Gli3 in fetal thymic epithelial cells promotes thymocyte positive selection and differentiation by repression of Shh

Anisha Solanki1, Diana C. Yanez1, Susan Ross1, Ching-In Lau1, Eleftheria Papaioannou1, Jiawei Li1, José Ignacio Saldaña1,2 and Tessa Crompton1,*

ABSTRACT

Gli3 is a Hedgehog (Hh)-responsive transcription factor that can function as a transcriptional repressor or activator. We show that Gli3 activity in mouse thymic epithelial cells (TECs) promotes positive selection and differentiation from CD4+CD8− to CD4+CD8+ single-positive (SP4) cells in the fetal thymus and that Gli3 represses Shh. Constitutive deletion of Gli3, and conditional deletion of Gli3 from TECs, reduced differentiation to SP4, whereas conditional deletion of Gli3 from thymocytes did not. Conditional deletion of Shh from TECs increased differentiation to SP4, and expression of Shh was upregulated in the Gli3-deficient thymus. Use of a transgenic Hh reporter showed that the differentiation to SP4, and expression of Shh was upregulated in the Gli3-deficient thymus. Use of a transgenic Hh reporter showed that the Hh pathway was active in thymocytes, and increased in the Gli3-deficient fetal thymus. Neutalisation of endogenous Hh proteins in the Gli3−/− thymus restored SP4 differentiation, indicating that Gli3 in TECs promotes SP4 differentiation by repression of Shh. Transcriptome analysis showed that Hh-mediated transcription was increased whereas TCR-mediated transcription was decreased in Gli3−/− thymocytes compared with wild type.

KEY WORDS: Shh, Gli3, Fetal thymus, Positive selection, CD4, T-cell development, Thymocyte, Thymic epithelial cell (TEC), Mouse

INTRODUCTION

Gli3 is a member of the Hedgehog (Hh)-responsive Gli family of transcription factors, mammalian orthologues of the Drosophila Ci protein (Ramsbottom and Pownall, 2016). The Gli proteins bind DNA in a sequence-specific manner, but have evolved different functions and distinct temporal and tissue-specific expression patterns. Gli3 can be processed to be a repressor of transcription (Gli3R) in the absence of Hh signalling, or an activator (Gli3A) upon Hh signal transduction (Sasaki et al., 1999). During development it can function before the expression of Hh genes, independently of Hh. In many tissues, Gli3R limits Shh signalling, and Gli3 and Shh have opposing functions, and Gli3 deficiency and Shh deficiency result in opposite phenotypes (Hager-Theodorides et al., 2005, 2009; Saldaña et al., 2016). Here, we investigate Gli3 function during αβ T-cell development in the embryonic thymus at the transition from the DP to SP cell.

Maturation from DP to SP follows successful rearrangement of the Tcra locus, and requires TCR signalling: positive selection results in appropriate MHC restriction of SP cells, followed by negative selection of potentially self-reactive clones (Klein et al., 2014; Starr et al., 2003). Many models have been proposed to describe how DP thymocytes commit to the SP4 and SP8 lineages, and how positive selection ensures that selected SP4 and SP8 populations express TCR appropriately restricted by MHCII and MHCII, respectively (Carpenter and Bosselut, 2010; Starr et al., 2003). The strength and duration of the TCR signal that a developing cell receives broadly determine its fate, with the strongest signals leading to negative selection, usually at the SP stage in the medulla (of TCR recognising self antigens), intermediate signals leading to positive selection, and weaker signals or lack of TCR signalling leading to cell death by neglect (Singer et al., 2008). For DP thymocytes undergoing positive selection, again TCR signal strength and duration influence SP4 and SP8 lineage choice. Those cells receiving stronger longer TCR signals tend towards the SP4 fate, weaker/more transient signals favour differentiation to SP8 SP, and additionally SP4/SP8 fate decisions may be influenced by the relative timing of cytokine signalling and TCR signalling that a developing cell receives (Bosselut, 2004; Klein et al., 2014; Starr et al., 2003). TCR signal strength and duration are dependent on avidity of the TCR for its ligand (and therefore on the TCR sequence), and may also be affected by other intracellular or extracellular influences on TCR signal transduction, in addition to cytokines. Thus, local thymic stromal factors, including Notch and morphogen signalling, may also influence SP lineage choice and selection (Brugnera et al., 2000; Crompton et al., 2007; Laky and Fowlkes, 2008; Park et al., 2010; Takahama, 2006). Several lineage-specific transcription factors are required for the SP4/SP8 lineage decision, including ThPok (Zbtb7b), Gata3, Runx1, Runx3 and Mazr (Carpenter and Bosselut, 2010; Naito et al., 2011). The ways in which the transcriptional regulation of lineage commitment and differentiation relate to extracellular signalling molecules and TCR signal transduction require further study.

In the thymus, Shh is expressed by TECs in the medulla and corticomedullary junction, and is required for normal medullary TEC development and maturation (El Andaloussi et al., 2006; Outram et al., 2000; Sacedón et al., 2003; Saldaña et al., 2016).
TECs provide MHC peptide ligands for developing thymocytes and are required for both positive and negative selection of the TCR repertoire (Klein et al., 2014).

Gli3R can suppress Hh pathway activation by at least two mechanisms. First, it can repress the expression of Hh genes in the Hh-secreting cell, hence reducing the overall Hh protein.

