACTGG3′;
Aqp5, F5′TCTACTTCTACTTGCTTTTCCCC TCCTC3′,
R5′ CGATGGTCTTCTTCCGCTCCTCTC3′;
Fgf10, F5′ TCTTCCTCCTCCTCGTCCTTCTC CTCTCCTTCC3′,
R5′ CCGCTGACCTTGCCGTTCTTCTCAATCG3′;
Dlk2, F5′GGCCAGTGTGTGTATGACGG3′,
R5′CGGCATGTGAAGTTGAGGG3′;
αSma, F5′CATCTTTCATTGGGATGGAG3′,
R5′TTAGCATAGAGATCCTTCCTG3′;
Hes1, F5′GTGCATGAACGAGGTGACCC3′,
R5′GTATTAACGCCCTCGCACGT3′;
*Gapdh*, F 5′ACGGCACAGTCAAGGCCGAG3′,
R5′CACCCTTCAAGTGGGCCCCG3′.

Quantification of the data obtained from the thermocycler, namely Ct values, was carried out by normalizing them to the house-keeping gene s29, calculated as ΔCt. ΔCt values were then transformed to relative fold changes of gene expression between the experimental conditions (Schmittgen and Livak 2008). The differences between the compared conditions were considered to be statistically significant at a confidence interval of 95 %. The experiment was repeated at least three times, and the graphs show means ± SEM.

Statistical analysis All values are expressed as means ± SEM. All data sets were subjected to the Kolmogórov-Smirnov or Shapiro-Wilk test prior to analysis in order to confirm that they followed a parametric distribution. Comparison between groups was performed by using the unpaired Student's t-test (two-tailed). A P-value ≤ 0.05 was considered to be statistically significant.

Results and discussion

Salivary gland size is reduced in $Dlk1^{(-/-)}$ mice

We compared the anatomy and morphology of the submandibular (SMG), sublingual (SLG), and parotid (PG) salivary glands of adult male and female $Dlk1^{(-/-)}$ and $Dlk1^{(+/+)}$ mice. We exposed the glands of the mice after perfusion and took photographs, after which we weighed the whole salivary gland complex after careful dissection (Fig. 1a–d). The $Dlk1^{(-/-)}$ glands were smaller than those from the $Dlk1^{(+/+)}$ control mice. In addition, glands from females were smaller than
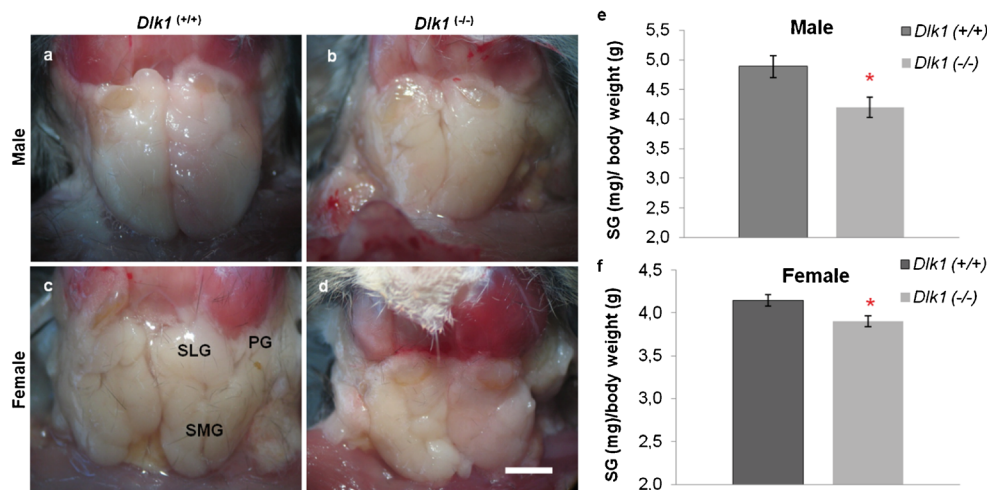
Fig. 1 Anatomy and weight of $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ adult salivary glands. Submandibular (*SMG*), sublingual (*SLG*), and parotid (*PG*) glands were photographed at both sides of the neck after perfusion. **a** $Dlk1^{(+/+)}$ male. **b** $Dlk1^{(-/-)}$ male. **c** $Dlk1^{(+/+)}$ female. **d** $Dlk1^{(-/-)}$ female. *Bar* 5 mm. **e, f** Graphical representations of weight (mg) of the salivary gland complex (*SG*) for male (**e**) and female (**f**) $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mice, with respect to mouse total body weight (in grams). Salivary glands were significantly smaller in both male and female $Dlk1^{(-/-)}$ mice. Data are means ± SEM ($n = 10$ of each group). Unpaired t-test, *$P \leq 0.05$

those from males. We attributed this gender size difference to the sexual dimorphism characteristic of rodent salivary gland (Atkinson et al. 1959; Gresik and Barka 1980). We weighed dissected glands from $Dlk1^{(-/-)}$ and $Dlk1^{(+/+)}$ mice ($n = 10$ males and females) and represented their relative weight with respect to total body weight of the mice (Fig. 1e, f). Salivary glands from knock-out mice were smaller and weighed significantly less, both for males and females, than those from wild-type mice.

$Dlk1$-null mice, including the mouse strain tested here, have previously been described as having a reduced body weight (Moon et al. 2002; Raghunandan et al. 2008; Cheung et al. 2013). We have confirmed here that these mice are definitely smaller than their wild-type counterparts (Electronic Supplementary Material, Fig. 2). However, this weight decrease appears to be particularly emphasized in the case of salivary glands, since their reduction in weight is significant, even when referred to the total body weight of each mouse genotype.

