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Fibroblast growth factors (FGFs) prime the limb specific Shh enhancer for chromatin changes that balance histone acetylation mediated by E26 transformation-specific (ETS) factors

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3 Fibroblast growth factors (FGFs) prime the limb specific *Shh* enhancer for chromatin changes
4   that balance histone acetylation mediated by E26 transformation-specific (ETS) factors

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29   **ABSTRACT**

30   Sonic hedgehog (*Shh*) expression in the limb bud organizing center called the ZPA is

31   regulated by the ZRS enhancer. Here, we examine in mouse and in a mouse limb-derived

32   cell line the dynamic events that activate and restrict the spatial activity of the ZRS. FGF

33   signalling in the distal limb primes the ZRS at early embryonic stages maintaining a poised,

34   but inactive state broadly across the distal limb mesenchyme. The ETS transcription factor,

35   ETV4, which is induced by FGF signalling and acts as a repressor of ZRS activity, interacts

36   with the histone deacetylase HDAC2 and ensures that the poised ZRS remains

37   transcriptionally inactive. Conversely, GABPα, an activator of the ZRS, recruits p300, which is

38   associated with histone acetylation (H3K27ac) indicative of an active enhancer. Hence, the

39   primed but inactive state of the ZRS is induced by FGF signalling and in combination with

40   balanced histone modification events establishes the restricted, active enhancer responsible

41   for patterning the limb bud during development.

42   **Key words**

43   Enhancer, ZRS, Shh, FGF, ETS factors, histone acetylation

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47   **INTRODUCTION**

48   Spatial specific gene expression is fundamental to controlling cell identity in

49   embryonic tissue. Early in the mesenchyme of the developing mammalian limb bud there

50   are no observable morphological differences or histological boundaries; nevertheless, the

51   limb bud is initially polarised along the anterior-posterior axis (Tickle et al 2015) establishing

52   a specialised compartment of cells at the posterior margin called the zone of polarising

53   activity (ZPA). The function of the ZPA is the expression of *Shh,* which operates as a

54   morphogen and a mitogen to coordinate digit formation by integrating growth with digit

55   specification during limb development (Towers et al., 2008; Zhu et al., 2008). A highly

56   conserved ~780 bp enhancer called the ZRS controls the spatiotemporal expression of the

57   *Shh* gene in the ZPA of both the fore and hind limbs (Lettice et al., 2002, 2003; Sagai et al.,

58   2005). The ZRS lies in an intron of the ubiquitously expressed *Lmbr1* gene at a distance of

59   1Mb from the *Shh* gene in human. Well over 20 different point mutations occurring in the

60   ZRS are associated with the misregulation of *Shh* and consequently, to limb skeletal defects.

61   These include preaxial polydactyly type 2 (PPD2), triphalangeal thumb polysyndactyly

62   (TPTPS), syndactyly type IV (SD4), and Werner's mesomelic syndrome (WMS), collectively

63   referred to as ZRS-associated syndromes (Lettice et al., 2003, 2008; Farooq et al., 2010;

64   Furniss et al., 2008; Gurnett et al., 2007; Semerci et al., 2009; Wieczorek et al., 2010).

65   The regulatory mechanisms that direct gene expression to embryonic compartments

66   are not well established in mammalian development; therefore, to understand the events

67   that occur, it is crucial to investigate the activation of spatiotemporal specific enhancers

68   during this process. We previously showed that members of the ETS transcription factor

69   family (Sharrocks, 2001) are involved in the spatial pattern of *Shh* expression (Lettice et al.

70 2012). Occupancy at multiple ETS sites, which bind the factors GABPα and ETS1, regulates

71 the position of the *Shh* expression boundary in the limb, thus defining the ZPA. In contrast,

72 binding sites for ETV4 and ETV5 in the ZRS, when occupied, repress ectopic *Shh* expression

73 outside the ZPA. A single base pair change is able to subvert this normal developmental

74 process to give rise to skeletal abnormalities. For example, two different human PPD2 point

75 mutations generate additional ETS binding sites, thereby, de-repressing expression of *Shh* in

76 the anterior limb bud. The balance between binding of the activators and repressors is

77 crucial for normal *Shh* expression and the mutations that disrupt this balance result in

78 skeletal defects.

79   Analysis of developmental gene regulation must also take into account the

80 mechanisms of long-range enhancer/promoter interactions. Previously, we showed that the

81 ZRS contains two domains with distinct activities; one domain, the 5' end of the ZRS

82 (472bp), directs the spatiotemporal activity and the second domain, the 3' half (308bp), is

83 required to mediate activity over long genomic distances (Lettice et al., 2014). Additionally

84 higher-order chromatin conformational changes that occur in the *Shh* locus play a role in

85 gene expression. Elevated frequencies of *Shh*/ZRS co-localisation were observed only in the

86 *Shh* expressing regions of the limb bud (Amano et al., 2009), in a conformation consistent

87 with enhancer-promoter loop formation (Williamson et al., 2016). However, the domain

88 between *Shh*-ZRS is highly compacted in all tissues and developmental stages analysed

89 independent of *Shh* expression.

90   Here, we investigate the stepwise events that mediate the spatiotemporal activation

91 of an enhancer during development. We demonstrate that even though the activity of the

92 ZRS is restricted to the ZPA, it retains features of a poised enhancer along the full distal

93 portion of the limb bud composed of the mesenchymal cells of the progress zone; whereas,

94 H3K27ac is enriched just in the distal-posterior limb region. We show that FGF signaling

95 plays a key role in priming the ZRS as indicated by an increase in H3K4me1 at the enhancer.

96 In addition, we show the mechanism of acetylation/deacetylation that GABPα, an activator,

97 and ETV4, a repressor, employ to restrict and promote ZRS activation in the distal limb bud.

98

99 **RESULTS**

100 **Modifications of chromatin at the ZRS differ in different regions of the limb bud**

101   In order to characterize specific features of the *Shh* limb enhancer, the ZRS, in

102 different regions of the developing limb, we micro-dissected the limb bud at embryonic day

103 11.5 (E11.5) into several defined segments. Firstly**,** we examined the distal region which

104 contained the specialised epithelial structure called the AER (apical ectodermal ridge), the

105 mesenchyme of both the progress zone, and the ZPA and in addition, the proximal region

106 which contained the shank of the limb bud (Fig. 1A). Chromatin immunoprecipitation

107 analysed by quantitative PCR (ChIP-qPCR) was performed on the dissected limb tissue for

108 the two modified histones, H3K4me1 and H3K27ac (Fig. 1B). Previous studies demonstrated

109 that these modifications are markers for enhancer activity. H3K4me1 is a predictive

110 chromatin signature for both poised and active enhancers in the human genome,

111 (Heintzman et al.,2007) but in association with H3K27Ac, these mark active regulatory

112 elements (Rada-Iglesias et al., 2012; Cotney et al., 2012). We found specific enrichment of

113 H3K4me1 at the ZRS in the distal region of the dissected E11.5 limb buds, which was

114 appreciably lower in the proximal region of the limb buds. Further dissections of the distal

115 limb bud into anterior and posterior halves enabled us to discern more precisely the

116 location of the H3K4me1 at the ZRS (Fig. 1B). Even though *Shh* was not expressed in the

117 anterior region of the limb bud, the H3K4me1 mark was enriched in both dissected halves

118 suggesting that the ZRS was poised across the distal compartment of the limb.

119   H3K27ac, in contrast, was differentially enriched in the distal mesenchyme greater in

120 the posterior portion of the limb bud. To investigate acetylation of histone H3 across the

121 large 770bp enhancer, two sets of PCR primers were used to amplify each end of the ZRS.

122 The ZRS encompasses two distinct regulatory activities; one residing in the 5' half driving

123 spatiotemporal expression and one residing in the 3' half mediating long range activation of

124 the *Shh* gene (Lettice et al., 2014) (Fig. 1C). Analysis of each half of the enhancer after ChIP

125 identified differential acetylation of H3 across the ZRS. PCR primers complementary to the

126 5' spatiotemporal half (5'ST primers) of the ZRS showed an increase in H3 acetylation

127 compared to the 3' long-range (3'LR primers) end. H3K27ac was associated with the 5' end

128 of the ZRS and was enriched in the distal region of the limb bud. H3K27ac was, therefore,

129 associated with the distal, posterior quadrant of the limb bud consistent with the presence

130 of the ZPA where the *Shh* gene is transcriptionally active (Fig. 1A). Hence, in the case of the

131 ZRS, the enhancer is poised throughout the posterior distal mesenchyme; further events

132 occur to activate the enhancer for productive transcription in the ZPA.

133   We next assessed the chromatin state of the ZRS in the distal region of the limb bud

134 using chromatin immunoprecipitation coupled with microarray called ChIP-on-chip. The

135 chromatin immunoprecipitation analysis using antibodies to H3K4me1 and H3K27ac

136 highlighted the specificity of the histone modifications over the ZRS (Fig. 1D). The pattern of

137 the H3K4me1 covered the extent of the ZRS; whereas, the pattern of H3K27ac, as predicted

138 by the PCR primers, was not centered over the ZRS but was skewed toward the 5' side.

139 Thus, the ZRS exists in differential chromatin states in the limb depending on position and

140 gene activity.

141 **A limb-derived cell line shows ZRS activation and *Shh* induction**

142   To investigate the dynamics of *Shh* regulation in relation to the ZRS, we generated

143 immortalised cell lines from early limb bud mesenchyme (Williamson et al., 2012). RNA-seq

144 analysis of the 14Fp cell line (cells derived from the distal/posterior part of the limb at E

145 11.5) shows that many of the key genes found in the posterior limb bud are expressed with

146 a notable exception being *Shh* (FIG. 2-SUPL. 1A). We performed ChIP-on-chip using

147 antibodies against H3K4me1 and H3K27ac which revealed that, H3K4me1 marked the full

148 extent of the ZRS in the cell line; whereas, H3K27ac was not enriched, demonstrating that

149 the ZRS resides in a poised state reflecting the origin of the cell line from the distal

150 mesenchyme of the limb bud (Fig. 2A and FIG. 2-SUPL. 1A).

151   The poised state of the ZRS in the cells indicated that *Shh* inactivation was due to the

152 lack of specific factors that are responsible for fully activating the ZRS. Previous attempts to

153 activate *Shh* expression in limb bud derived cells using cocktails of known developmental

154 activators such as FGFs and retinoic acid or transfection with HoxD genes have shown that

155 *Shh* is refractory to activation (Kimura et al., 1998). Our attempts with known *Shh* activators

156 confirmed these observations (FIG. 2-SUPL. 2A); however, the drug tricostatin A (TSA), a

157 histone deacetylase (HDAC) inhibitor, stimulated expression (FIG. 2-SUPL. 2B-C). *Shh*

158 expression was detectable within 6h of treatment (FIG. 2B) reaching a maximum at ~24hrs.