Fig. 1. See next page for legend.
Fig. 1. T-cell development in Gli3<sup>+/−</sup>, Gli3<sup>−/−</sup> and Gli3<sup>−/-</sup> E17.5 plus 4 days in FTOC and E18.5 thymus. Flow cytometry analysis. Scatter plots show means±s.e.m. Each point represents thymus from an individual mouse embryo. (A-H) E17.5 FTOC for 4 days. Gli3<sup>−/−</sup> (n=14), Gli3<sup>−/−</sup> (n=14) and Gli3<sup>−/-</sup> (n=11). (I,J) E18.5 Gli3<sup>−/−</sup> (n=3) and Gli3<sup>−/-</sup> (n=3) thymus. (A) CD8 against CD4. (B) Percentage of populations (relative to mean of WT) giving significance by Student’s t-test compared with WT for SP4 (Gli3<sup>−/−</sup>, P<0.03; Gli3<sup>−/-</sup>, P<0.001), SP8 (Gli3<sup>−/−</sup>, P<0.03) and SP4/SP8 ratio (Gli3<sup>−/−</sup>, P<0.02; Gli3<sup>−/-</sup>, P=0.0006). (C) CD3 staining on Gli3<sup>−/−</sup>, Gli3<sup>−/-</sup> and Gli3<sup>−/-</sup> thymocytes, giving the percentage of CD3<sup>hi</sup> cells. (D) Percentage of CD3<sup>hi</sup> thymocytes in Gli3<sup>−/−</sup>, Gli3<sup>−/-</sup> and Gli3<sup>−/-</sup> (relative to mean of WT littermate). (E) CD8 against CD4, gated on Gli3<sup>−/-</sup>. (F) Percentage of populations gated on CD3<sup>−</sup>, giving significance by Student’s t-test compared with WT for CD3<sup>−</sup> SP4 (Gli3<sup>−/−</sup>, P=0.002; Gli3<sup>−/-</sup>, P=0.0004), CD3<sup>−</sup> SP4/SP8 ratio (Gli3<sup>−/−</sup>, P=0.002) and CD3<sup>−</sup> DP (Gli3<sup>−/−</sup>, P=0.05; Gli3<sup>−/-</sup>, P=0.02). (G) HSA against Qa2 expression, gated on SP4 cells from Gli3<sup>−/−</sup> and Gli3<sup>−/-</sup>. (H) Relative percentage of HSA + Qa2<sup>+</sup> cells in the SP4 population, giving significance by Student’s t-test compared with WT (P<0.002). (I) Percentage of CD69<sup>+</sup> cells in SP4 and DP populations, giving significance by Student’s t-test for SP4 (P<0.07) and DP (P<0.05). (J) CD69 expression on SP4 and DP cells in a representative experiment.

concentration in a tissue. Second, expression of Gli3 in the signal-receiving cell will be processed to Gli3R in the absence of Hh proteins, which will transcriptionally repress Hh target genes.

In the thymus stroma, Gli3 has both Hh-independent and Hh-dependent functions, and Gli3 deficiency leads to Hh-dependent upregulation of the Hh target gene Gli1 (Hager-Theodorides et al., 2009). In the fetal thymus, Gli3 deficiency leads to reduced pre-TCR-mediated signalling and transcription. This culture period was significantly increased in mutant thymus compared with WT (Fig. 1G,H). CD69 expression was significantly decreased in SP4 thymocytes in the Gli3<sup>−/−</sup> thymus compared with WT (Fig. 1J), indicating that fewer DP cells were undergoing positive selection, and consistent with the overall reduction in SP4 maturation. CD69 expression was also on average lower on the Gli3<sup>−/−</sup> SP4 population than WT, and there was greater variation in expression levels in Gli3<sup>−/−</sup> compared with WT, suggesting dysregulated maturation in the absence of Gli3.

Positive and negative selection and SP4/8 lineage commitment are determined by many factors, including transcriptional regulators of signal transduction and the TCR signal strength itself. A longer and stronger TCR signal promotes differentiation towards SP4, whereas a weaker signal favours SP8 (Bosselut, 2004). Since Gli3 deficiency suppressed lineage commitment towards SP4 cells and biased the SP4/SP8 ratio, we investigated whether the TCR signal strength was affected in the Gli3<sup>−/−</sup> mutant FTOC by measuring cell-surface CD5 expression, which correlates with TCR and pre-TCR signal strength (Azzam et al., 2001, 1998). The mean fluorescence intensity (MFI) of CD5 on the DP, SP4 and SP8 cells was significantly decreased in the Gli3<sup>−/−</sup> thymus compared with WT (Fig. 2A-C). This suggested that reduced TCR signal strength might be one factor responsible for the decreased commitment to SP4 in the Gli3 mutant.

**RESULTS**

**Impaired development of mature SP4 T-cells in the Gli3 mutant thymus**

Gli3 deficiency is embryonic lethal, so to investigate whether Gli3 is required for differentiation of thymocytes from the DP to SP stage, we cultured wild-type (WT) and Gli3 mutant E17.5 mouse fetal thymus organ culture (FTOC) for 4 days and assessed changes in developmentally regulated cell-surface markers. This culture period enabled us to measure the rate of differentiation of the mature SP populations, as they are first produced. We observed a significant gene dose-dependent decrease in the proportion of SP4 cells and in the SP4:SP8 ratio in the Gli3 mutant FTOC compared with WT (Fig. 1A,B). The SP4 population was TCRβ<sup>+</sup> and TCRγδ<sup>+</sup> in both WT and Gli3 mutant thymus (Fig. S1), consistent with normal divergence of the γδ lineage at the DN stage in the Gli3<sup>−/-</sup> thymus (Vantourout and Hayday, 2013). The proportion of CD4<sup>+</sup> CD8<sup>+</sup> cells was significantly increased in Gli3<sup>−/-</sup> compared with WT (Fig. 1A,B). This suggests that efficient differentiation from DP to SP4 cells required Gli3, and that Gli3 deficiency favoured lineage commitment to SP8 over SP4. However, the embryonic CD8<sup>+</sup> CD4<sup>+</sup> population also contains immature single positive (ISP) cells, so we gated on the CD3<sup>hi</sup> population, and analysed the distribution of DP and SP thymocytes. We found no significant difference in the proportion of CD3<sup>hi</sup> thymocytes between WT, Gli3<sup>−/-</sup> and Gli3<sup>−/-</sup> (Fig. 1C,D). Gating on CD3<sup>hi</sup> cells confirmed the requirement for Gli3 for normal differentiation from CD3<sup>hi</sup> DP to CD3<sup>hi</sup> SP4 cell, as the proportion of CD3<sup>hi</sup> DP cells was significantly increased and the proportion of CD3<sup>hi</sup> SP4 cells was significantly decreased, whereas the proportion of the CD3<sup>hi</sup> SP8 population was not significantly different between the three genotypes of embryo (Fig. 1E,F).