Several possible explanations can be proposed for this specific effect. In this context, we have recently observed that $Dlk1$ is highly expressed during salivary gland development (Garcia-Gallastegui et al. 2014). DLK1 is a regulator of the growth hormone (GH)/IGF-1 somatotroph axis (Abdallah et al. 2007), which has a key role in the regulation of body size in growing animals (Olney 2003; Ohlsson et al. 2000). DLK1 colocalizes with GH-secreting cells in the pituitary, and $Dlk1$ overexpression has been shown to inhibit GH secretion in GH3 pituitary cell lines (Ansell et al. 2007). Accordingly, serum DLK1 and GH levels correlate inversely in adult mice (Abdallah et al. 2007), and the same $Dlk1^{(-/-)}$ mouse strain that we have used here has previously been reported to present increased pituitary mRNA levels for GH (Puertas-Avendaño et al. 2011). However, if increased levels of systemic GH were responsible for the salivary gland phenotype in these animals, one might expect that these would be larger, rather than smaller, making this a highly unlikely explanation. Additionally, conditional $Dlk1^{(-/-)}$ mice in somatotroph cells notably present no alteration in their growth phenotype at all (Appelbe et al. 2013), making altered levels of GH unlikely to account for the growth-regulatory effect of $Dlk1$ (Cheung et al. 2013).

A more plausible scenario would be that DLK1 also regulates IGF-1-binding proteins locally (Nueda et al. 2008; Charalambous et al. 2014a), making its growth-promoting or

Fig. 2 $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ adult and embryonic salivary gland. **a–f** Hematoxylin and eosin (H/E) staining of section of adult submandibular (*SMG*), sublingual (*SLG*), and parotid (*PG*) glands, showing mature mucous, seromucous, and serous acini, respectively, together with some larger striated secretory ducts. **g–n** H/E staining of E15 and E17 SMG and of E19 SMG and SLG. Differentiating SLG and SMG of mouse salivary glands are distinguishable by E19. *Bars* 20 μm (**a–f**, 100 μm(**g–n**)
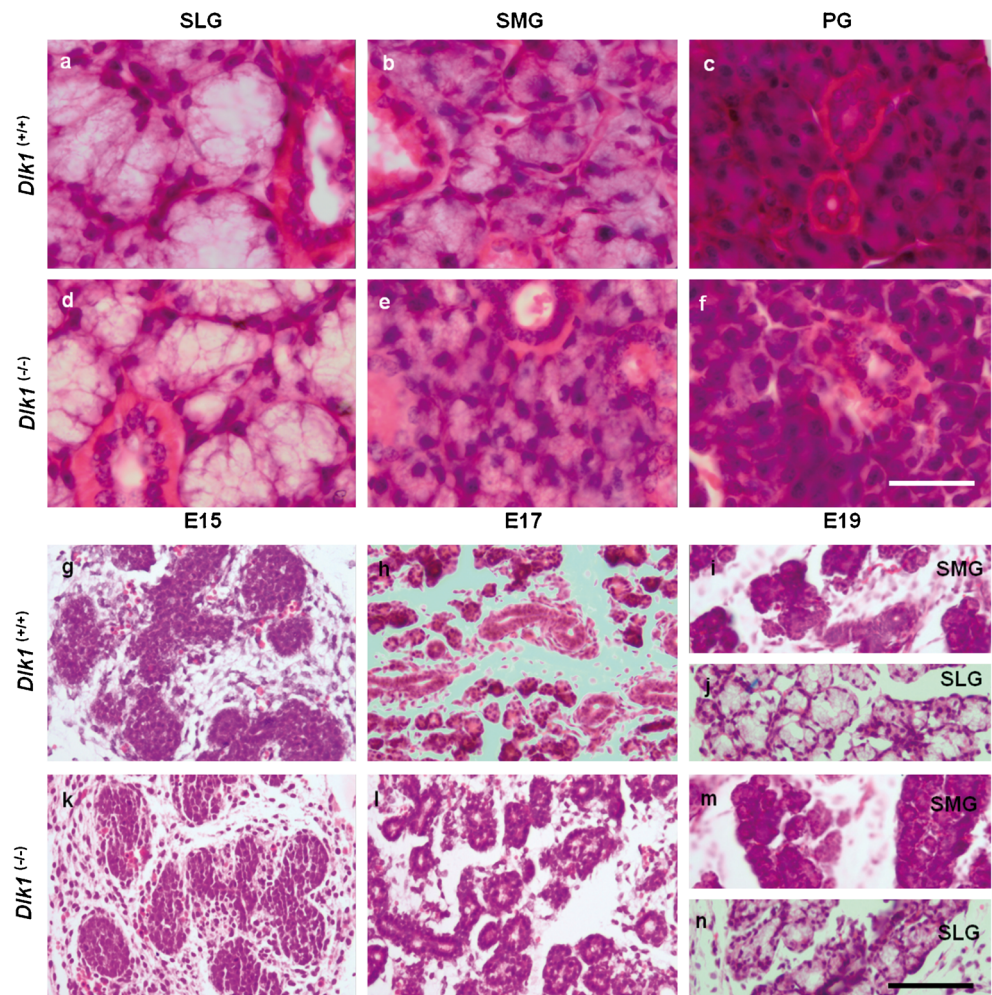
growth-repressing effect context-dependent. In this regard, interestingly, IGF-1 is present throughout salivary gland branching morphogenesis stages (Jaskoll and Melnick 1999), and its deregulation could lead to this reduction in organ size. Furthermore, these mice have also been reported to present increased serum levels of other endocrine hormones, such as leptin (Puertas-Avendaño et al. 2011), which might reflect a systemic metabolic alteration in these animals. Of note, salivary gland development is highly sensitive to endocrine action, in particular to that of sex hormones (Konttinen et al. 2010), and any perturbation of the global endocrine-metabolic balance at this level might affect salivary gland size.