159 To further investigate *Shh* activation in the cell line, we carried out a TSA time course to

160 assay H3K27ac enrichment at the ZRS. H3K27ac enrichment was observed over both the ZRS

161 and the *Shh* promoter at 16h of treatment, reaching a maximum at 24h (Fig. 2C),

162 accordingly, with the *Shh* expression time course (Fig. 2B). The fold enrichment of H3K27ac

163 in the limb buds [Fig. 1B] and cell lines [Fig. 2C] seemed dramatically different; therefore,

164 enrichment of H3K27ac was directly compared between the limbs buds and the cells in the

165 same experiment showing that the magnitude of enrichment is comparable [FIG. 2-SUPL.

166 3A].) In the cell lines, the correlation between *Shh* induction of expression and recruitment

167 of H3K27ac suggests that the ZRS is involved in the activation of the transcriptional process.

168 In addition H3K27ac was more highly associated with the region involved in the

169 spatiotemporal activity of the ZRS (as shown using the 5'ST primers). Two control mouse cell

170 lines were used to investigate the specificity of *Shh* activation in the 14Fp cells. Firstly, the

171 mouse ES cell line, E14, which in response to retinoic acid induces *Shh* expression and

172 secondly, an immortalised mesenchymal cell line from the embryonic mandible at E11.5,

173 called the MD cell line were used. In both cell lines, the histone mark, H3K4me1, was not

174 enriched over the ZRS, and TSA treatment did not lead to *Shh* induction after 24h of

175 treatment (FIG. 2-SUPL. 3B-C).

176   Extragenic transcription sites correlate with active regulatory elements and in accord

177 are occupied by RNA Pol II (De Santa et al., 2010, Kim et al., 2010). Genome-wide studies

178 highlighted Pol II occupancy and the synthesis of noncoding transcripts at active enhancer

179 sites, the roles of which, so far, remain unclear. In order to confirm the limb specific

180 enhancer activation, PolII ChIP-qPCR was carried out in limb cells after TSA treatment. In the

181 untreated 14Fp cell line, even though the enhancer is in a poised state, PolII was not

182 appreciably enriched at the *Shh* promoter nor at the ZRS (Fig. 2D). After activation with TSA,

183 PolII was detected at both sites confirming the association of PolII recruitment to the

184 enhancer and the promoter, further suggesting that TSA mediated *Shh* activation in 14Fp

185 involves the ZRS. Thus, this cell line derived from early limb bud mesenchyme showed that

186 the ZRS could be induced to undergo modifications consistent with enhancer activation and

187 concomitantly, *Shh* expression was activated. To further establish the role for ZRS in the

188 activation of Shh expression in the cell line, the interaction of the ZRS with its target

189 promoter was analysed.

190 **Promoter-enhancer contacts are established during *Shh* gene activation**

191 *Shh* gene activation is linked to an increase in the level of the H3K27ac histone mark

192 over the ZRS. In order to investigate the reorganization of chromatin structure after TSA

193 treatment and to confirm the involvement of the ZRS in *Shh* activation induced by TSA,

194 circularized chromosome conformation capture (4C-seq) (Stadhouders et al., 2013) analysis

195 was carried out at 18 and 24 hours after TSA treatment (FIG. 2-SUPL. 3D-F). Evidence of

196 increased colocalisation of the ZRS and the *Shh* gene in the expressing region of the limb

197 bud was demonstrated previously by FISH and 3C analysis (Amano et al., 2009, Williamson

198 et al., 2015). 4C-seq analysis in the 14Fp cell line (Fig. 3A) showed marked and highly

199 significant ZRS–*Shh* interactions in the TSA treated cells. The interaction between

200 the *Shh* gene and the ZRS was confirmed by 3C-qPCR (FIG. 3-SUPL. 1E).

201 The fragment containing the promoter site of *Shh* colocalizes with the ZRS and was

202 detected at 18 hours and at 24 hours after TSA treatment (TSA minus : q < 1.5 x 10^{-5}; TSA^{18h} :

203 q-value < 5 x 10^{-10} ; TSA^{+24h} : q < 7.8 x 10^{-35}); whereas, significant contact in the fragment

204 containing the promoter was undetectable in the untreated cells. *Shh*/ZRS proximity in the

205 nucleus occurs regardless of whether the gene or enhancer is active (Williamson et al.,

206 2016); however, in TSA treated cells reorganization in chromatin structure occurs due to

207 activation of the ZRS increasing interactions with the promoter.

208 **FGF signalling is responsible for priming the ZRS**

209   At an early stage of limb development, *Fgf10* expression in the distal limb bud mesenchyme

210   is important for both limb bud outgrowth and induction of FGF signalling from the AER

211   (Ohuchi et al. 1997) which, in turn, maintains the cells of the progress zone. Subsequently,

212   the FGFs function to maintain *Shh* expression in the ZPA (Laufer et al. 1994; Niswander et al.

213   1994; Crossley et al. 1996; Vogel et al. 1996; Ohuchi et al. 1997). We tested the hypothesis

214   that localized FGF expression is correlated with the poised state of the ZRS. Firstly, the 14Fp

215   cell line pre-treated with nintedanib (NIN), a potent broad spectrum inhibitor of FGFR1/2/3,

216   VEGFR1/2/3 and PDGFRα/β (Hillberg et al., 2008) showed a significant reduction in *Shh* after

217   TSA treatment (Fig. 4A). Previous studies shows that an increase in AER-FGF levels leads to

218   gradual repression of *Grem1* in the distal mesenchyme as part of an inhibitory feedback

219   loop (Fgf/Grem1 loop) (Verheyden et al., 2008) and promotes expression of *Etv4* (Mao J et

220   al., 2009). Hence, as control for the efficiency of the FGF inhibition, *Grem1* and *Etv4* levels

221   were evaluated (Fig. 4B) after 4h incubation with NIN or after 4h NIN plus FGF8/10

222   incubation. Since nintedanib can also inhibit VEGF and PDGF receptors, another FGFR

223   inhibitor, BGJ398 (BGJ), was also tested for *Grem1* and *Etv4* expression. Increased levels of

224   *Grem1* were observed after 4h of NIN and BGJ incubations while Etv4 levels were reduced.

225   The FGF8/10 treatment was sufficient to restore the original levels of both *Grem1* and *Etv4*.

226   To investigate the action of FGF signalling at the ZRS, ChIP for H3K4me1 in the limb specific

227   cell line exposed to NIN was performed (Fig. 4C). As control, a region of the first intron of

228   *Rbm33*, a neighbouring gene, which displays open chromatin coincident with a peak of

229   H3K4me1 was examined. The H3K4me1 enrichment over the ZRS was dramatically reduced

230   after inhibition of FGF activity and TSA treatment did not rescue the presence of this histone

231   modification; whereas, *Rbm33* intron 1 was not significantly affected. The same effect was

232   caused by BGJ on H3K4me1 enrichment (FIG. 4-SUPL. 1D-E), and no differences were

233   observed in comparison with NIN treatment. To assess whether the NIN or BGJ treatment

234   would affect cell survival or cause other abnormality trypan blue staining was performed

235   and no alterations were observed after 4h treatment (FIG. 4-SUPL. 1A).

236   To determine if FGF plays a similar role in effecting the poised state of the ZRS in the

237   embryo, we developed a short-term organ culture approach (Havis E. et al. 2014). Distal tips

238   of E11.5 limb buds were dissected, maintained in media and exposed to NIN for 4h to

239   examine the state of endogenous ZRS. In agreement with the cell line results, the distal tips

240   lost ZRS enrichment of H3K4me1 and higher levels of *Grem1* expression were observed (Fig.

241   4F-G). On the other hand, when proximal dissections of E11.5 limb bud, where the enhancer

242   is in an inactive state, are exposed to a combination of FGF8 and FGF10 the ZRS displays the

243   poised state (Fig. 4H, FIG. 4-SUPL. 1F) showing enrichment of H3K4me1. These data suggest

244   that FGF signalling has a key role in priming and maintaining the ZRS as a poised enhancer in

245   the distal mesenchyme of the limb bud, delineating the boundaries where *Shh* can be

246   potentially expressed. Trypan blue staining was performed on limb dissections by following

247   a reported protocol with slight modifications (Siddique Y.H 2012) (FIG. 4-SUPL. 1B). No signs

248   of increased cell death was observed after 4h of NIN treatment.

249

250   **GABPα activates *Shh* expression**

251   The 14Fp cells retain the expression of some of the ETS genes (FIG. 2-SUPL. 1A)

252   which were shown to play a regulatory role at the ZRS (Lettice et al., 2012). One of these,

253   GABPα, interacts with the ZRS in the limb bud and activates *Shh* expression. In addition

254   others have demonstrated (Kang et al., 2008) that GABPα recruits the co-activator histone

255   acetyltransferase CBP/p300. Therefore, binding of GABPα to the ZRS and recruitment of

256   p300 was examined in the 14Fp cell line (Fig. 5A). ChIP-on-chip analysis on untreated cells

257   for both GABPα and p300, showed an overlapping peak of enrichment for both factors,

258   suggesting a co-occupancy over the ZRS. To further study the interaction between these TFs,

259   we performed co-immunoprecipitation experiments on nuclear extracts from 14Fp cells

260   transfected with GABPα tagged with three copies of the flag epitope. Immunoprecipitation

261   of endogenous p300 co-precipitates the flag-GABPα indicating an association between these

262   two factors (FIG. 5B). Western blot for p300, normalised against the histone H3 showed that

263   p300 levels were not affected (FIG. 5-SUPL. 1B). Based on the assumption that GABPα and

264   p300 together have an important, yet undefined role in the activation of the regulatory

265   element, we next addressed whether the presence of GABPα/p300 influenced ZRS

266   activation. *Shh* expression was examined in cells induced with TSA after reduction of *Gabpa*

267   expression*. Gabpα* siRNA knockdown (Fig. 5C) revealed decreased *Shh* expression after TSA

268   treatment (Fig. 5D). Conversely, overexpression of *Gabpα*, using a doxycycline inducible

269   vector leads to activation of *Shh* (Fig. 5E, FIG. 5-SUPL. 1A). In addition, ChIP analysis for

270   H3K27ac in cells overexpressing GABPα showed an appreciable enrichment over the ZRS;

271   whereas, the transcription factor ETV4, a repressor (see below) which restricts expression

272   outside the ZPA, is displaced from the ZRS (Fig. 5F). Under these conditions, enrichment of

273   GABPα and p300 over the ZRS is also observed (Fig. 5G). These data suggest that GABPα

274   regulates *Shh* expression by modulating the acetylation status of H3K27 of the ZRS.