In order to dissect further the stages of maturity affected by Gli3, we stained thymocytes for the surface markers CD69, HSA (CD24) and Qa2. DP thymocytes express high levels of HSA and then acquire CD69 expression as a result of TCR signalling for positive selection (Ge and Chen, 1999). Newly positively selected SP thymocytes also express high levels of HSA and CD69, and as they mature they downregulate HSA and CD69 and gain expression of Qa2 (Ge and Chen, 1999; Weinreich and Hogquist, 2008).

The proportion of mature HSA + Qa2<sup>+</sup> cells in the SP4 population, although low in both genotypes, was significantly decreased in Gli3<sup>−/-</sup> compared with WT (Fig. 1G,H). CD69 expression was significantly decreased on DP thymocytes in the Gli3<sup>−/-</sup> thymus compared with WT (Fig. 1J), indicating that fewer DP cells were undergoing positive selection, and consistent with the overall reduction in SP4 maturation. CD69 expression was also on average lower on the Gli3<sup>−/−</sup> SP4 population than WT, and there was greater variation in expression levels in Gli3<sup>−/−</sup> compared with WT, suggesting dysregulated maturation in the absence of Gli3.

**Increased Shh signalling in Gli3 mutant thymocytes**

The Gli3 mutant fetal thymus has increased expression of the Hh target gene Gli1 in stroma (Hager-Theodorides et al., 2009), indicating that, overall, Gli3 acts as a repressor of Hh pathway activation in the stroma. Since Gli3 can repress Shh expression by repression of an intermediate transcriptional activator of Shh in other tissues (te Welscher et al., 2002; Rowbotham et al., 2007; Saldaña et al., 2016; Shah et al., 2004), we tested whether more Shh protein was present in the Gli3-deficient fetal thymus compared with WT by ELISA. Shh protein was significantly increased in Gli3<sup>−/−</sup> compared with WT (Fig. 2D). To test if Shh was signalling directly to developing T-cells, we used Gli binding site (GBS)-GFP transgenic (tg) reporter mice to measure active Hh-dependent transcription in DP, SP4 and SP8 populations in the Gli3 mutants (Fig. 2E-I). The GBS-GFP-tg express GFP when activator forms of Gli proteins bind to the GBS transgene (Balaskas et al., 2012). We observed significant increases in GFP expression in SP4, SP4 and SP8/ISP populations in GBS-GFP-tg Gli3 mutants compared with GBS-GFP-tg Gli3<sup>+/−</sup> (Fig. 2E,F). The MFI of
GFP on the SP4, DP and SP8 populations was significantly higher in Gli3−/− than in Gli3+/+ (Fig. 2G). Gating on CD3hi thymocytes, the proportion of GFP+ mature CD3hi SP4 cells was significantly higher in the Gli3 mutants compared with Gli3+/+, and the MFI of CD3hi SP4 and CD3hi SP8 cells was also significantly increased (Fig. 2H,I). This increase in GFP expression showed that Hh pathway activation is
Fig. 2. Expression of CD5 on thymocytes, ELISA for Shh in E17.5 thymus, and detection of Hh pathway activation in E17.5 WT and Gli3 mutant thymocytes. (A-C) Flow cytometry analysis of E17.5 FTOC +4 days, with scatter plots showing relative mean + s.e.m., where each point represents thymus from an individual embryo from Gli3+/− (WT; n=14, circles), Gli3−/− (n=14, squares), Gli3−/− (n=11, triangles). (A) CD5 staining on DP, SP4 and SP8 populations, showing MFIs from a representative experiment. (B) MFI of CD5 on SP4, DP and SP8 populations, showing significance by Student’s t-test compared with WT for SP4 (Gli3+/−, P<0.007; Gli3−/−, P<0.004), DP (Gli3+/−, P<0.001) and SP8 (Gli3+/−, P<0.004). (C) MFI of CD5, gated on CD3hi on SP4, DP and SP8, giving significance by Student’s t-test for CD3hi SP4 (Gli3+/−, P<0.03), CD3hi DP (Gli3+/−, P<0.04) and CD3hi SP8 (Gli3+/−, P<0.04). (D) Shh protein measured by ELISA in Gli3+/− (n=3) and Gli3−/− (n=3) E17.5 thymus (P<0.003). (E-I) Flow cytometry analysis of GFP expression in SP4, DP and SP8 populations from GBS-GFP-tg E17.5 FTOC +4 days from Gli3+/− (n=3), Gli3−/− (n=4) and Gli3−/− (n=3) littermates. Scatter plots show relative mean + s.e.m., where each point represents thymus from a different embryo. (E) SSC versus GFP, gated on SP4 (top row), DP (middle row) and SP8 (bottom row) cells from Gli3+/− (left), Gli3−/− (middle) and Gli3−/− (right) littermates, with percentage in region shown. (F) Percentage GFP+, giving significance by Student’s t-test compared with WT for SP4 (Gli3+/−, P<0.004), DP (Gli3+/−, P<0.003) and SP8 (Gli3−/−, P=0.02; Gli3+/−, P=0.02). (G) MFI of GFP, giving significance by Student’s t-test compared with WT for SP4 (Gli3+/−, P<0.003; Gli3−/−, P<0.02), DP (Gli3+/−, P<0.04) and SP8 (Gli3+/−, P<0.004). (H) Percentage of GFP+ cells, gated on CD3hi, giving significance by Student’s t-test compared with WT for SP4 (Gli3+/−, P<0.003; Gli3−/−, P<0.01) and CD3hi SP8 (Gli3+/−, P<0.05). (I) MFI of GFP, gated on CD3hi, giving significance by Student’s t-test compared with WT for SP4 (Gli3+/−, P<0.01) and SP8 (Gli3+/−, P<0.01; Gli3−/−, P=0.01).

increased in thymocytes in Gli3−/− and therefore suggests that the increased Shh protein level is signalling directly to developing T-cells in the Gli3 mutant thymus.