Salivary gland histology is normal in both adult and developing $Dlk1^{(-/-)}$ mice

In spite of their reduced size, the macroscopic morphology of adult salivary glands from $Dlk1^{(-/-)}$ mice appeared to be normal. However, we wished to assess whether malformations occurred at the tissue level. We therefore stained sections of $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ adult salivary glands (SMG, SLG, and PG) with H/E. In Fig. 2, we show representative histological images of the SLG, SMG, and PG, with a predominance of mucous, seromucous, and serous acini, respectively. A detailed histological examination did not reveal any difference between $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ salivary glands. We did however observe a clear gender difference in the abundance of granular convoluted tubules (GCT), which were much more prominent in males than females, but no histological differences were otherwise found between adult wild-type and $Dlk1$-null mice, in either H/E-stained (Fig. 2a-f) or Alcian-blue-stained (Electronic Supplementary Material, Fig. 3) sections.

Since $Dlk1$ expression in the salivary gland takes place almost exclusively during embryonic development, we wondered whether we could detect histological alterations in salivary glands at various stages of organogenesis. Thus, we stained sections of $Dlk1^{(+/+)}$ and $^{(-/-)}$ salivary glands at E15 (canalicular stage), E17 (terminal bud stage), and E19 (differentiation stage) with H/E. Progressive differentiation of SMG and SLG acini and tubuli was observed during this time sequence (Fig. 2g-n). At E15, the morphology of the glands was typical for the canalicular stage, with the epithelia being more intensely stained with H/E and the less compact mesenchyme being paler. At E17, the future acini and tubuli begin to differentiate. At E19, we could clearly distinguish the SMG portion, with a predominance of seromucous acini, and the SLG, with mostly mucous acini, that were less stained because of their cytoplasmic mucin content. However, again, no histological differences could be observed between $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mice.

In a previous study, we found that DLK1 inhibited the NOTCH signaling pathway and critically influenced SMG organogenesis (Garcia-Gallastegui et al. 2014). We checked the activity level of the NOTCH signaling pathway in E14 SMGs by examining the expression of its downstream target $Hes1$ by qPCR (Electronic Supplementary Material, Fig. 4f). We found no difference in NOTCH activity between SMG from $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mice. Thus, because an absence of $Dlk1$ does not seem to affect NOTCH pathway activity in $Dlk1^{(-/-)}$ mouse embryonic salivary glands, the most likely explanation for the lack of a strong salivary phenotype in $Dlk1^{(-/-)}$ mice is genetic compensation. Recently, we described that the related protein DLK2, which is structurally and functionally homologous to DLK1 (Nueda et al. 2007a), also inhibited NOTCH signaling and was present during salivary gland development (Garcia-Gallastegui et al. 2014). For this reason, we wished to evaluate whether DLK2 upregulation could account for a possible compensatory effect in the absence of DLK1 in knock-out mice. Thus, we performed both RT-PCR and qPCR to check $Dlk2$ mRNA expresion in E14 and P1 SMG (Electronic Supplementary Material, Fig. 4e, f), and a immunocytochemical analysis of DLK2 protein expression in E13 and P1 SMG samples, but again we found no differences between $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mice (Electronic Supplementary Material, Fig. 4a-d). Therefore, the upregulation of $Dlk2$ expression does not occur in $Dlk1^{(-/-)}$ salivary glands, ruling out this mechanism of compensation. We could not discard that compensation is exerted at the protein activity levels (e.g., by regulating the generation of membrane-bound and soluble DLK2 isoforms), but whether DLK2 can be proteolitically processed in a similar way to DLK1 remains unknown.

$Dlk1^{(-/-)}$ mice secrete less saliva after pharmacological stimulation

Taking into account that $Dlk1^{(-/-)}$ mice presented smaller salivary glands with no alterations in their histological structure, we carried out a functional analysis. We measured the total amount of secreted saliva in $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mice, after stimulation by the injection of pilocarpine. We observed that $Dlk1^{(-/-)}$ mice, both male and female, produced a smaller amount of saliva as a function of time (0–22 min) compared with $Dlk1^{(+/+)}$ mice. For instance, after 22 min, $Dlk1^{(+/+)}$ males reached a secretion rate of 12 mg saliva/g body weight. By contrast, $Dlk1^{(-/-)}$ males only produced an average of 8 mg/g body weight (Fig. 3a, c). Likewise, WT females reached a saliva secretion rate of 8 mg/g body weight, but the KO female only an average of 5 mg/g body weight (Fig. 3b, d). The rate of secretion between normal $Dlk1^{(+/+)}$ males and females was also different, with females comparatively producing less saliva than males, a finding that we attribute to sexual dimorphism.