275   Furthermore, FGF signalling plays a central role, since both GABPα and p300 are released

276   from the ZRS when FGF signalling is inhibited by NIN and the enhancer is no longer poised

277   (Fig. 4D,E). FGF, therefore, mediates priming of the ZRS enabling the binding of GABPα

278   which, in turn, recruits p300.

279   **ETV4 carries out its repressive role via interacting with HDAC2**

280   ETV4/ETV5 binding represses *Shh* expression outside the ZPA in the limb bud (Lettice

281   2012). Since there is a close association between GABPα and p300, we investigated the

282   possibility that the repressive role of ETV4/ETV5 was related to HDAC activity. In order to

283   investigate specific HDAC candidates, RNA-seq data obtained from the cell line and the

284   distal and proximal portions of the limb bud showed nearly all the HDAC classes are

285   represented in both 14Fp and in limb tissue between the cells and the tissue, with the

286   exception of HDAC9 (FIG. 2-SUPL. 1B). Most of the HDACs represented were subjected to

287   ChIP in 14Fp (HDAC1,2,3,4,5,6,8 and 9) and HDAC2 appeared significantly enriched over the

288   ZRS (data not shown) and is one of the most abundant *Hdac* in the RNA samples analysed

289   (FIG. 2-SUPL. 1B)**.** To further examine the role of HDACs in ZRS activity, *Hdac2* and *Hdac1*

290   expression levels were reduced by specific siRNAs in 14Fp cells (Fig. 6A). Both are class I

291   HDACs and often work in concert. *Hdac1* down-regulation had no effect on *Shh* expression,

292   while the reduction of *Hdac2* levels (by siRNA) in 14Fp showed an increase in *Shh* levels.

293   Simultaneous reduction of both *Hdac1* and *Hdac2* did not have an additive effect on the

294   induction of *Shh* (Fig. 6A). To investigate the relationship between the repressor activity of

295   the ETV genes and HDAC2, we performed ChIP-on-chip for these two factors to visualize

296   their distribution over the ZRS (Fig. 6B). Since in the 14Fp cell line ETV5 expression is low

297   (approx. 8-10 fold lower than ETV4) (FIG. 2-SUPL. 1A-B), depletion of ETV5 activity was not

298   necessary in this analysis. ETV4 exhibits two peaks encompassing the ZRS which correspond

299   to the two ETV4 binding sites (Lettice et al., 2012); interestingly, the HDAC2 peak

300   overlapped one of these peaks located at the 3' end which encodes the long range activity

301   of the regulatory element (Lettice et al., 2014). We next tested if HDAC2 and ETV4 were able

302   to physically interact; analysis showed that endogenous ETV4 co-immunoprecipitated with

303   HDAC2 (FIG. 6C). In addition, the negative role of ETV4 on *Shh* expression in 14Fp cells (Fig.

304 6D) was investigated. *Etv4* levels were reduced with siRNA, resulting in a ~40% decrease in

305 *ETV4* and a significant activation of *Shh* expression (Fig. 6D). ChIP using anti-ETV4 and

306 HDAC2 antibodies performed on TSA-treated cells showed that ETV4 together with HDAC2

307 are displaced from the ZRS (Fig. 6E), suggesting that ETV4 opposes GABPα activity by

308 maintaining lower levels of H3K27ac**.**

309 **DISCUSSION**

310 **The distal limb bud is poised for expression**

311 The ZPA is the organizing center of the early developing limb bud and the restricted

312 expression of *Shh* at this location along the posterior boundary is crucial for correct

313 specification of digit identity and number. Various regulatory inputs are essential to acquire

314 this spatial specific pattern of expression. We showed that an initial input is the event that

315 primes the ZRS, such that the enhancer is poised but transcriptionally inactive. The ZRS

316 priming occurs in a broad region of the distal limb bud mesenchyme that includes tissue that

317 will not express *Shh* in addition to the ZPA. Furthermore, we undertook studies to identify

318 the signalling pathway involved in the induction of ZRS priming. Distal limb mesenchyme,

319 referred to as the progress zone, is known to be under the influence of the FGFs produced in

320 the AER (Laufer et al. 1994; Niswander et al. 1994; Crossley et al. 1996; Vogel et al. 1996;

321 Ohuchi et al. 1997) and we showed that FGFs can induce ZRS priming in distal mesenchyme;

322 whereas, inhibition of FGF signalling results in chromatin changes and loss of H3K4me1 and

323 loss of transcription factor binding suggesting that the ZRS is no longer recognized as a

324 poised enhancer and is in a 'closed' configuration. Thus, one role of FGF signalling is the

325 establishment and maintenance of ZRS priming. Activation of the ZRS, therefore, appears to

326 be a two-step process; ZRS priming occurring broadly in the distal mesenchyme which is a

327   prerequisite for subsequent action by other signals in the posterior region containing the

328   ZPA to activate the ZRS.

329   The poised state of the enhancer is notable in light of the response of the ZRS to the

330   point mutations that cause preaxial polydactyly type 2 and other associated skeletal

331   abnormalities (Anderson 2012). These mutations cause mis-regulation of *Shh* expression in

332   the developing limb bud such that *Shh* expression occurs at an ectopic anterior site in the

333   distal mesenchyme in addition to the ZPA. Ectopic expression of all ZRS mutations

334   examined, thus far, is restricted to the distal mesenchyme suggesting that there is a

335   requirement for the ZRS to be in a poised state in order to be activated and demonstrates a

336   mechanism for *Shh* ectopic activation restricted to the distal mesenchyme of the limb bud.

337   **Levels of GABPα and ETV4 regulate *Shh* expression**

338   Attempts to understand how developmental enhancers operate over such large

339   distances have led to the general idea that chromatin looping assists the interactions of a

340   long-range enhancer with its target promoter (Sanyal et al., 2012). In the limb bud, 3C assay

341   revealed a physical interaction between the ZRS and the promoter (Amano et al., 2009).

342   Here, we confirmed , that upon activation of *Shh* expression in the limb-derived cell line,

343   there is highly specific co-localization of the ZRS with the *Shh* promoter fragment. Thus, the

344   *Shh* limb regulator in the cell line exists in two states; one in which the ZRS is poised

345   reflecting its origin from the distal mesenchyme of the developing limb bud and secondly, in

346   an active state in which the ZRS and the promoter recruit PolII and interact. Hence, the cell

347   line reveals the chromatin dynamics that occur during long-range activation.

348   GABPα/ETS1 and ETV4/ETV5 have antagonistic effects on *Shh* activity in the limb.

349   Occupancy at multiple GABPα/ETS1 sites in the ZRS regulates the position of the ZPA

350   boundary, whereas ETV4/ETV5 binding acts as repressors to restrict expression outside the

351 ZPA. We have hypothesized that the balance between these activator and repressor factors

352 are responsible for regulating activity levels in the developing limb bud (Lettice et al, 2012).

353 Here, we showed that GABPα and ETV4 bind to the ZRS in the cell lines similar to that in the

354 limb bud. GABPα is associated with p300 histone acetyltransferase at the ZRS in the cell

355 lines. Previous reports demonstrated that GABPα, physically interacts with p300 in myeloid

356 cells (Resendes et al., 2006). Activation of AChR (nicotinic acetylcholine receptor) genes in

357 subsynaptic nuclei in adult skeletal muscle is mediated by GABPα, which recruits the histone

358 acetyl transferase (HAT) p300 to the AChR ε-subunit promoter (Ravel-Chapuis et al., 2007).

359 Furthermore, the surface of the GABPα OST domain binds to the CH1 and CH3 domains of

360 the co-activator histone acetyltransferase CBP/p300 (Kang H-S, 2008). Here, we showed in

361 the limb-derived cells that a similar interaction of these two factors occurs. In addition,

362 overexpression of GABPα results in an increase in the levels of H3K27ac at the ZRS. A

363 correlation between *Gabpα* expression and the presence of ETV4 at the ZRS is also

364 observed; in that, expression of *Gabpα* results in a displacement of ETV4 at the enhancer.

365 Thus GABPα operates at the ZRS to increase levels of H3K27ac by recruiting p300 and

366 decreasing levels of the repressor ETV4.

367   ETV4 appears to operate in the opposite manner and is one of the dominant factors

368 in maintaining the ZRS in a poised state. Reduction of ETV4 levels is sufficient to activate *Shh*

369 expression. ETV4 interacts with HDAC2 and we further showed the co-localisation of HDAC2

370 and ETV4 at the ZRS in the cell line. Similar to the downregulation of ETV4 the knockdown of

371 HDAC2 induces *Shh*. Activation of *Shh* by TSA releases ETV4 and HDAC2 from the ZRS.

372 Hence activation of the ZRS by both the overexpression of GABPα and treatment with TSA is

373 associated with loss of ETV4 binding which acts directly on histone deacetylase activity.

374   The FGFs play a central role in *Shh* expression by ensuring that the ZRS maintains its

375   primed state throughout the distal limb mesenchyme. This widespread priming, however,

376   opens the ZRS for potential ectopic activation as occurs in preaxial polydactyly and

377   associated phenotypes (Anderson 2012). ETV4 is a repressor that restricts this activity and

378   FGF signalling also induces the levels of ETV4 in the distal mesenchyme of the limb bud

379   (Mao et al., 2009; Zhang et al., 2009). The FGFs, therefore, operate by regulating two

380   contrasting events; firstly, by effecting the chromatin in the distal mesenchyme ensuring

381   that the ZRS is maintained in a poised state and secondly, acting counter to enhancer

382   activation by inducing the level of a repressor that ensures *Shh* expression does not occur

383   outside the region of the ZPA.