**Attenuation of Hh signalling in Gli3−/− thymus reverses the decrease in the SP4 population**

Since the Gli3-deficient thymus has increased Hh signalling, loss of Gli3 in the thymus could cause changes that are directly dependent on the increase in the Hh signal or, alternatively, that are dependent on Gli3 but independent of the increase in Hh pathway activation. To investigate whether the differences in the Gli3−/− thymus were directly due to increased Hh signalling, we attenuated Hh signalling by treatment with recombinant (r) Hhip to neutralise endogenous Hh proteins in FTOC. As expected, rHhip-treated WT FTOC had a higher proportion of SP4 and SP8 cells but a decreased percentage of DP cells than untreated controls (Fig. 3A,B) (Lau et al., 2017). rHhip-treated Gli3−/− FTOC had a significantly higher proportion of SP4 cells (Fig. 3A,B). The mature CD3hi SP4 and CD3hi SP8 populations were significantly increased and the CD3hi DP population decreased in the rHhip-treated WT FTOC relative to their controls, whereas in the rHhip-treated Gli3−/− FTOC, although the mean proportional change in both the CD3hi SP4 and CD3hi SP8 populations was greater than in the WT, only the increase in the CD3hi SP4 was significant, and variability was greater (Fig. 3C,D). This increased variability in response to Hh neutralisation in the Gli3−/− compared with WT suggested that Gli3 might be required for normal interpretation of changes in the Hh signal.

Neutralisation of Hh proteins also increased cell-surface CD5 expression in WT FTOC (Fig. 3E-G). As expected, the highest cell-surface CD5 expression was observed in the SP4 population in all cultures, and gating on CD3hi cells showed that the CD3hi DP populations expressed lower levels of cell-surface CD5 than the WT CD3hi SP4 and CD3hi SP8 populations (Fig. 3E). Interestingly, rHhip treatment significantly increased the MFI of CD5 on the CD3hi DP population in the Gli3−/− FTOC, whereas in WT FTOC MFI of CD3hi SP4 and CD3hi SP8 populations was significantly increased (Fig. 3E-G).

These experiments suggest that the decrease in SP4 differentiation in the Gli3−/− FTOC at this developmental transition is a direct result of the increase in Shh, but that Gli3 might also be required to respond to changes in the Shh signal. Consistent with this, previous studies showed that Shh treatment of WT FTOC decreases the SP4 population, the SP4:SP8 ratio and cell-surface CD5 expression, and that in mature T-cells constitutive activation of Gli2-mediated transcription reduces TCR signal transduction (Furmanski et al., 2015, 2012; Rowbotham et al., 2007). By contrast, constitutive loss of Shh, Gli1 or Gli2 from fetal thymus increases differentiation from the DP to SP stage (Drapko-Boulot et al., 2010; Rowbotham et al., 2007).

**Gli3 expression in TECs plays a key role in T-cell development**

We next tested if the changes in thymocyte selection and maturation were the result of the activity of Gli3 expressed in TECs, rather than of cell-intrinsic Gli3 activity in developing thymocytes. We compared fetal thymocyte development in conditional knockouts, in which Gli3 is conditionally deleted from TECs (Gli3fl/fl FoxN1Cre+), and in which Gli3 is specifically deleted from all haematopoietic cells including all thymocytes (Gli3fl/fl VavCre+). Most interestingly, most of the changes observed in the Gli3-deficient thymus were due to loss of Gli3 expression from TECs.

We first analysed thymocyte development at E17.5, the day on which mature SP4 cells first arise. Conditional deletion of thymocyte-intrinsic Gli3 in fresh E17.5 Gli3fl/fl VavCre+ thymus did not result in significant changes in the proportion of thymocyte populations or in cell-surface CD5 expression compared with littermate control thymus (Fig. 4A-D). By contrast, conditional deletion of Gli3 from TECs in Gli3fl/fl FoxN1Cre+ embryos resulted in a significant decrease in the differentiation of SP4 cells (Fig. 4E,F). On E17.5, in a significant reduction in the emerging SP4 population, we observed a significant decrease in the DP population, and a concomitant increase in the DN population, as previously described in the constitutive Gli3−/− thymus (Hager-Theodorides et al., 2005) (Fig. 4E,F). Cell-surface CD5 expression was significantly lower on the DP and DN cells in Cre+ than in the control, indicating lower pre-TCR and/or TCR signal strength (Fig. 4G,H) (Azzam et al., 1998; Rowbotham et al., 2009; Sahni et al., 2015).

We then cultured E17.5 Gli3fl/fl FoxN1Cre+ and Gli3fl/fl FoxN1Cre FTOCs for 4 days to investigate the rate of differentiation at the transition from DP to SP4 cells (Fig. 4I-M). Conditional deletion of Gli3 from TECs resulted in a significant decrease in the proportion of SP4 cells and in the SP4:SP8 ratio, while the proportion of DP cells significantly increased compared with the Cre− littermate control (Fig. 4I,L). Gating on CD3hi cells, we observed a significant decrease in the SP4 population and in the SP4:SP8 ratio (Fig. 4J,L). Cell-surface CD5 expression was significantly decreased in the DP population from Gli3fl/fl FoxN1Cre Cre FTOC compared with Cre− littermates (Fig. 4M). By contrast, FTOC from Gli3fl/fl VavCre+ showed no differences in the rate of differentiation, distribution of thymocyte subsets, or cell-surface CD5 expression compared with control, confirming the importance of Gli3 expression in TECs, rather than in the haematopoietic compartment of the thymus, for the normal regulation of thymocyte differentiation (Fig. 4N-R).

Since constitutive loss of Gli3 led to Hh-dependent changes in thymocyte differentiation and maturation, we tested whether the changes that resulted from conditional deletion of Gli3 specifically from TECs were also Hh dependent. We treated the Gli3fl/fl
FoxN1Cre<sup>+</sup> FTOC with Hhip and observed a significant increase in the SP4 population and a significant decrease in the DN population compared with untreated FTOC (Fig. 5A,B). Gating on CD3<sup>hi</sup> cells also showed that the Hhip-treated Gli3<sup>fl/fl</sup> FoxN1Cre<sup>+</sup> FTOC had an increased proportion of CD3<sup>hi</sup> DP cells compared with untreated controls (Fig. 5C,D). The MFI of CD5 on SP4 and DP cells in the Hhip-treated Gli3<sup>fl/fl</sup> FoxN1Cre<sup>+</sup> FTOC was significantly increased compared with the untreated control (Fig. 5E,F). This was in contrast to the effect of Hhip treatment on constitutive Gli3<sup>−/−</sup> FTOC, in which, although Hhip treatment increased the proportion of SP4 cells, it only increased the MFI of anti-CD5 staining in the CD3<sup>hi</sup> DP population (Fig. 3G). As thymocytes in the Gli3<sup>fl/fl</sup> FoxN1Cre<sup>+</sup> FTOC express Gli3, whereas those in the Gli3<sup>−/−</sup> FTOC do not, this difference suggests that Gli3 activity in developing fetal thymocytes is also required for interpretation of the change in the Shh signal, when the high Shh signal caused by Gli3 deficiency is neutralised by Hhip treatment.