Fig. 3 Measurement of saliva production in *Dlk1* [(+/+)] and *Dlk1* [(−/−)] mice. **a, b** Amount of saliva (mg) produced per body weight (g) as a function of time for *Dlk1* [(+/+)] and *Dlk1* [(−/−)] male and female mice, after stimulation with pilocarpine. **c, d** Amount of saliva (mg) produced per body weight (g) after 22 min post-stimulation, for *Dlk1* [(+/+)] and *Dlk1* [(−/−)] male and female mice. *Dlk1* [(−/−)] female and male mice produce less saliva than *Dlk1* [(+/+)] mice. Data are means ± SEM ($n = 5$ each group); unpaired *t*-test, *$P \leq 0.05$
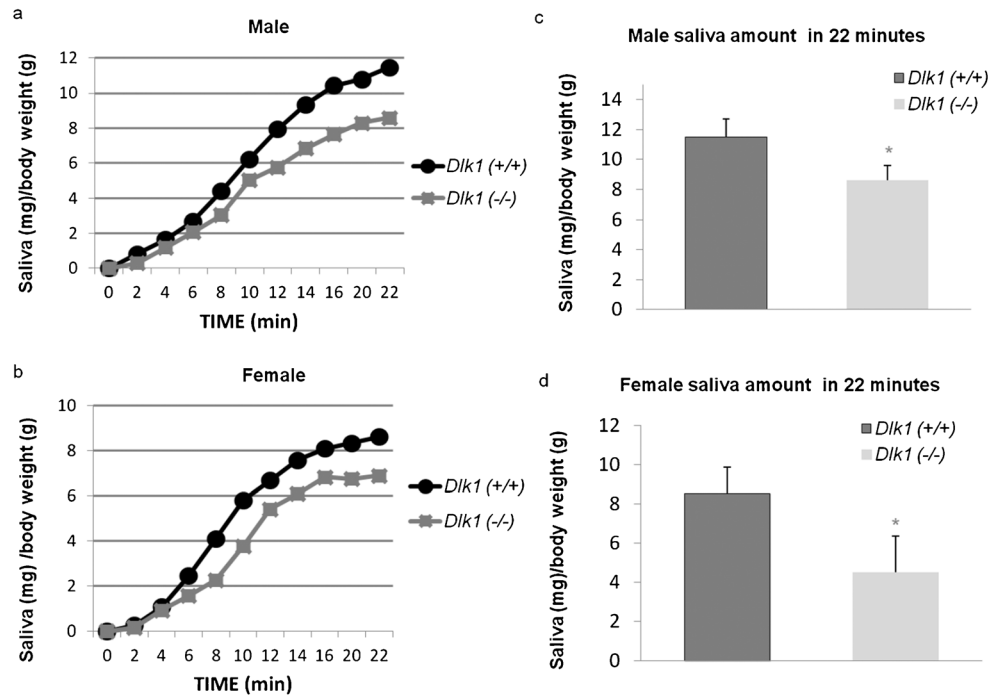
Considering this result, we wished to determine the reason that *Dlk1* [(−/−)] mice produced less saliva. The SMG is the gland that, in rodents, produces the largest amount of saliva. Although we detected no histological anomalies in the acini and tubuli in H/E-stained sections, the possibility remained that SMG secretory units, especially acini and GCTs, had some type of ultrastructural defect, which was not detectable by H/E staining, but which would relate to the reduced saliva secretion in *Dlk1* [(−/−)] mice. GCTs are the main histological feature distinguishing male and female SMGs (Gresik and Barka 1980). Thus, as shown in Fig. 3, saliva secretion is significantly higher in males compared with females, a finding that can be directly attributed to differences in GCT function. We wished to assess whether an alteration in GCT, the surrounding acini, or the striated duct ultrastructure might be responsible for a similar decrease on saliva secretion in *Dlk1* [(−/−)] mice.

To address this question, we visualized *Dlk1* [(+/+)] and *Dlk1* [(−/−)] SMG by TEM, which enabled us to visualize fine cellular details at high magnification. As the sexual dimorphism is very marked in the mouse SMG, we examined males and females separately. TEM images revealed that sexual dimorphism involved differences in GCTs, which were larger and had more secretory vesicles in males (Fig. 4a, b, white arrowheads) than in females. We also observed that the female SMG had larger mitochondria accumulated in the basal side of striated duct cells, compared with the male SMG (Fig. 4g, h, black arrowheads). Striated ducts in the male SMG still show some secretory vesicles in the apical part of the cells, as these ducts are the continuation of GCT. However, we were not able to detect any difference, by

TEM, in the structure of either acinar or ductal cells between *Dlk1* [(−/−)] and *Dlk1* [(+/+)] SMG.

Considering all these data, we attribute the difference in saliva secretion between *Dlk1* [(+/+)] and *Dlk1* [(−/−)] mice to a size difference in the salivary glands themselves. In males, the total amount of saliva produced after stimulation decreases 33.4 % in the knock-out mice, and the weight of the glands is 35.2 % lower than that in controls for these animals. For the females, the reduction in saliva production is 37.5 %, and the weight of the glands is 25.5 % lower in *Dlk1* [(−/−)] mice. These data suggest that both variables, namely salivary gland size and saliva secretion, are effectively related. We assume that the larger the size of the glands, the higher their capacity to secrete saliva. Thus, smaller salivary glands in *Dlk1* [(−/−)] mice lead to a reduction in saliva production (Teshima et al. 2010). Salivary gland size/body weight ratio is a fundamental factor for explaining the higher production of saliva after stimulation in males than in females. Some other authors have also described a gender-dependent difference in saliva flow rate in humans and have attributed this to a difference in salivary gland size (Inoue et al. 2006). Therefore, as salivary glands of *Dlk1* [(−/−)] mice are smaller than those of *Dlk1* [(+/+)], this seems to be the most likely explanation of their production of less saliva.

Innervation and CK5+ epithelial progenitors are normal in *Dlk1* [(−/−)] SMG

Innervation is a crucial factor for regulating not only the physiology and secretion rate of the adult salivary gland, but also its proper organogenesis (Knox et al. 2013; Knosp et al.