384

385   **MATERIALS AND METHODS**

386   **Cell Lines, Transfections, and Treatments.**

387   The 14FP cell line was derived from the posterior third of distal forelimb buds from an

388   Immortomouse (H-2k-tsA58)(Jat et al. 1991). Cells are plated in DMEM (Invitrogen) with

389   10% Foetal Calf Serum (Sigma), Penicillin/Streptomycin and 20 ng/ml Interferon

390   (Peprotech). Cells are grown at 33°C the permissive temperature for the temperature-

391   sensitive T antigen and were passaged as necessary but no later than passage 12. Cells

392   biological replicates are intended as cells cultured separately and treated/analysed at a

393   different passage. Cellular identity was confirmed by RNA expression analysis of specific

394   genes and immortomouse markers and verified to be mycoplasma free. Knock-down of

395   endogenous proteins was performed in 14fp cells after siRNA transfection using Dharmafect

396   1 solution (Dharmacon). Briefly, cells were seeded in 6-well plates to 40% confluence and

397 after 24 hr were transfected using 25 nM of each siRNA pool and 10 µl of the transfection

398 reagent. The transfection medium was replaced after 12 hr and cells were grown for

399 another 12 hr. Cells were collected 24 hr after the transfection for analyses. siRNA pools

400 were purchased from Thermo Fisher Scientific (Ambion): Gabpα (s66354,s66355),

401 Etv4(s71463, s201776), Hdac2 (s67417, s67416), Hdac1(s119557, s119558) and

402 nontargeting siRNAs (control) (D-001810-02). The siRNA were used separately. Unless

403 otherwise specified, the cells were treated with HDAC inhibitors trichostatin A (TSA; 1 µM)

404 for 24h before cell harvest and nintedanib (NIN; 100 nM) and BGJ398 (BGJ; 2.5 µM) for 4h.

405 Cell cultures where incubated, when necessary with 500 ng/ml of FGF10 (R&D Systems, cat.

406 no. 345-FG) and 100 ng/ml of FGF8 (ABCAM, ab205522) combination for 6h after NIN

407 treatments.

408 **Mouse Limb Cultures**

409 Limb buds were dissected from E11.5 mouse embryos and cultured as described by Havis E.

410 et al. (2014) with the following modifications. Limb dissections obtained from different

411 embryos were considered as biological replicates. Briefly distal dissections were treated

412 with nintedanib (NIN.; 100 nM) for 4h. Proximal dissections were incubated with 500 ng/ml

413 of FGF10(R&D Systems, cat. no. 345-FG) and 100 ng/ml of FGF8 (ABCAM, ab205522)

414 combination for 6h. inhibitors were diluted in DMSO. Media with buffers only were used as

415 controls. After treatments, explants were processed for RT-q-PCR or ChIP.

416 Trypan blue staining was performed on limb dissections by following a reported protocol

417 with slight modifications (Siddique Y.H 2012). Total limb dissections were collected and

418 treated 4h with NIN. or media alone. The limbs were then transferred to trypan blue

419 stain and kept in shaking condition for 20 min. 8 limb dissections were directly stained after

420   collection (T0). The samples were washed thoroughly with PBS solution again for 15 more

421   minutes and observed by microscope and imaged to check for any cell damage

422   **Chromatin Imunoprecipitation and antibodies**

423   Crosslinked ChIP was performed as described (Stock et al., 2007) from approximately 10^7

424   cells per experiment. All antibodies used in this study have been previously reported as

425   suitable for ChIP and/or ChIP-seq, p300 (sc-585, Santa Cruz Biotechnology), H3K4me1

426   (ab8895, Abcam), H3K27ac (s39133, Active motif), HDAC2 (ab16032), GABPα (sc-22810,

427   Santa Cruz), with the exception at ETV4 (ABE635, Millipore). All statistical analyses were

428   performed using a two-tailed Student's *t*-test.

429

430   **3C and 4C**

431   3C was conducted according to the protocol described by Stadhouders (2014) et al with

432   minor alterations. In brief cells were treated with 1 mM TSA for 18 and 24 hr or DMSO as a

433   control and fixed in 2 % formaldehyde solution for 10 min. 0.125 M glycine was used to

434   quench the reaction. After a PBS wash and 15 min incubation in lysis buffer, the solution

435   was spun down and nuclei stored at -80 °C until needed. If more than 5×10^5 cells were

436   used, pellets were made up in 1.2 × restriction buffer and divided into 4 aliquotes to reduce

437   formation of aggregates. Primary restriction enzyme digestion was conducted using 800 U

438   Hind III-HF restriction enzyme (NEB) at 37 °C on each aliquot. Before ligation aliquots were

439   combined and T4 DNA ligase (NEB) added and incubated at 16 °C overnight. Hind III digested

440   samples were analysed on a 0.6 % agarose gel and appear as high molecular weight smear

441   running from roughly 4 - 12 kb (FIG. 2-SUPL. 3D- FIG. 3-SUPL. 1A). 3C libraries were analysed

442   on 0.6 % agarose gel and appear as a high molecular weight band (around 12 kb) (FIG. 2-

443   SUPL. 3E- FIG. 3-SUPL. 1B). To this point the 3C libraries were analysed (see below). In order

444   to make 4C libraries a second restriction digest was performed using the 4-cutter MluCI

445   (Roche) at 37 °C overnight and second ligation with T4 DNA ligase (NEB). MlucI digested

446   samples were analysed on a 1.5 % gel appearing as smear between roughly 0.3 - 1kb (FIG. 2-

447   SUPL. 3F). Finally, 4C libraries were purified using QIA quick PCR purification kit (Qiagen) to

448   produce final purified 4C libraries. After PCR amplification and purification (table S2),

449   sequencing adaptors were ligated and 4C libraries sequenced using in-house Ion ProtonTM

450   sequencing.

451   **Analysis of 4C libraries**

452   De-multiplexed sequencing reads (fastq files) can be summarised: first we trimmed known

453   bait sequence using cutadapt (Martin M 2011) and selected only those reads where known

454   viewpoint-associated sequence was present. Reads were mapped to the mouse reference

455   genome (build mm9) using bowtie2 (Langmead B et al. 2012) with the very-sensitive flag 3.

456   Alignments were filtered with a MAPQ score > 30 to select for high-confidence alignments

457   using samtools (Li H et al. 2009). Contacts were then normalised using the r3cseq R package

458   and assigned FDR q-values to interactions, with the aim of finding those significantly over-

459   represented relative to expectation. The normalisation procedure for 4C data is adapted

460   from a previous method for normalising deepCAGE data between samples (Balwierz PJ et al.

461   2009).

462   **Analysis of 3C libraries**

463 Digestion efficiency and sample purity was assessed as described previously (Hagege H et al.

464 2007). Primers were designed using Primer3 with an anchor primer in the fragment at the 5'

465 end of the ZRS and in potential interacting fragments around the Shh promoter, gene body,

466 3' end and gene desert. Quantitative PCR was carried out using the Roche LightCycler 480

467 SYBR Green I Master and Roche LightCycler 480 probe Master on a Roche LC480 according

468 to the instructions of the manufacturer (Roche). Two PACs, RCPI21-542n10 (148kb long

469 covering Rnf32 to 5' of LMBR1) and RCPI21-508F15 (203kb long covering Shh and 150kb 5')

470 obtained from RPCI21 library (HGMP Resource Centre, Cambridge, U.K.) were used as as a

471 PCR control template. The PAC clones were cut with *Hin*dIII and equimolar amounts re-

472 ligated by T4 DNA ligase. All primer pairs were tested on a standard curve of the BAC control

473 library and yielded PCR efficiencies >1.7. The presence of a single PCR product was

474 confirmed and melting curves analysed. Cycling conditions were: 95 °C for 5 min, 40 cycles

475 of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s for the. Quantitative PCR data were

476 normalized to ZRS 3'LR ChIP oligos as a loading control. 3C oligos from Splinter E et al (2006)

477 were used to control for interaction frequencies between samples. ZRS 3'LR ChIP

478 oligos cycling conditions were 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C

479 for 30 s. The cycling conditions for the interacting fragments and the anchor were 95 °C for

480 5 min, 45 cycles of 95 °C for 15 s, 60 °C for 1', 72 °C for 1 s. Data analysis was carried out

481 according to Hagege (2007) and is presented as relative crosslinking frequency. The primers

482 used for the chromatin conformation capture interaction studies are listed in table S2.

483 **Chromatin Immunoprecipitation and Tiling Microarrays**

484 Cells from dissected E11.5 limbs and 14fp were fixed with 1% formaldehyde (25°C, 10 min)

485 and stopped with 0.125 M glycine. Crosslinked ChIP was performed as described (Stock et

486   al., 2007). In brief, the nuclei were sonicated using a Diagenode Bioruptor (Leige, full power

487   30s on, 30s off, in an ice bath for 50 min) to produce fragments of <300 bp. Chromatin (350

488   mg) was incubated with 5 mg prebound (to Protein A or G magnetic beads, Invitrogen) IgG

489   (Santa Cruz, sc-2025) or the previously mentioned antibodies, raised to in the presence of

490   50 mg of BSA, washed, and eluted. Reverse crosslinked DNA was purified with Proteinase K

491   (Glenaxxon) and QIAGEN PCR purification kit. qPCR of the ChIP experiments were carried

492   out using equal concentrations of input, IgG and Chip DNA using a Sybr Green (Roche)

493   reaction. From each biological replicate three technical replicates were analysed.

494   Enrichment values for ChIP samples from limb bud sections extracts are presented either as

495   percentage of input or as fold differences relative to IgG and normalized to input with the

496   formula $2[(Ct_{IgG}-Ct_{Input})-(Ct_{Ab}-Ct_{Input})]$ where Ct values are threshold cycles. All biological

497   replicates were carried out in duplicate unless stated. Primers used for ZRS and the controls

498   are shown in the table TS1. For the custom Nimblegen tilling arrays ChIP DNA and input DNA

499   ChIP DNA and input DNA were amplified (WGA2 kit, Sigma), labelled, and hybridized

500   according to the manufacturer's protocol to a 354,999 unique probe custom microarray

501   containing specific tiled regions of the mouse genome (Nimblegen). For the Agilent arrays a

502   custom tiling array was designed including some of the genes involved in limb development,

503   including gene deserts associated with such genes. GEO accession number for the ChIP data

504   is GSE104074.

505   **Nimblegen Arrays**

506   Microarray data were analyzed in R/Bioconductor

507   (http://genomebiology.com/2004/5/10/R80) with the Epigenome (PROT43) protocol

508   (http://www.epigenome-noe.net/WWW/researchtools/protocol.php?protid = 43) with the

509   following parameters. The mean signal intensity of the two replicate probes on each array

510   was taken. Loess normalization was used within arrays to correct for the dye bias, and scale

511   normalization was used within the replicates group to control interarray variability using the

512   R package (Limma et al., 2015). The log_2 enrichment for each ChIP group was calculated by

513   subtracting the mean of log_2 input intensities from the mean of d enriched intensities and

514   averaging over the two biological replicates.