Conditional deletion of Shh from TECs (Shh<sup>fl/fl</sup> FoxN1Cre<sup>+</sup>) led to the opposite phenotype to conditional deletion of Gli3 from TECs (Fig. 5G-L). There was a significant increase in the proportion of SP4 cells and in the SP4:SP8 ratio, but a significant decrease in the DP population compared with Cre<sup>−</sup> control FTOC (Fig. 5G,H). The proportion of mature CD3<sup>hi</sup> SP4 cells was also significantly higher, whereas the proportion of CD3<sup>hi</sup> DP cells was significantly lower, in Shh<sup>fl/fl</sup> FoxN1Cre<sup>+</sup> FTOC compared with Cre<sup>−</sup> controls (Fig. 5G,I). Treatment of Shh<sup>fl/fl</sup> FoxN1Cre<sup>+</sup> FTOC with rShh reversed this phenotype.
Fig. 4. See next page for legend.
Fig. 4. Gli3 expression in TECs and not thymocyte-intrinsic Gli3 expression is required for SP4 development. (A–D) Flow cytometry of fresh E17.5 Gli3fl/fl FoxN1Cre− control (n=7) and Gli3fl/fl VavCre− (n=6) thymus. Scatter plots show relative mean±s.e.m., where each point represents thymus from a different embryo for control littermate and Gli3fl/fl VavCre−. (A) CD4 and CD8 expression. (B) Relative percentage of thymocyte subsets. (C) Relative MFI of anti-CD5 staining on thymocyte subsets. (D) Representative histogram showing anti-CD5 staining on the DP population in control (solid line) and Gli3fl/fl VavCre− (dotted line), showing MFIs. No significant differences were found between control and Cre− thymus. (E–H) Flow cytometry of fresh E17.5 thymus from Gli3fl/fl FoxN1Cre− (control, n=4) and Gli3fl/fl FoxN1Cre− (n=4) littermates. Scatter plots show relative mean±s.e.m., where each point represents thymus from a different embryo for control and Gli3fl/fl FoxN1Cre−. (E) CD8 against CD4. (F) Relative mean percentage of SP4, DP, SP8 and DN populations, giving significance by Student’s t-tests compared with control littermate for SP4 (P<0.01), DP (P<0.05) and DN (P<0.05). (G) Representative histogram shows CD5 staining on DP thymocytes from control (Cre−, solid line) and Cre− (dotted line), MFI of CD5 fluorescence is given. (H) Relative mean MFI of CD5 on CD5 on SP4, DP, SP8 and DN populations. Differences are significant between Cre+ and control for DP (P<0.03) and DN (P<0.05). (E–H) Flow cytometry analysis of E17.5 FTOC +4 days from Gli3fl/fl FoxN1Cre− (n=8) and Gli3fl/fl FoxN1Cre− (n=10) littermates. Scatter plots show relative mean±s.e.m., where each point represents thymus from a different embryo for control FTOC and Gli3fl/fl FoxN1Cre− FTOC. (I) CD8 against CD4. (J) CD8 against CD8, gated on CD3hi. (K) Relative percentage of SP4, DP and SP8 populations and SP4:SP8 ratio, with significance by Student’s t-tests compared with Cre− littermate for SP4 (P<0.02), DP (P<0.007) and SP4:SP8 ratio (P<0.05). (L) Relative percentage of thymocyte populations and CD4:CD8 ratio, gated on CD3hi, with significance compared with Cre− littermate, for Gli3fl/fl SP4 (P<0.05) and Gli3fl/fl SP4:SP8 ratio (P<0.04). (M) Relative MFI of CD5 on thymocyte populations, with significance compared with Cre+ for DP (P<0.007). (N) Flow cytometry of E17.5 FTOC +4 days from control Gli3fl/fl VavCre− (n=7) and Gli3fl/fl VavCre− (n=6) littermates. No significant differences were found between Cre+ and Cre−. (N) CD4 against CD8. (O) CD4 against CD8, gated on CD3hi. (P) Relative percentage of SP4, DP and SP8 populations and SP4:SP8 ratio. (Q) Gated on CD3hi, relative percentage of SP4, DP and SP8 populations and SP4:SP8 ratio. (R) MFI of CD5, gated on thymocyte populations.

effect and significantly reduced differentiation to SP4 and CD3hi SP8 but increased the proportion of CD3hi DP cells (Fig. 5J-L).

Transcriptional mechanisms at the DP to SP transition in the Gli3-deficient thymus

In order to understand the transcriptional mechanisms by which Gli3 regulates the DP to SP transition and thymic selection, we performed microarrays on FACS-sorted CD69− DP, CD69− DP and SP thymocytes from E18.5 WT and Gli3 mutant thymus (Fig. 6A-C, Fig. S2; GEO accession GSE87499). We identified differentially expressed genes (DEGs) by EBayes between Gli3+/− and WT samples for each sorted population, and used principal component analysis (PCA) to investigate the datasets in an unbiased way (Fig. S2, Tables S1 and S2). For each sorted population dataset, we intersected the 2000 most significant DEGs with the 4000 genes that contributed most to the principal component (PC) axis in which Gli3+/− samples segregated from WT, in order to identify genes of interest.

PCA on the CD69− DP microarray data segregated the samples by genotype on PC2, which accounted for 19% of variability (Fig. 6A, Fig. S2A). Interestingly, genes that contributed to PC2, which were higher in the Gli3+/− samples than in WT, included many Hh signalling and target genes, consistent with increased Hh signalling in Gli3+/−. These included Hoxd13, Hoxa13, Hoxd8, Smoc2, H19, S100a9 and Ihh (Chan et al., 2014; Lu et al., 2015; Mandhan et al., 2006; Outram et al., 2009; Pazin and Albrecht, 2009). Runx3 and Notch1, which both promote SP8 differentiation over SP4, also contributed to PC2, with higher expression in Gli3+/− than in WT (Bosselut, 2004; Fowlkes and Robey, 2002).