2012). During embryonic development, at the branching morphogenesis stages, communication is established between growing axon terminals and the branching salivary epithelium. In the developing SMG, this innervation comes from a local PSG, which is located proximally, next to the main SMG duct, and whose axonal fibers extend through the secondary

ducts finally to reach the end buds where they expand and make contacts with the epithelial cells. Acetylcholine secretion by these nerve terminals is fundamental for the maintenance of epithelial salivary progenitors (Knox et al. 2010), and if the PSG is experimentally removed, salivary glands fail to complete branching morphogenesis. We recently discovered

Fig. 4 Images of granular convoluted tubules (GCT) and striated ducts from adult SMG of *Dlk1* $^{(+/+)}$ and *Dlk1* $^{(-/-)}$ mice. Transmission electron microscopy. Representative images of (**a–d**) *Dlk1* $^{(+/+)}$ and *Dlk1* $^{(-/-)}$ GCT, striated ducts, and some surrounding mixed acini in males and of (**e–h**) *Dlk1* $^{(+/+)}$ and *Dlk1* $^{(-/-)}$ GCT and striated ducts in females. GCT cells show a characteristic accumulation of electrodense secretory vesicles at their apical side (*white arrowheads*); these vesicles appear more prominently in males than in females. Striated duct cells show mitochondria accumulating at their basal side (*black arrowheads*); the mitochondria appear more prominently in females than in males. No differences in tissue ultrastructure were found between SMGs of *Dlk1* $^{(-/-)}$ and $^{(+/+)}$ mice. *Bars* 5 μm
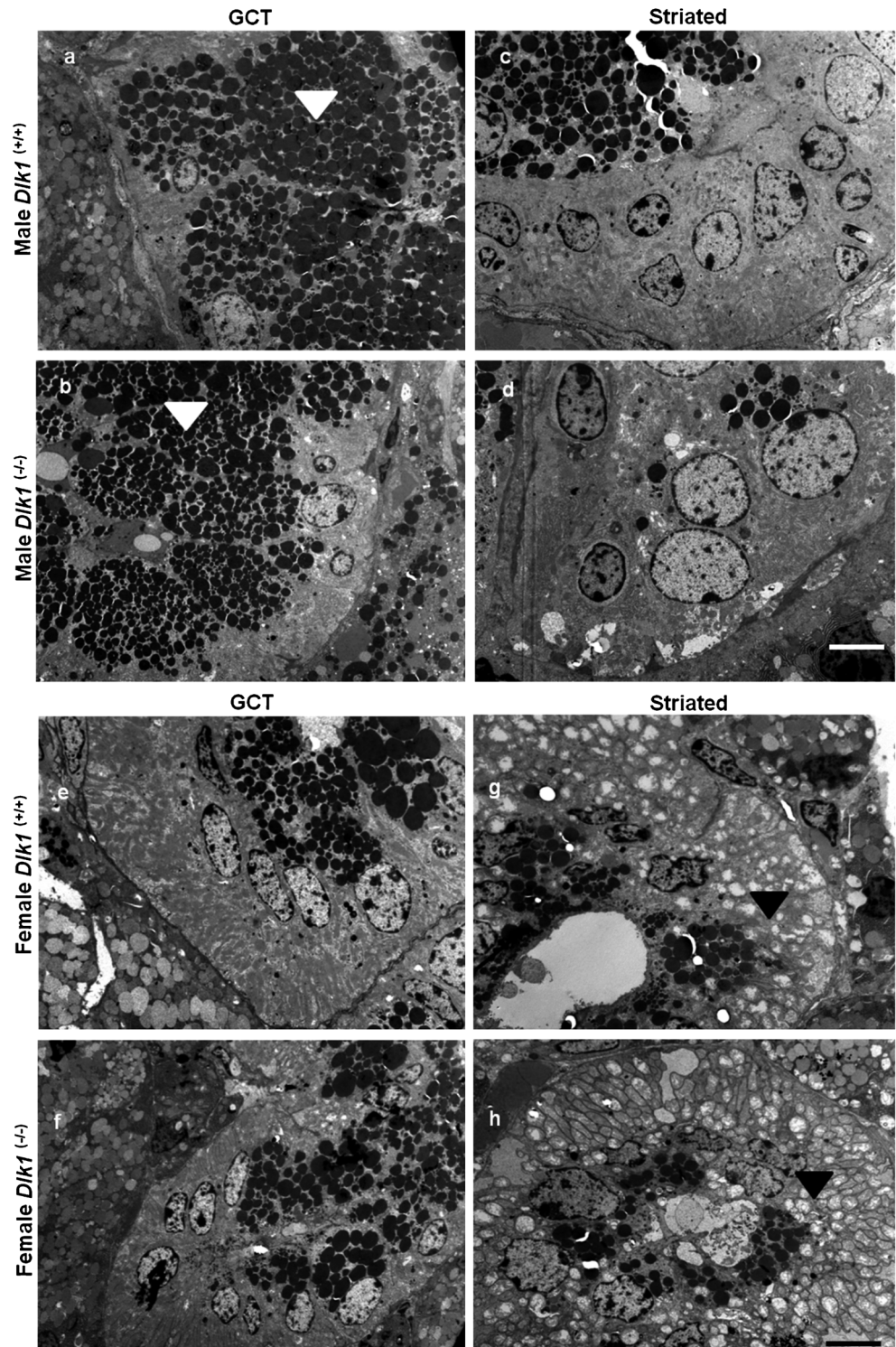
that the application of sDLK1 to mouse SMG organotypic cultures caused a similar effect to the loss of innervation (Garcia-Gallastegui et al. 2014). Thus, sDLK1 blocked branching morphogenesis in the SMG, which was associated with a loss of PSG innervation and a fall in the number of cytokeratin-5-positive (CK5+) epithelial cells. This could not be attributed to any systemic endocrine mechanism, since sDLK1 applications were made in isolated SMG in culture. We found that sDLK1 inhibited the NOTCH pathway in the isolated SMG, and likewise, NOTCH inhibitors mimicked the branching-inhibitory effect of sDLK1 (Garcia-Gallastegui et al. 2014).