515   **Agilent Arrays**

516   The Median Signal was extracted from the scanned image files and processed using the R

517   package (Ringo et al., 2007). Probe intensities were transformed from raw values into

518   background-corrected normalised log ratios (ChIP/Input) using Ringo's preprocess method

519   (VSN normalisation). Smoothing over individual probe intensities was performed using a

520   sliding window of 1000 bp along the chromosome and replacing the intensity at the

521   genomic position by the median over the intensities of those reporters inside the window.

522   **Immunoprecipitation of FLAG Fusion Proteins**

523   The pSV40-Tet3G- pTRE3G-mCherry- Gabpα plasmid was generated using the In-Fusion®

524   cloning technology (Clontech, catalogue No 639649). First, the pTRE3G-mCherry vector

525   (Clontech, catalogue Nos 631160 & 631175) was linearized using a unique restriction

526   site between the origin of replication and the Tet-responsive promoter (pTRE3G). Second,

527   the sequences encoding for SV40 promoter, Kanamycin/Neomycin resistance, internal

528   ribosome entry site IRES2 and Tet-ON 3G transactivator were inserted. Third, we removed

529   the Ampicillin resistance cassette. Lastly, the sequence of the self-cleaving peptide P2A was

530   cloned between the mCherry fluorescent marker and the 3xFLAG tagged Gabpα gene, in

531   order to generate a bicistronic expression under the control of the TRE3G promoter and an

532   empty control vector was also used. Overexpression analyses were performed in 14fp cells

533   by transfecting plasmids using Lipofectamine LTX with plus reagent (Thermofisher) and

534   following standard manufacturer's protocol. Nuclear extracts used in the

535   immunoprecipitation were prepared from 14fp using a NE-PER nuclear and cytoplasmic

536   extraction kit (Thermo Scientific). The nuclear extract was incubated with anti-FLAG M2

537   affinity gel (Sigma) for 3h at 4°C. The beads were washed three times with washing buffer.

538   The immunoprecipitates were eluted with 1× SDS buffer, separated on a 4%–20% Novex

539   Tris-Glycine SDS-PAGE gel (Invitrogen), transferred to a PVDF membrane (Millipore),

540   incubated with anti-FLAG (Sigma) or with anti-GABPα (Santa Cruz, sc-22810).

541

542   **Gene expression analysis, RNA library Preparation, Sequencing and Analysis**

543   Total RNA was prepared using Trizol reagent (Invitrogen) according to manufacturer's

544   protocol (for limb buds, dissected anterior, posterior distal and proximal tissue was

545   dissociated into single cell suspensions in Trizol using a syringe fitted with a 25G (0.5

546   mm) needle (BD Microlance), followed by acid phenol:chloroform:isopropyl alcohol

547   extraction and then digested with 2U DNaseI (Ambion) for 30 minutes at 37°. RNA was

548   reverse-transcribed to cDNA using QuantiTect Reverse Transcription Kit (QIAGEN). The

549   quantitative real-time PCR reactions were performed in a 7300 system (Applied Biosystems,

550   Life Technologies) by using LightCycler® 480 SYBR Green I Master (Roche) and gene specific

551   primer sets for shh, Gabpα, Etv4. The cycle threshold (CT) values form all quantitative real-

552   time PCR experiments were analysed using ΔΔCT method. Data were normalized to

553   glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as fold changes over

554 that in control treatment group. From each biological replicate three technical replicates

555 were analysed. All statistical analyses were performed using a two-tailed Student's *t*-test.

556 RNA sequencing was conducted by GATC Biotech. Samples were only submitted with an OD

557 260/280 ratio ≥ 1.8, a 260/230 ratio ≥ 1.7 and a RNA Integrity Number (RIN) value ≥ 8 as

558 detected by Agilent Technologies 2100 Bionalayser. The InViewTM Transcriptome Explore

559 service provided by GATC was used to provide a randomly primed and amplified cDNA

560 library with Illumina adaptors ready for sequencing. Illumina sequencing was conducted

561 producing 50 bp single end reads and a guarantee of over 30 million reads per sample. All

562 samples were analysed on the main Galaxy server by first checking sequence quality by

563 FastQC. Reads were then trimmed and any Illumina sequencing adaptors removed as

564 appropriate and aligned to the mouse genome (mm9, NCBI 37) using Tophat2 (Galaxy Tool

565 Version 0.9). The results for each condition were fed into Cuffdiff (Galaxy Tool Version

566 2.2.1.3) and visualised using the R Bioconductor package CummeRbund (Release 3.2). This

567 process was repeated using RNA isolated from both immortalized limb cell lines and isolated

568 limb tissue. Two biological replicates were analysed for each condition.

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720 (WMS) while complete ZRS duplications underlie Haas type polysyndactyly and

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739 **Figure legends**

740 **Figure 1. H3K4me1 and H3K27Ac distribution over the ZRS in E11.5 limb buds**

741 (A) Representation of an E11.5 mouse limb bud. The AER (orange), the ZPA (yellow) and

742 FGF10 (cyan) are responsible for the anterior/posterior limb patterning and for directing the

743 proliferation of the distal portion of the limb. The limb buds were dissected into anterior-

744 posterior or distal-proximal regions. (B) Chromatin from E11.5 dissected limb buds tissue

745 was enriched by chromatin immunoprecipitation (ChIP) for H3K4me1 and H3K27ac histone

746 modifications. DNA was quantified by qRT-PCR. Means of fold enrichment over nonspecific

747 IgG recoveries and ± SEM from two independent experiments are plotted. (C) Schematic of

748 the ZRS (dark blue box) lying in intron 5 of *Lmbr1* (blue box), 800kb away from *Shh* gene.

749 Within the ZRS (dark blue box) five ETS binding sites (light blue) and two ETV binding sites

750 (orange) are highlighted. The primers used to evaluate the ChIP experiments are localised

751 over the long-range activity and the spatio-temporal activity sequences, both indicated with

752 a blue line (complete list of oligos in Supplementary file 1). (D) ChIP-on-chip analysis of distal

753 mesenchyme from two biological replicates E11.5 limb buds using antibodies to two

754 different histone modifications (H3K4me1 and H3K27ac). Data for two different genomic

755 regions, the fifth intron of Lmbr1 gene and the *Shh* gene are shown The y axis is the Log_2 for

756 each ChIP/input DNA and the x axis represents a segment of DNA. The DNA region

757 containing the ZRS is highlighted by the grey shading. As controls, the whole of the Shh

758 coding region plus promoter (Shh) is shown.

759 **Figure 2. TSA treatment activates *Shh* in a limb derived cell line (14Fp)**

760 (A) ChIP from two biological replicates of E11.5 limb derived cell line (14Fp cell line) using

761 antibodies to two different histone modifications (H3K4me1 and H3K27Ac) analysed by

762 hybridizing to tiling microarrays. Summary is presented using two different genomic regions,

763   the y axis is log$_2$ for each ChIP/input DNA and the x axis represents a segment of DNA from

764   the microarray. The DNA region containing the ZRS is highlighted by the grey shading. As

765   controls, the whole of the *Shh* coding region plus promoter is shown. (B) Time course of the

766   expression of *Shh* in E11.5 limb derived cell line after TSA treatment detected by

767   quantitative RT-PCR. The Shh levels were evaluated relative to control and normalized to

768   GAPDH expression levels. Data points represent the average of triplicate determinations ±

769   SEM. (C) Chromatin from 14Fp was harvested 3, 6, 18 and 24 h after TSA treatment or 24h

770   with DMSO as control (ctr). Shown are results from ChIP analysis using anti-H3K27ac

771   antibody. Enrichment of H3K27ac at the 5'ST (grey), 3'LR (blue) and promoter (black) was

772   detected by qPCR and represented as mean of fold enrichment/background (IgG) ± SEM

773   over three biological replicates; a negative control region was analysed and did not give an

774   appreciable signal (data not shown). (D) Shown are results from ChIP analysis using anti-RNA

775   Pol II antibody after 24h of TSA treatment. Indicated are the *shh* promoter (black), the 5'ST

776   (grey) and control region (yellow). Recovered DNA sequences were quantified as percentage

777   of input and +/- SEM from two independent experiments and are plotted. The IgG did give

778   no detectable signal.

779   **Figure 3. TSA treatment induces a ZRS–SHH interaction**.

780   (A) The profile of 4C-Seq at the ZRS locus in 14Fp cells. Control and TSA treatment after 18h

781   and 24h are shown. ZRS shown as the enhancer (anchor) bait fragment along an

782   approximately 2Mb region of chromosome 5 (UCSC genome browser view of

783   chr5:28,000,000–30,000,000 (mm9). The x axis represents the position on chromosome 5

784   and the y axis the normalised reads as read per million sequences (RPMs). Only highly

785   significant interactions are shown (FDR q-value < 5$\rightarrow$10^5). The blue bar represents the

786   location of the ZRS (bait) and the red bar represents the *Shh* gene. Each rectangle is a

787   restriction fragment, the dots coloured at the top of each rectangle reflect the FDR q-value

788   indicating the significance of the interaction (legend between A and B). (B) Focus on the Shh

789   region (red bar in the zoomed-in view) shows the number of interactions of the bait region

790   with SHH in both untreated and TSA treated (after 24h) samples.

791   **Figure 4. FGFs are crucial in priming *Shh* limb specific enhancer**.

792   (A) qRT-PCR was used to detect the expression levels of *Shh* in 14Fp cell line after TSA and

793   nintedanib (NIN) treatment. The *Shh* levels were evaluated relative to control and

794   normalized to GAPDH levels. Data points represent the mean of three biological replicates

795   +/- SEM. (B) qRT-PCR to detect the expression of *Grem1* (grey bars) and *Etv4* (black bars)

796   after 4 h of nintedanib (NIN), with or without supplement of FGF8/10 for 6 h. Aleso shown is

797   BGJ treatment. Data points represent the average of duplicate determinations +/- SEM. (C)

798   Chromatin from the 14Fp cell line was harvest after TSA and NIN treatment and ChIP for

799   H3K4me1 was carried out. DNA was quantified by qRT-PCR using the ZRS 3'LR and *Rbm33*

800   oligos. Data are represented as mean ± SEM of the fold enrichment over nonspecific IgG

801   recoveries from two independent experiments. (D, E) ChIP analyses after NIN treatment

802   where performed to further analyse the enrichment of the transcription factor GABPα and

803   P300 over the ZRS (black) and on a specific genomic control region, Rbm33 intron (grey).

804   DNA was quantified by qRT-PCR. Mean (± SEM) of the fold enrichment over nonspecific IgG

805   recoveries from two independent experiments are plotted. (F) qRT-PCR to detect the

806   expression of *Grem1* in the distal limb bud after 4h of NIN treatment. (G) ChIP of distal and

807   proximal limb tissue from limb buds using an antibody against H3K4me1. Distal limb tissue

808   was treated for 4h with or without NIN. Proximal limb tissue was used as negative control.