Genes that contributed to PC2, which were more highly expressed in WT samples, included genes important in selection and SP4 maturation, including Map3k1, which is required for positive selection (Germain, 2002; Xue et al., 2008) (Fig. 6A).

The intersection analysis of the CD69− DP data highlighted 1083 genes, including the Hh target genes Fgf1 and Kit24, the Wnt pathway activator BcI91 (Martin, 1998; Sarkar et al., 2010), and Cldn3, which is induced upon a lower affinity TCR-MHC interaction (Puls et al., 2002). These were more highly expressed in Gli3+/− than in WT (Fig. 6A, Table S2). In addition, many genes involved in TCR signalling and apoptosis during repertoire selection were identified, including Fas, Tcerg1, Libr, Pien, Camk4 and Cifc (Castro et al., 1996; Heath et al., 2008; Krebs et al., 1997; Montes et al., 2015; Zhu et al., 2010), all of which showed lower expression in Gli3+/− than in WT.

PCA on the microarray data from the CD69+ DP population, which are cells that have received the TCR signal for positive selection (Ge and Chen, 1999), segregated the Gli3+/− samples from the WT on PC3, which accounted for 20% of variability (Fig. 6B, Fig. S2B). Again, Hh signalling and target genes, such as Foxa1, Klf3a, Hdac2 and Tgb2, contributed to this PC, with higher expression in Gli3+/− than in WT (Furmanski et al., 2013; Katoh and Katoh, 2009) (Fig. 6B). By contrast, genes that contributed to PC3 and were more highly expressed in WT than in Gli3+/− again included several genes involved in selection and SP4 commitment, including: Egr2, which is downstream of TCR signalling and involved in positive selection (Lauritsen et al., 2008); and Gata3, an SP4 commitment gene (Hernández-Hoyos et al., 2003).

The intersection analysis of the CD69+ DP data identified 1150 genes (Fig. 6B), including some known Hh targets, which were more highly expressed in Gli3+/− than WT, such as Hoxa7, Bmp2, Tulp3, Itgav and H19 (Chan et al., 2014). Many genes crucial for thymic selection showed lower expression in Gli3+/− than in WT, including: Ikb, a tyrosine kinase downstream of TCR that is required for positive selection and the SP4:SP8 lineage decision (Lucas et al., 2003); Egr1, which is downstream of TCR signalling and involved in positive selection (Lauritsen et al., 2008); and Socs1, the deficiency of which promotes SP8 differentiation (Ilangumaran et al., 2010); Anxa5, which promotes apoptosis during negative selection (Rosenbaum et al., 2011); and Cldn6, a co-stimulatory molecule that interacts with its ligand on TECs to promote differentiation to SP (Singer et al., 2002).

Finally, we carried out PCA and DEG analysis of the SP4 microarray data. PCA showed that the Gli3+/− SP4 data segregated from the WT on PC2, which accounted for 22% of variability (Fig. 6C, Fig. S2). The intersection analysis of the SP4 data highlighted 703 genes. Egr2 and Nab2, which are required for selection, Anxa6, which regulates selection-related apoptosis, and Lef1 and Tocx, which promote SP4 lineage commitment and maturation, were expressed at lower levels in Gli3+/− than in WT (Collins et al., 2006; Rosenbaum et al., 2011). Nr4a1, Nr4a3 and Cld5, which are transcriptional targets of TCR signal transduction with a level of expression that correlates with TCR signal strength, were also lower in Gli3+/− than in WT (Moran et al., 2011; Sekiya et al., 2013). Several Hh target genes, such as Itgav, Smmn1, Itgax and Nedd9, were more highly expressed in Gli3+/− than in WT (Aquino et al., 2009; Lu et al., 2015) (Fig. 6C).

Since Hh target genes were upregulated in the Gli3+/− CD69+ DP population (which is undergoing positive selection), and the FTOC experiments suggested that the reduced DP to SP4 transition in the Gli3+/− thymus was due to increased Hh signalling, we tested...
whether the transcription of genes required for differentiation to SP could be modulated by rShh treatment. We chose *Egr2* and *Tox* for this experiment because both were expressed at very similar low levels in WT and *Gli3*−/− in the CD69+ DP dataset, but upregulated in the CD69+ DP and SP4 populations. Furthermore, both transcripts were significantly lower in the *Gli3*−/− CD69+ DP and
Fig. 5. Neutralisation of Hh in Gli3<sup>fl/fl</sup> FoxN1Cre<sup>+</sup> FTOC and conditional deletion of Shh from TECs increase SP4 differentiation. (A-L) Flow cytometry analysis with scatter plots showing relative mean±s.e.m., where each point represents thymus from a different embryo. (A-E) E17.5 Gli3<sup>fl/fl</sup> FoxN1Cre<sup>+</sup> FTOC treated with rHhip for 4 days (n=7) compared with the untreated lobe (control) from the same thymus (n=7). (A) CD8 against CD4. (B) Percentage of thymocyte populations, showing significance by Student’s t-test for rHhip treated versus control untreated for SP4 (P<0.02) and DN (P<0.006). (C) The percentage of DP thymocytes, gated on CD3<sup>hi</sup>. The difference in percentage of CD3<sup>hi</sup> DP cells between rHhip treated and control was statistically significant by Student’s t-test (P<0.02). (D) CD8 versus CD4, gated on CD3<sup>hi</sup>. (E) MFI of CD5 on rHhip-treated FTOC showing significance by Student’s t-test versus control untreated FTOC for SP4 (P<0.02) and DP (P<0.007). (F) Gated on CD3<sup>hi</sup>, MFI of CD5 on rHhip treated showing significance by Student’s t-test versus control untreated FTOC for CD3<sup>hi</sup> SP4 (P<0.01) and CD3<sup>hi</sup> DP (P<0.02). (G-I) Flow cytometry of E17.5 FTOC +4 days from Shh<sup>−/−</sup> FoxN1Cre<sup>+</sup> (n=4, control) and Shh<sup>−/−</sup> FoxN1Cre<sup>+</sup> (n=6) littermates. Scatter plots show relative mean±s.e.m., where each point represents thymus from a different embryo. (H) Percentage of thymocyte populations and SP4:SP8 ratio, in control and Shh<sup>−/−</sup> FoxN1Cre<sup>+</sup>, giving significance by Student’s t-test compared with Cre<sup>+</sup> for SP4 (P<0.02), DP (P<0.04) and SP4:SP8 ratio (P<0.05). (I) Percentage of thymocyte populations and SP4:SP8 ratio gated on CD3<sup>hi</sup>, in control and Shh<sup>−/−</sup> FoxN1Cre<sup>+</sup>, giving significance by Student’s t-test compared with Cre<sup>+</sup> for CD3<sup>hi</sup> SP4 (P<0.003) and CD3<sup>hi</sup> DP (P<0.002). (J-L) E17.5 Shh<sup>−/−</sup> FoxN1Cre<sup>+</sup> FTOC treated with 1 μM Shh for 4 days compared with control untreated Shh<sup>−/−</sup> FoxN1Cre<sup>+</sup> FTOC thymus lobe from the same thymus (n=5). (J) CD8 against CD4 staining (top); CD8 against CD4 staining, gated on CD3<sup>hi</sup> (bottom). (K) Percentage of thymocyte population, divided by the percentage from the control untreated cultured lobe from the same thymus, giving significance compared with untreated for SP4 (P<0.04). (L) Percentage of thymocyte population, gated on CD3<sup>hi</sup>, divided by the percentage from the control untreated cultured lobe from the same thymus, giving significance compared with untreated for CD3<sup>hi</sup> DP (P<0.02) and CD3<sup>hi</sup> SP8 (P<0.03).