Consequently, since sDLK1 affected innervation and the pool of CK5+ epithelial progenitors in isolated SMGs, we decided to analyze both these developmental features in the SMG of $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mice. E16 $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ mouse SMGs were whole-mount immunostained for the PSG axonal marker βIII-tubulin. We observed that axons were properly extended through epithelial end buds at this stage (Fig. 5a, d). Moreover, we immunostained for CK5+ epithelial progenitor cells at E16 (Fig. 5b, e) and P1 (Fig. 5c, f). These cells are typically and most abundantly located in the excretory ducts, as we found for both the E16 and P1 SMG samples. However, no differences were detected, either for PSG innervation (βIII-tubulin) or for epithelial progenitor cell populations (CK5), when we compared the localization and the intensity of immunostaining in $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ SMGs (Fig. 5g, h). Thus, we conclude that the absence of

$Dlk1$ does not affect these developmental characteristics of the SMG in genetically modified mice.

Epithelial progenitor cell marker CK14 is upregulated in $Dlk1^{(-/-)}$ SMG

$Dlk1^{(-/-)}$ mice have smaller salivary glands that produce less saliva but which appear otherwise to be normal. Given that we had not found any histological or ultrastructural anomaly in SMG from $Dlk1^{(-/-)}$ mice, we decided to look for changes in gene expression that might shed light on some aspects of the salivary phenotype in these animals. Thus, we sought comparative changes in the expression of several genes by qPCR (Fig. 6a, b). First, we decided to analyze the expression of aquaporin 5 ($Aqp5$), which codes for a fundamental water channel for saliva secretion. $Aqp5^{(-/-)}$ mice present a drastic decrease in saliva production, with a higher susceptibility to caries and other oral diseases (Culp et al. 2005), and thus we considered $Aqp5$ to be a good marker for salivary gland cell differentiation and functionality. Next, we also analyzed $Fgf10$, a key regulator of salivary gland development (Janebodin et al. 2013; Jaskoll et al. 2005), to address whether a putative upregulation of this gene was involved with compensation in the $Dlk1^{(-/-)}$ SMG. Finally, we also decided to address whether salivary epithelial cell progenitors were present in normal amounts in the $Dlk1^{(-/-)}$ SMG, and for this, we studied the expression of their specific markers $Ck5$ and $Ck14$ (Lombaert and Hoffman 2010). We analyzed all these markers