809   DNA was quantified by qRT-PCR and fold enrichment over nonspecific IgG recoveries using

810   the ZRS 3'LR oligos and ± SEM from two independent experiments were plotted. (H)

811   H3K4me1 ChIP of the proximal limb tissue at E11.5 after 4h of exposure to FGF8 and FGF10.

812   Fold enrichment over nonspecific IgG recoveries and ± SEM from two independent

813   experiments are plotted. DNA was quantified by qRT-PCR using the ZRS 3'LR oligos.

814   **Figure 5. GABPα co-localises with P300 and modulates ZRS acetylation status.**

815   A) Chromatin immunoprecipitation (ChIP) analysis from two biological replicates on the

816   14Fp cell line using GABPα and p300 antibodies using tiling microarrays. Summary is

817   presented using two different genomic regions, the y axis is Log$_2$ for each ChIP/input DNA

818   and the x axis represents a segment of DNA from the microarray. The DNA region containing

819   the ZRS is highlighted by the grey shading. As controls, the whole of the *Shh* coding region

820   plus promoter is shown. (B) 14Fp nuclear cell extracts from cells stably transfected with

821   3Xflag-Gabpα (FIG. 5-SUPL. 1E) treated with or without doxycycline were analysed by

822   immuno-precipitation with anti-p300 antibody followed by Western blot analysis with anti

823   flag-tag antibody. As control the empty vector plus doxycycline was used. (C) Western blot

824   analysis with anti-GABPα of 14Fp nuclear cell extracts transiently transfected with Gabpα

825   siRNA (siGabp-α) or nonspecific siRNA (siCTR) and TSA treated. (D) Quantitative RT-PCR was

826   used to detect the mRNA levels of *Shh* (grey box) and *Gabpα* (black box) in 14Fp cells

827   transfected with *Gabpα* siRNA or nonspecific siRNA. 18 hours after transfection, the cells

828   were treated with 1μM TSA for 24 hours. *Shh* and *Gabpα* levels were evaluated relative to

829   control and normalized to GAPDH levels from two biological replicates. (E) Quantitative RT-

830   PCR to detect the mRNA levels of *Shh* (black box) and *Gabpα* (grey box) in 14Fp cells stably

831   transfected with 3Xflag-Gabpα vector and an empty vector as control. Data points represent

832   the mean ± SEM of three biological replicate. (F) Chromatin from 14Fp cells stably

833   transfected with 3Xflag-*Gabpα* vector and an empty vector as control was analysed by ChIP

834   for H3K27Ac histone modification and ETV4 enrichment. DNA was quantified by qRT-PCR

835   using the ZRS 5'ST activity oligo set. Data are represented as mean ± SEM of the fold

836   enrichment over nonspecific IgG recoveries from two independent experiments. (G)

837   Chromatin from 14Fp cells stably transfected with 3Xflag-*Gabpα* vector and an empty vector

838   as control was analysed by chromatin immunoprecipitation for GABPα and P300. DNA was

839   quantified by qRT-PCR using the ZRS 3'LR and 5'ST activity oligos sets. Average of

840   percentage of input +/- SEM from two independent experiments are plotted.

841   **Figure 6. ETV4 acts as a repressor via interactions with HDAC2**

842   (A) Quantitative RT-PCR to detect the mRNA levels of *Shh* (black box) in 14Fp cells

843   transfected with HDAC1 and HDAC2 siRNA either alone or combined and with nonspecific

844   siRNA as control. Data were collected after 18 hours of transfection. *Shh* levels were

845   evaluated relative to control and normalized to GAPDH levels. Data points represent the

846   average of triplicate determinations +/- SEM. (B) ChIP from two biological replicates using

847   the 14Fp cell line and anti- ETV4 and HDAC2 antibodies analysed by hybridizing to tiling

848   microarrays (FIG. 5-SUPL. 1B). Summary is presented using two different genomic regions,

849   the y axis is $\log_2$ for each ChIP/input DNA and the x axis represents a segment of DNA from

850   the microarray. The DNA region containing the ZRS is highlighted by the grey shading. As

851   controls, the whole of the *Shh* coding region plus promoter is shown. (C) 14Fp nuclear cell

852   extracts were analysed by immuno-precipitation with anti- ETV4 and IgG antibodies

853   followed by Western blot analysis with anti-HDAC2. (D) Quantitative RT-PCR to detect the

854   mRNA levels of *Shh* (black box) and *Etv4* (grey box) in 14Fp cells transiently transfected with

855 ETV4 siRNA (siETV4) or nonspecific siRNA (siCTR). Data points represent the average of

856 triplicate determinations +/- SEM. (E) Shown are results from ChIP analysis using anti-HDAC2

857 and ETV4 antibody after 24h of TSA treatment. Recovered DNA sequences were quantified

858 by qPCR using 5'ST oligo set. Average percentage of input and +/- SEM from two

859 independent experiments are plotted. The IgG did not give detectable signal.

860 **Figure 7. FGF signaling is responsible for priming the ZRS for local chromatin changes**

861 **mediated by ETS factors.**

862 Summary model showing how FGF signaling in the distal mesenchyme regulates the ZRS

863 poised state and that this allows the stepwise recruitment of transcriptional regulators to

864 the ZRS. FGF signaling has a dual fundamental role; firstly, as an activator it is responsible for

865 priming and maintaining the ZRS and secondly, as a repressor inducing the expression on

866 ETV4 which restricts the expression of the ZRS. ETV4 (red oval) interacts with HDAC2 (light

867 blue rectangle) to maintain the inactivity of the poised ZRS enhancer throughout the distal

868 mesenchyme, while GABPα (blue oval) acts within the ZPA domain to recruit p300 (yellow

869 rectangle), trigger H3K27 (orange circle) acetylation, and thereby activate *Shh* transcription.

870 **Figure 2-Figure supplement 1**

871 (A) RNA-seq analysis for 14Fp cell line and E11.5 distal limb bud. Fragments per kilobase per

872 million reads (FPKM+1) are plotted for a selected number of genes involved in limb

873 development. (B) RNA-seq analysis of E11.5 limb bud distal and proximal tissue and 14Fp

874 cell line. The relative expression FPKM+1 of *Hdacs* genes are represented in the chart. Two

875 biological replicates where analysed for each sample.

876 **Figure 2-Figure supplement 2**

877   (A) RT-PCR and quantitative (q) RT-PCR to detect the expression of Shh in E11.5 limb specific

878   cell line 14fp after treatment with TSA, FGF8, RA, and their combinations. The Shh levels

879   were evaluated relative to control and normalized to GAPDH levels. RT-PCR was normalised

880   to HGRPT. Data points represent the average of triplicate determinations +/- SEM. (B) RT-

881   PCR from 14Fp harvested 3, 6, 16 and 24 h after TSA treatment or 24h after DMSO

882   treatment as control (ctr). The limb specific markers Hand2, Gremlin, Alx4 levels were

883   evaluated and normalised to HGPRT. (C) Immunofluorescence analysis of endogenous SHH

884   in 14Fp cells treated without and with TSA. Cells were stained with antibodies directed

885   against the SHH C-terminal and SHH N-terminal portion of the protein (ABCAM) 1/500,

886   nuclei were counterstained with DAPI. An Alexa Fluor® 488 (1/1000) (ThermoFisher) was

887   used as the secondary antibody.

888

889   **Figure 2-Figure supplement 3**

890   (A) Chromatin was prepared from E11.5 dissected limb buds (distal, proximal, anterior and

891   posterior) and from 14Fp cells 18, 24, 30 h after TSA treatment, 24h treatment with DMSO

892   was used as control (ctr). Shown are results from ChIP analysis using anti-H3K27ac antibody.

893   Enrichment of H3K27ac at the 5'ST region was detected by qPCR and represented as mean

894   of fold enrichment/background (IgG) ± SEM over three biological replicates. (B) Quantitative

895   (q) RT-PCR to detect the expression of *Shh* in embryonic cell lines at E11.5 from the limb

896   (14Fp) and the mandibule (MD) after TSA treatment. MDs do not express *Shh* in response to

897   the treatment. The *Shh* levels were evaluated relative to control and normalized to *Gapdh*

898   levels. (C) ChIP of 14Fp and MD cell line using antibodies to two different histone

899   modifications (H3K4me1 and H3K27Ac) analysed by qPCR. Low enrichment of H3K4me1

900 with the 3'LR oligos set was observed in MD in comparison to 14Fp and no enrichment of

901 H3K27Ac with the oligos set 5'ST was observed after TSA treatment. (D) Gel with three 3C

902 samples for the 4C experiment (ctr, 18h TSA and 24h TSA). Undigested and *Hin*dIII digested

903 DNA after cross linking was run on a 0.6% agarose gel and appears as a high molecular

904 weight smear running from roughly 12-4 Kb showing that they were all efficiently digested.

905 (E) Final 3C templates run as one tight band above 10kb in size on 0.6% agarose gel for the

906 ctr, 18h TSA and 24h TSA ligated samples. On the first lane as control a digested template is

907 loaded. (F) Final 3C libraries are redigested with *Mlu*CI and the products were run on a 1.2%

908 agarose gel appearing as smear between roughly 0.3 - 1kb. As control the ligated products

909 are run together with the samples.

910 **Figure 3-Figure supplement 1**

911 (A) Gel with four 3C samples (ctr and 24h TSA, in biological replicates). Cross-linked DNA

912 was either undigested or *Hin*dIII digested cross linked DNA was run on 0.6% agarose gel.

913 DNA appears as a high molecular weight smear running from roughly 12-4 Kb showing that

914 those were all efficiently digested. (B) Final 3C templates run as one tight band above 10kb

915 on a 0.6% agarose gel for the two biological replicates for samples either treated (+) or

916 untreated (-) with TSA. (C) 3C analysis of TSA (black lines) and no TSA treated 14Fp cell line

917 (red lines). The relative level of each ligation product (fragments −1 to 5) has been plotted

918 according to its distance (in kb) from the 5' end of the ZRS (see map below graphs). The bait

919 primer and the TaqMan probe are listed in TS2 which were designed according to the

920 position of the *Hin*dIII fragment at the 5'end of the ZRS. The mean of two biological

921 replicates is plotted. The data were normalized to an internal region of the ZRS included in

922 between two *Hin*dIII fragments (ZRS 3'LR ChIP, TS1). Below the graphs, the *Hin*dIII restriction

923   fragments are indicated. *Hin*dIII fragments are numbered from fragment −1 to 5. The

924   locations of the *Shh* gene and the promoter are indicated (black rectangles, black rectangle

925   with arrow). The position of the bait is also indicated (blacks circle). (D) 3C analysis of

926   posterior (black lines) and anterior (red lines) limb tissue dissected at E11.5. (E)

927   Representation of fold change of expressing *Shh* samples versus non expressing *Shh*

928   samples. TSA treated 14Fp over untreated 14Fp relative crosslinking frequencies are

929   represented with black bars and E11.5 posterior limb tissue over E11.5 anterior limb tissue

930   are represented with grey bars. The means ± SEM are plotted.