**Gli3<sup>−/−</sup>** SP4 datasets than in their WT counterparts, and these are the populations that contain cells that are undergoing selection (Fig. 7A,B). After 2 days of rShh treatment of WT E17.5 FTOC, the levels of Egr2 and Tox were reduced compared with the untreated control (Fig. 7C,D).

In the microarray data, we observed the same pattern of expression for the known transcriptional target genes of TCR signal transduction, Nr4a1 and Cdl5 (Fig. 7E). Both were low in CD69<sup>−</sup> DP cells, upregulated in the CD69<sup>+</sup> DP population, and again in the SP4 population, but were significantly lower in the Gli3<sup>−/−</sup> CD69<sup>+</sup> DP and SP4 datasets than in WT. Thus, the differences in expression in these genes only became apparent after TCR signalling for positive selection, in support of the idea that Gli3 mutation influences SP4 T-cell development by increasing Shh, which signals to DP thymocytes to dampen TCR signal transduction during positive selection.

Taken together, the transcriptome data suggest that cells undergoing selection in the Gli3<sup>−/−</sup> thymus have a lower average strength of TCR signal. To test this, we used canonical correspondence analysis (CCA) to compare the patterns of gene expression in our SP4 datasets with transcriptome data from publicly available datasets that were prepared from thymocytes that were receiving different strengths of TCR signals during selection (GSE38909; Lo et al., 2012). We selected the 1500 most significant DEG genes from the GSE38909 dataset and used these to generate a scale of strong to weak TCR signalling and plotted our SP4 datasets against this scale. This analysis showed that the SP4 Gli3<sup>−/−</sup> samples have the transcriptional signature of thymocytes receiving a lower TCR signal than those of WT, whereas the transcriptome of the SP4 population from the Gli3<sup>−/−</sup> thymus showed an intermediate transcriptional signature (Fig. 7F). The CCA therefore confirms that Gli3 is important for selection and differentiation from DP to SP4, and is consistent with higher Shh expression dampening the TCR signal during repertoire selection in the Gli3<sup>−/−</sup> thymus.

**DISCUSSION**

Here, we showed that expression of the transcription factor Gli3 in TECs is necessary for normal differentiation from DP to mature SP4 thymocyte in the fetal thymus. Constitutive deletion of Gli3 reduced differentiation and maturation of SP4 T-cells, and this reduction in differentiation to SP4 was also seen when Gli3 was conditionally deleted from TECs only, but not when Gli3 was conditionally deleted from thymocytes. Gli3 repressed expression of Shh, and analysis of an Hh reporter line showed that the Hh signalling pathway was active in developing thymocytes, and that activation of the pathway in thymocytes was increased when Gli3 was deleted. DP and SP thymocyte populations from Gli3<sup>−/−</sup> had reduced levels of cell-surface CD5, indicative of lower TCR signalling, and consistent with the fact that rShh treatment of WT FTOC and constitutive activation of Hh-mediated transcription both reduced cell-surface CD5 expression (Furmanski et al., 2012; Rowbotham et al., 2007).

Conditional deletion of Shh from TECs increased differentiation from DP to SP, whereas differentiation from DP to SP4 in the Gli3 mutant fetal thymus was restored by neutralisation of endogenous Hh proteins. Taken together, our findings indicate that Gli3 activity in TECs promotes SP4 T-cell development by repression of Shh, which signals directly to developing T-cells to reduce TCR signal strength. In support of this, the transcriptome data showed reduced expression of genes important for TCR signalling and positive selection, and of transcriptional targets of TCR signal transduction in the Gli3<sup>−/−</sup> populations compared with their WT counterparts, whereas the WT samples had decreased expression of Hh target and signalling genes. Overall, the Gli3<sup>−/−</sup> SP4 cells had the transcriptional signature of thymocytes that have received a weaker TCR signal than their WT counterparts. The influence of Gli3 mutation on the SP4 population is consistent with this mechanism, as positive selection to the SP4 lineage requires stronger and longer TCR signals (Bosselut, 2004; Klein et al., 2014; Stall et al., 2003).

Gli3 is expressed in fetal but not adult thymocytes (Hager-Theodorides et al., 2005), and although conditional deletion of Gli3 from thymocytes did not significantly influence the proportions of DP and SP populations, our experiments showed that Gli3 activity in thymocytes is required for the normal interpretation of changes in the Hh signal. In the constitutive Gli3<sup>−/−</sup> thymus, where increased Shh signalling to developing thymocytes reduced differentiation from DP to SP, although neutralisation of the Hh signal with rHhip treatment was able to restore the proportion of the SP4 population, it only significantly increased cell-surface expression of CD5 in the CD3<sup>hi</sup> DP population. In contrast to the constitutive Gli3<sup>−/−</sup> thymus, rHhip treatment in Gli3<sup>−/−</sup> FoxN1Cre<sup>+</sup> FTOC increased cell-surface CD5 expression on SP4 and CD3<sup>hi</sup> DP populations, suggesting that the ability of developing fetal thymocytes to respond to the decrease in the Shh signal upon rHhip treatment requires thymocyte intrinsic Gli3 activity.