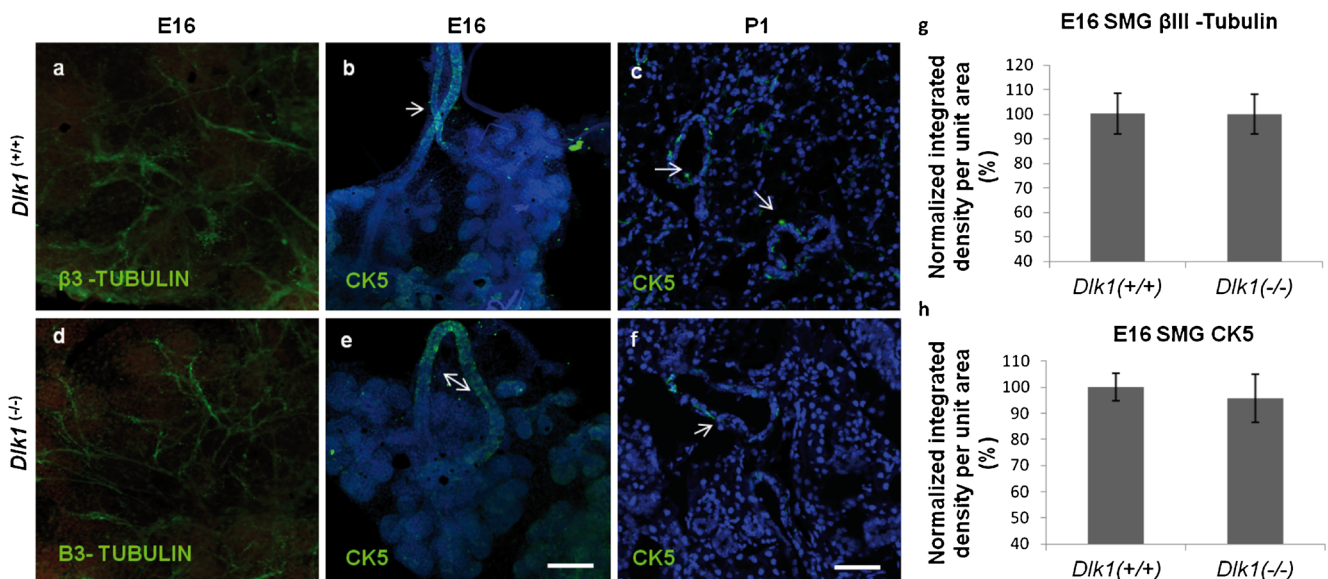
Fig. 5 Immunostaining of parasympathetic ganglia (PSG) innervation and cytokeratin 5 (CK5)-positive progenitor cells in $Dlk1^{(+/+)}$ vs. $Dlk1^{(-/-)}$ SMG. a, d Immunostaining against βIII-tubulin (green) for innervation shows no differences between $Dlk1^{(+/+)}$ and $Dlk1^{(-/-)}$ E16 SMG. b, e Immunostaining for epithelial progenitor cell marker CK5 (green) in E16 SMG from $Dlk1^{(-/-)}$ and $Dlk1^{(+/+)}$ mice. The immunostained cells are located mainly in the excretory ducts (arrows). c, f CK5 marker staining in P1 SMG. The marker is mainly found in the basal epithelial cells of the ducts. g,h Quantification of fluorescent labeling density for βIII-tubulin (a, d) and CK5 (b, e) staining, respectively. Data are means ± SEM. No differences were found between $Dlk1^{(-/-)}$ and $Dlk1^{(+/+)}$ mice. Bars 50 μm (a, d, c, f), 150 μm (b, e)
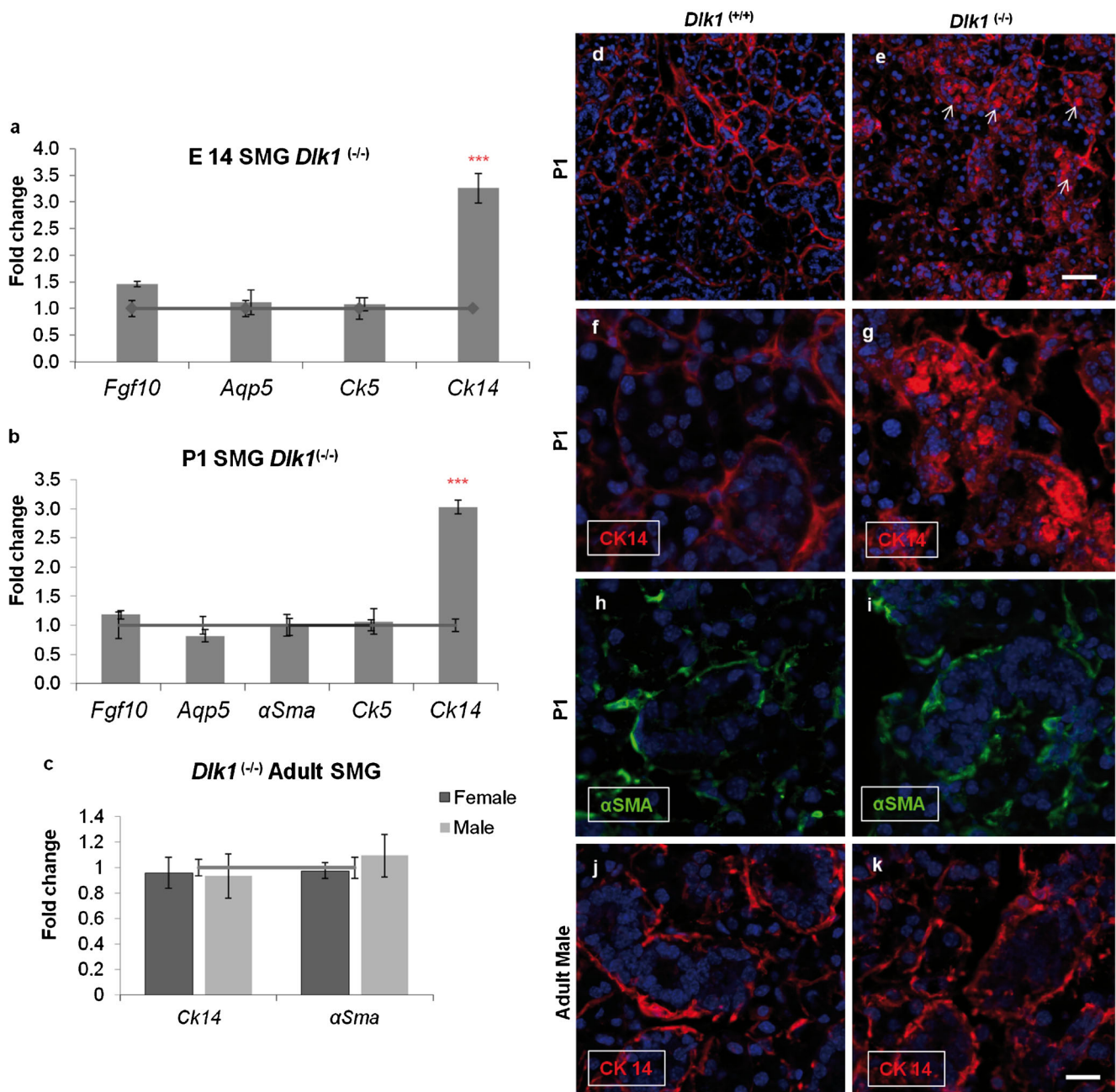
Fig. 6 CK14 progenitor/stem cell marker expression is upregulated during development and early postnatal stages in SMG from *Dlk1* $^{(-/-)}$ mice. Quantitative polymerase chain reaction (qPCR) analysis of gene expression in SMG at (**a**) E14, (**b**) P1, and (**c**) in adult male and female glands. No difference is apparent between *Dlk1* $^{(+/+)}$ and *Dlk1* $^{(-/-)}$ SMG with regard to *Fgf10*, *Aqp5*, *αSma*, and *Ck5* expression. However, *Ck14* mRNA levels are increased about three-fold of the levels in the *Dlk1* $^{(+/+)}$ control SMGs in both the E14 and P1 stages of the *Dlk1* $^{(-/-)}$ mice and return to normal levels in the adult. Data are means ± SEM. Unpaired *t*-test; ***$P \leq 0.05$. **d–g** Immunostaining for CK14 in P1 *Dlk1* $^{(+/+)}$ and

Dlk1 $^{(-/-)}$ SMG. An increased amount of CK14+ epithelial cells (*arrows*) is seen inside the ducts and acini of developing *Dlk1* $^{(-/-)}$ SMG. **h, i** P1 SMG stained for αSMA highlights myoepithelial cells (*green*), and no differences in αSMA immunoreactivity are found between developing SMG from *Dlk1* $^{(-/-)}$ and *Dlk1* $^{(+/+)}$ mice. **j, k** Adult male SMG immunostained for CK14 showing the typical grid-like labeling pattern of the stellate myoepithelial cells surrounding the ducts and acini. No differences in CK14 immunoreactivity are found between adult SMG from *Dlk1* $^{(-/-)}$ and *Dlk1* $^{(+/+)}$ mice. *Bars* 50 μm (**d, e**), 20 μm (**f–k**

by qPCR in SMG at two developmental stages: (1) early in development at E14 and (2) during late stages at P1.