931

932   **Figure 4-Figure supplement 1**

933   (A) Cell morphology of 14Fp was assessed after NIN and BGJ treatments by microscopy. Cells

934   were stained with 1X trypan blue and the number of blue nuclei assessed (red circles). No

935   differences were observed between the different treatments and the control. (B) Trypan

936   blue staining was performed on limb tissue dissected following a treatment of 4h with NIN

937   and analysed by microscopy. Represented are time 0, 4h media alone or 4h with NIN. No

938   differences were observed between the media alone or NIN treatment, (C) qRT-PCR to

939   detect the expression of Dusp6 after 4 h of nintedanib (NIN), with or without supplement of

940   FGF8/10 for 6 h, and BGJ treatment. Data points represent the average of duplicate

941   determinations +/- SEM. (D) Chromatin from 14Fp was harvest after BGJ treatments and

942   ChIP for H3K4me1 was carried out. DNA was quantified by qRT-PCR using the ZRS 3'LR (D)

943   and *Shh* promoter oligos (E) as control. Data are represented as mean ± SEM of the fold

944   enrichment over nonspecific IgG recoveries from two independent experiments. (F)

945   H3K4me1 ChIP of the proximal mesenchyme of E11.5 limb buds after 6h of exposure to

946 FGF8 and FGF10. Fold enrichment over nonspecific IgG recoveries and ± SEM from 2

947 independent experiments are plotted. DNA was quantified by qRT-PCR using Shh promoter

948 oligos.

949 **Figure 5-Figure supplement 1**

950 (A) Map of the plasmid pSV40-Tet3G- pTRE3G-mCherry-Gabpα, showing in schematic

951 fashion the relative positions of notable features of the plasmid. (B) 14Fp nuclear cell

952 extracts stably transfected with 3Xflag-Gabpα and with an empty vector treated with or

953 without doxycycline were analysed by Western blot analysis with anti P300 and normalised

954 with anti H3. (C-D) Zoom out of the ChIP-chip in 14Fp cell line using H3K27Ac and H3K4me1

955 antibodies (C) and ETV4 and HDAC2 antibodies (D). Visualised is the region included

956 between Shh gene and Lmbr1 gene.

957

958 **Supplementary File 1. (A)** List of oligos used for qRT-PCR, q-PCR and ChIP. **(B)** List of oligos

959 used for 3C and 4C analysis.

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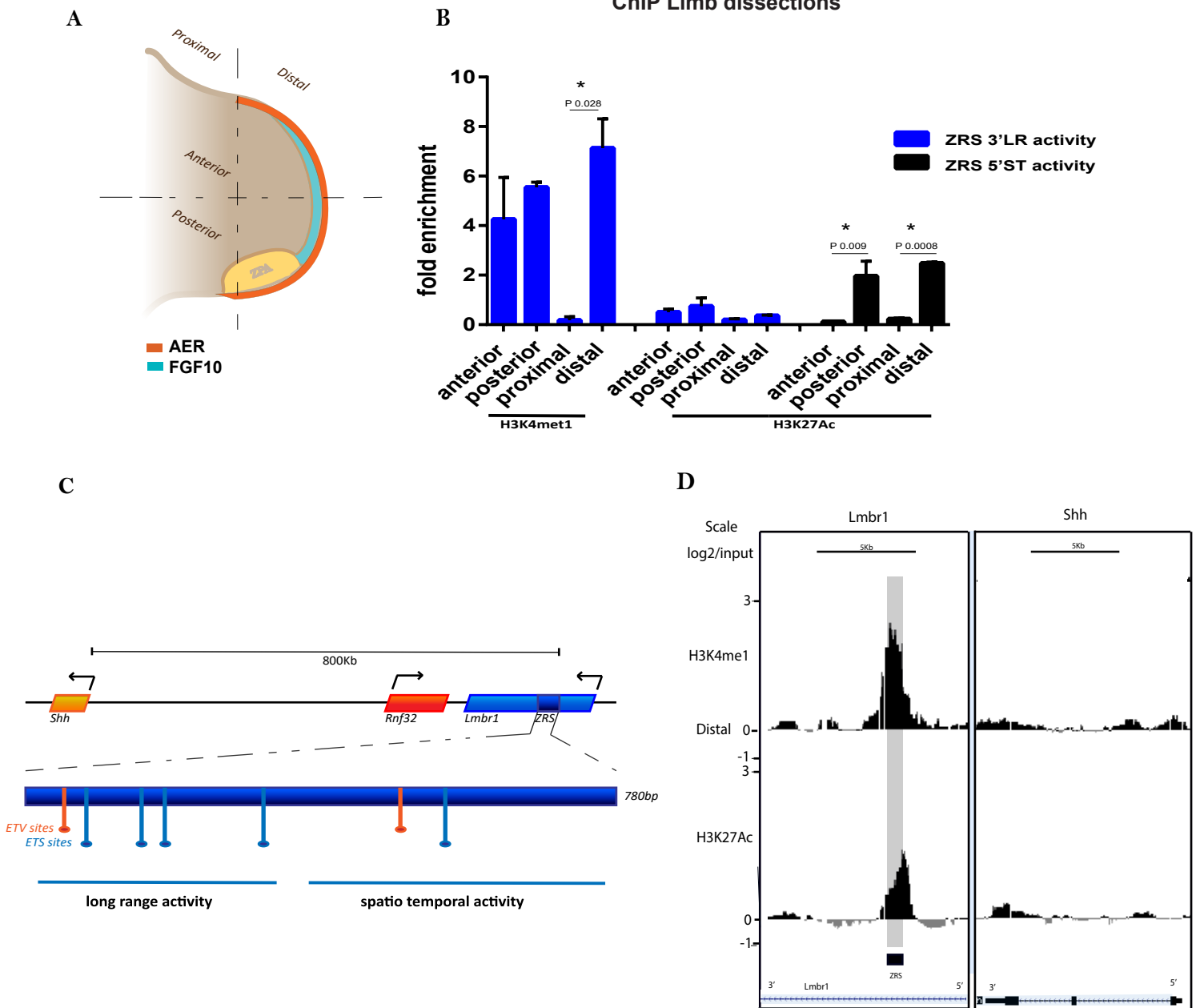
Peluso_Fig. 1

Peluso_Fig. 2

A

14fp

B
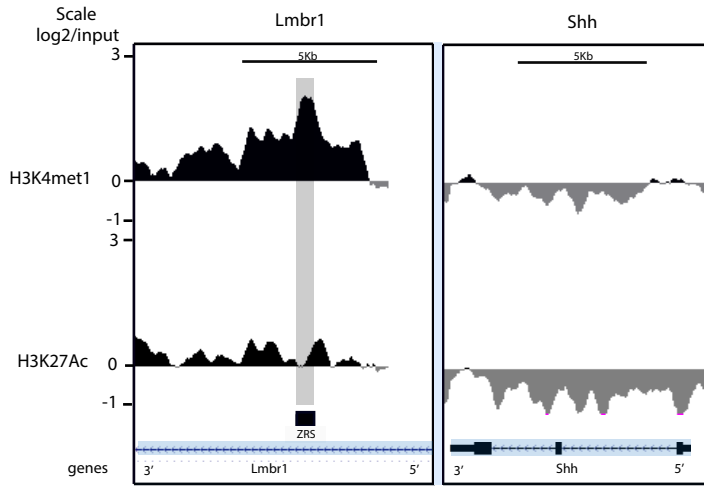
Shh expression 14fp
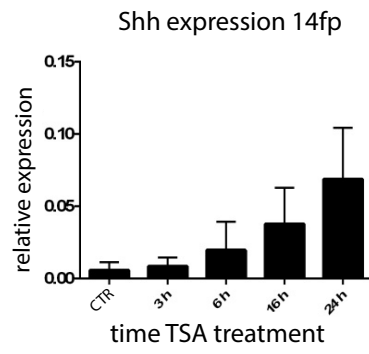
C