In summary, we showed that expression of the transcription factor Gli3 by TECs is required for normal SP4 T-cell development in the fetal thymus. As Gli3 deficiency increases Shh expression in the thymus, and Hh plays a role in T-cell acute lymphoblastic leukaemia (T-ALL), which arises in the thymus, it will be important to investigate the impact of Gli3 activity in the thymic stromal environment on T-ALL (Dagklis et al., 2016, 2015; González-Gugel et al., 2013; Hou et al., 2014). It will also be important to investigate the influence of Gli3 on shaping the TCR repertoire and in autoimmune disease.
MATERIALS AND METHODS

Mice
Mice were on a C57BL/6 background. C57BL/6 mice were purchased from Charles River; Gli3\(^{fl/fl}\), Gli3\(^{+/−}\) and Shh\(^{fl/fl}\) from The Jackson Laboratories; GBS-GFP-tg was provided by J. Briscoe (Balaskas et al., 2012); Vav-iCre-tg was provided by D. Kioussis (de Boer et al., 2003); and FoxN1Cre-tg by G. Hollander (Zuklys et al., 2009). As FoxN1-Cre-tg and Vav-Cre-tg can be expressed in the male germline, all conditional knockouts were generated by crossing Cre\(^{+/−}\) females with Cre\(^{−/−}\) males (Joseph et al., 2013; Shi et al., 2016). Mice were bred and maintained at UCL under UK Home Office regulations.

Flow cytometry, antibodies and cell purification
Thymus cell suspensions were prepared and stained as described (Hager-Theodorides et al., 2005) using combinations of the following directly conjugated antibodies at concentration of 1:100: (from BD Pharmingen) anti-γ\(^{δ}\)PE (catalogue no. 553178); from eBioscience: anti-TCR\(β\)FITC (catalogue no. 11-5961-85), anti-CD3PE (catalogue no. 12-0031-82), anti-CD24PE (catalogue no. 12-0241-82) and anti-CD69FITC (catalogue no. 11-0691-85); (from Biolegend) anti-CD3FITC (catalogue no. 100204), anti-CD5FITC (catalogue no. 100605), anti-Qa2FITC (catalogue no. 121709), anti-CD4APC (catalogue no. 116014), anti-CD5PE (catalogue no. 100607), anti-CD8PerCP/Cy5.5 (catalogue no. 100734), anti-CD4PerCP/Cy5.5 (catalogue no. 100734). Data were acquired on a C6 Accuri flow cytometer (BD Biosciences) and analysed using FlowJo software. Live cells were gated by FSC and SSC profiles. Data represent at least three experiments.

Fetal thymus organ cultures
FTOCs were carried out as described (Saldaña et al., 2016). In some experiments, rHhip or rShh (R&D Systems) was added at 1 μg/ml. To allow comparison between litters for statistical analysis, relative numbers or

**Fig. 6. Microarray datasets show transcriptional differences in Hh signalling genes and thymic selection genes between E18.5 WT and Gli3 mutant sorted CD69\(^−\) DP, CD69\(^+\) DP and SP4 populations.**

(A) (Left) PCA axis 2 (PC2) for WT and Gli3\(^{−/−}\) CD69\(^−\) DP, showing some genes that contributed to the negative (Gli3\(^{−/−}\)) and positive (WT) PC2 axis. (Right) Heatmap of DEGs identified by intersection analysis between WT and Gli3\(^{−/−}\) CD69\(^−\) DP. (B) (Left) PC3 for WT and Gli3\(^{−/−}\) CD69\(^+\) DP, showing some genes that contributed to the negative (Gli3\(^{−/−}\)) and positive (WT) axis. (Right) Heatmap of DEGs identified by intersection analysis between WT and Gli3\(^{−/−}\) CD69\(^−\) DP. (C) (Left) PC2 for WT and Gli3\(^{−/−}\) SP4, showing some genes that contributed to the negative (WT) and positive (Gli3\(^{−/−}\)) axis. (Right) Heatmap of DEGs highlighted in intersection analysis between WT and Gli3\(^{−/−}\) SP4.
percentages for each genotype or treatment were calculated by dividing by the mean of controls from the same litter (untreated control or WT littermates).

**Microarray and data analysis**

E18.5 WT, Gli3+/− and Gli3−/− thymocytes (n=2) were stained for CD4, CD8 and CD69. SP4, CD69− DP and CD69+ DP populations were FACS sorted. RNA was extracted using the Arcturus PicoPure RNA Isolation kit (Applied Biosystems) and quantity and quality determined by Bioanalyzer 2100 (Agilent).

Microarrays were performed by UCL Genomics on the Affymetrix GeneChip Mouse Gene 2.0ST Array using standard Ambion (Invitrogen) chemistry. Array data were normalised using the oligo package from Bioconductor. DEGs were determined using the moderated Ebayes t-statistic \( P<0.05 \) from the limma package in Bioconductor. Principal component analysis (PCA) was performed using normalised microarray transcript expression values, using the CRAN package ade4. PCA is a multivariate statistical method, which can be used to segregate genome-wide transcription datasets according to variability in transcript expression values, taking into account all genes (Ringnér, 2008). PCA can thus cluster microarray datasets to detect dominant patterns of gene expression, as represented by the principal components (PCs).

Canonical correspondence analysis (CCA) is a multivariate analysis that allows the comparison of experimental transcriptome data with publically available datasets from other laboratories (Ono et al., 2014). CCA was performed using the CRAN package vegan. The GSE38909 dataset was used as the environmental variable and our dataset was regressed onto it. It contains thymocytes stimulated with a positively selecting peptide gp250 and the non-selecting control peptide Hb (Lo et al., 2012). We created a strong TCR signalling axis using the DEGs between the control peptide and the positively selecting peptide and regressed our samples onto this axis (Sahni et al., 2015).
**References**