Our results showed no difference in *Aqp5* or *Fgf10* expression in *Dlk1* $^{(-/-)}$ SMG compared with normal SMG, either at

E14 or at P1, suggesting no functional defects in salivary secretion or any compensatory switch mediated by *Fgf10* in the absence of *Dlk1* expression in the SMG. With regard to the epithelial progenitor markers, *Ck5* expression in *Dlk1* $^{(-/-)}$

SMG was similar to that in controls, corroborating the previous results obtained by immunocytochemistry. However, mRNA levels for *Ck14* were increased three-fold in *Dlk1* [(−/−)] SMG, at both developmental stages, namely E14 and P1 (Fig. 6a, b). This striking result prompted us to examine CK14 protein expression by immunocytochemistry in SMG from *Dlk1* [(+/+)] and *Dlk1* [(−/−)] mice. We found that *Dlk1* [(−/−)] SMG possessed more CK14-positive cells (Fig. 6e, g) than WT. Thus, in the WT SMG at P1, CK14 labeling displayed a characteristic grid-like pattern, showing the long and thin cytoplasm of myoepithelial cells surrounding the round acinar and ductal structures (Fig. 6d, f). By contrast, in *Dlk1* [(−/−)] SMGs of the same age, in addition to the typical myoepithelial labeling pattern, we could also identify many other CK14-positive cells, some of them intensely labeled, inside the ducts and the acini.

CK14, apart from being an epithelial progenitor marker, also labels adult myoepithelial cells (Grandi et al. 2000). To verify whether the increase in CK14 protein and gene expression was restricted to progenitor cells or, alternatively, related to an expansion of the myoepithelial cell population, we performed immunostaining of SMG sections for α-smooth muscle actin (αSMA), together with an assessment of *αSma* expression by qPCR in SMG at various developmental stages. Contrary to CK14, αSMA is a specific marker for myoepithelial cells in the salivary gland. We observed that αSMA immunostaining was restricted to the periphery of ducts and acini in SMGs at P1, generating a grid-like staining pattern characteristic of myoepithelial cells, which was partly similar to the one found for CK14 in *Dlk1* [(+/+)] mice. However, in this case, a complete absence of αSMA-positive labeled cells was seen inside the ducts and acini, both in *Dlk1* [(+/+)] and *Dlk1* [(−/−)] samples (Fig. 6h, i), suggesting that the previously detected CK14+ cell population in Dlk1 [(−/−)] SMG was not related to myoepithelial cells. Consistently, qPCR analysis revealed no significant differences in *αSma* expression between SMG from *Dlk1* [(+/+)] and *Dlk1* [(−/−)] mice, either at E14 or at P1, confirming that the expanded CK14+ cell population found in the *Dlk1* [(−/−)] SMG corresponded to genuine salivary progenitor cells.

We wished to assess whether the expansion of salivary progenitor cells would persist and be maintained in the adult salivary gland. To this end, we performed CK14 immunostaining of sections of SMG from adult *Dlk1* [(+/+)] and *Dlk1* [(−/−)] mice, together with a *Ck14* expression analysis of adult male and female SMGs by qPCR. Surprisingly, we found that the expanded non-myoepithelial CK14+ cell population that was detected at P1 had disappeared from adult SMG samples (Fig. 6j, k). CK14 immunostaining gave identical results for both *Dlk1* [(+/+)] and *Dlk1* [(−/−)] adult SMGs, showing a purely myoepithelial labeling pattern in both cases. No increased populations of CK14+ cells could be detected inside the ducts and acini in the SMG of adult *Dlk1* [(−/−)] mice, contrary to our

findings at P1. Furthermore, *Ck14* and *αSma* expression levels in the adult SMG, as assessed by qPCR, were not altered either (Fig. 6c).

Together, these results confirm that the CK14+ epithelial progenitor cell pool is expanded in the SMG of *Dlk1* [(−/−)] mice. However, this effect is transient and restricted exclusively to embryonic development and early postnatal stages and cannot be detected in the adult salivary glands.

As reported elsewhere, CK5 and CK14 label different cell populations of salivary progenitors: whereas CK5 is predominantly a ductal marker, CK14 is more widespread and extends through both ducts and acini, with a peak of expression during the canalicular stage, around E15 (Lombaert and Hoffman 2010). We have no explanation for the findings that *Dlk1* elimination in genetically modified mice affects the one population but not the other or that these changes are restricted to developmental and early postnatal stages. Both *Ck5* and *Ck14* are downregulated during differentiation and salivary gland maturation (Lombaert and Hoffman 2010), and an increase of a particular progenitor population (in this case, CK14+ cells) in the developing salivary gland of *Dlk1* [(−/−)] mice indicates a failure of these salivary progenitor cells properly to differentiate in the absence of DLK1. We are tempted to speculate that a failure in progenitor cell differentiation during development negatively affects the final salivary gland size and ultimately causes a reduction in saliva secretion, as we have observed in *Dlk1* [(−/−)] animals, but this hypothesis remains as yet unproven. Other studies have established that DLK1 is an important regulator of stem cell renewal, and changes in its expression levels are associated with processes such as adipogenesis, angiogenesis, neurogenesis, or hematopoiesis (Al Haj Zen and Madeddu 2012; Ferrón et al. 2011; Mirshekar-Syahkal et al. 2013). In our study, the absence of DLK1 in the SMG leads to an increase in the population of CK14+ progenitor cells, a finding that again points to DLK1 as a regulator of stemness balance.

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