ChIP H3K27Ac 14Fp

D

ChIP PolII 14Fp

A

B

A

B

C

A

ChIP H3K27Ac 5'ST activity

B

Shh expression

C

ChIP MD cell line

D

4C libraries digestion efficiency

E

4C libraries ligation efficiency

F

4C libraries II digestion efficiency

Peluso_Fig. 3

A

3C libraries digestion efficiency

B

C

3C qPCR 14Fp

D

3C qPCR Limb bud dissections
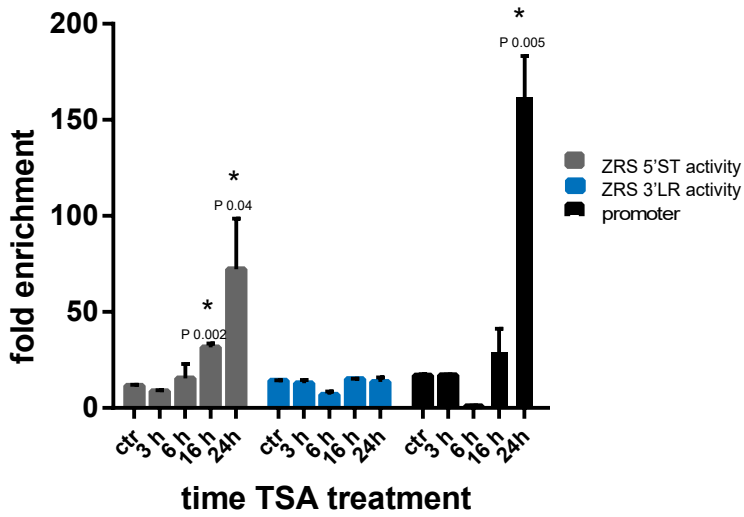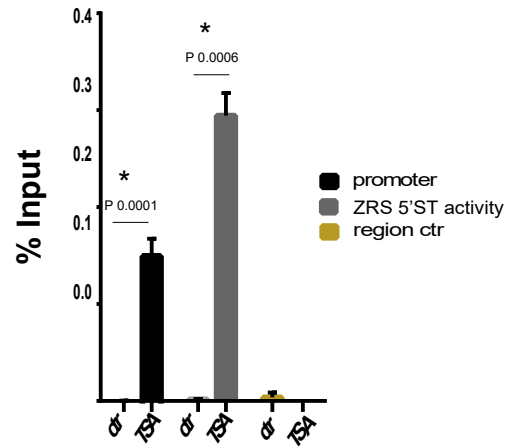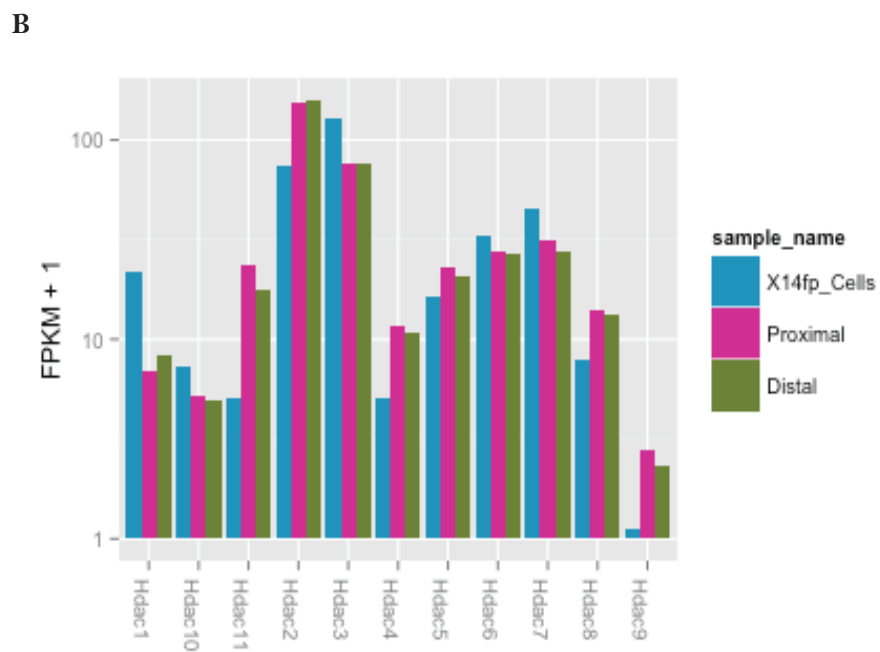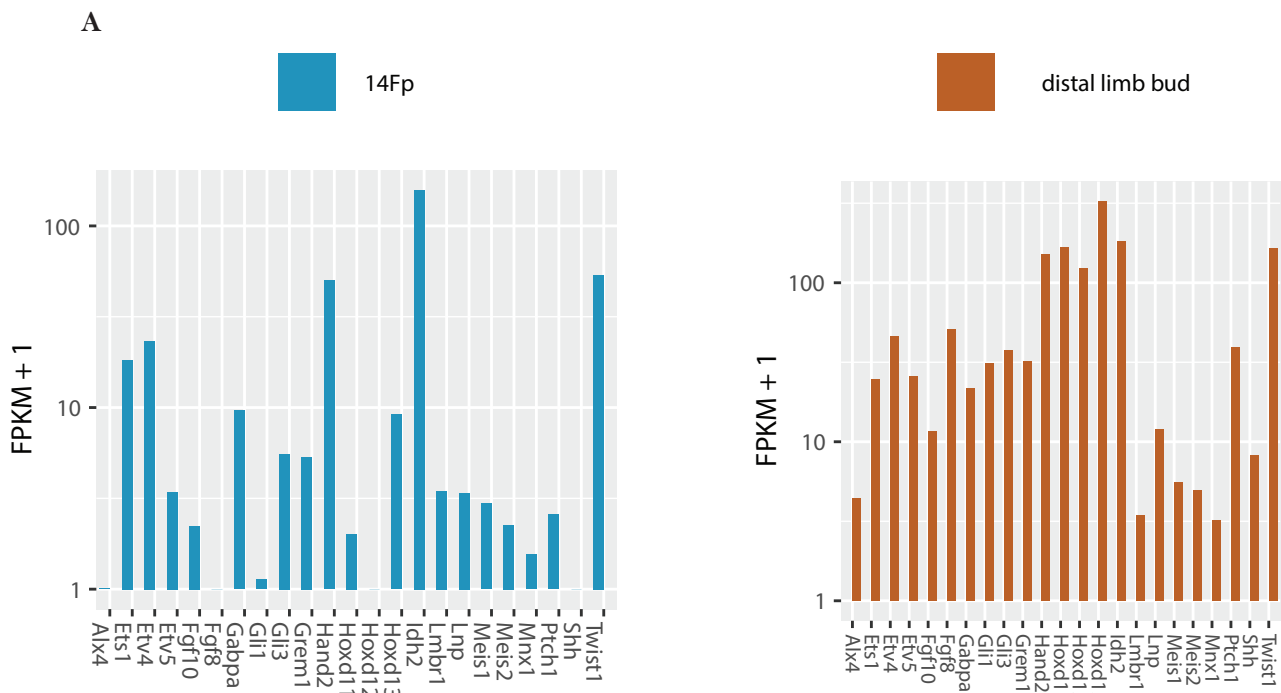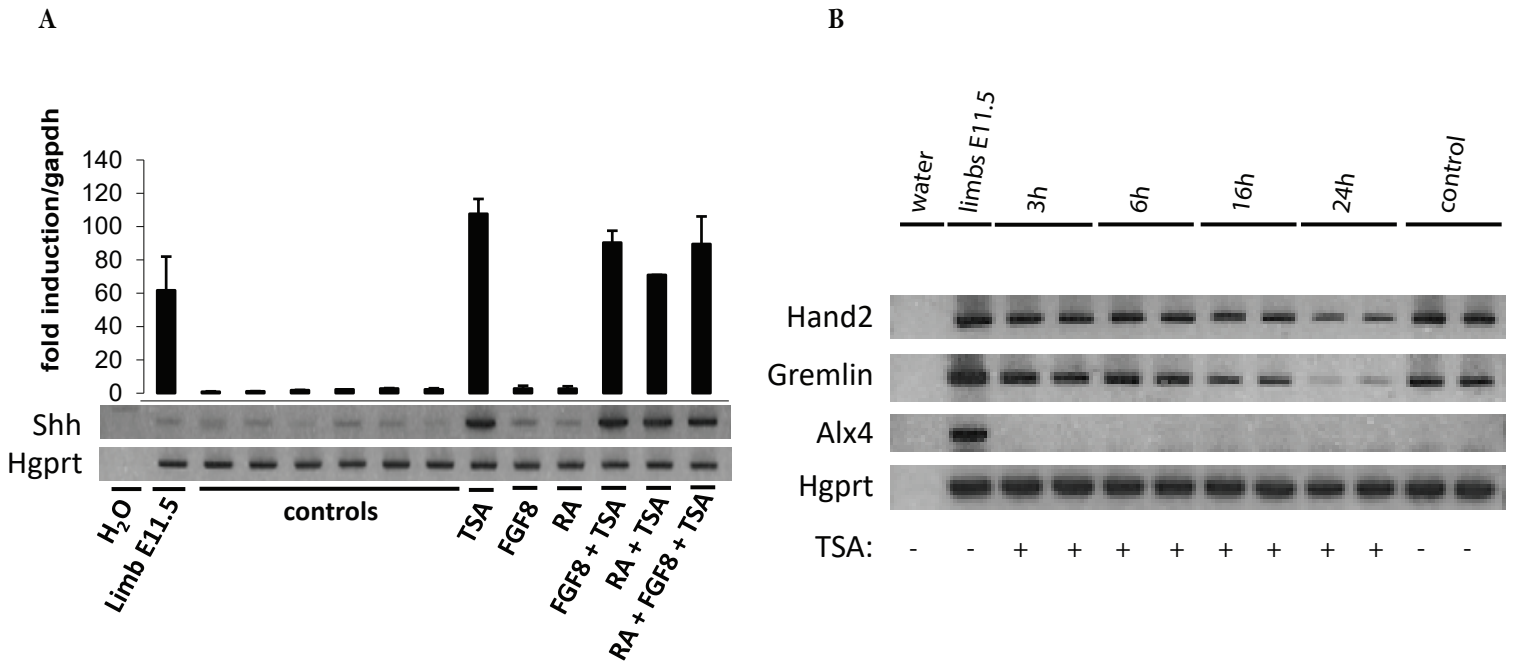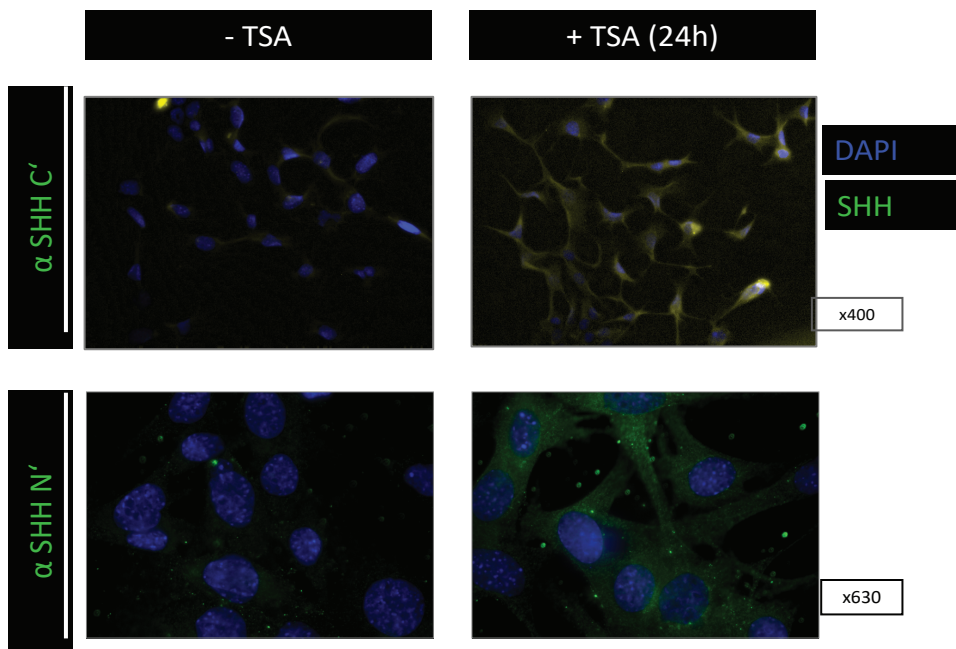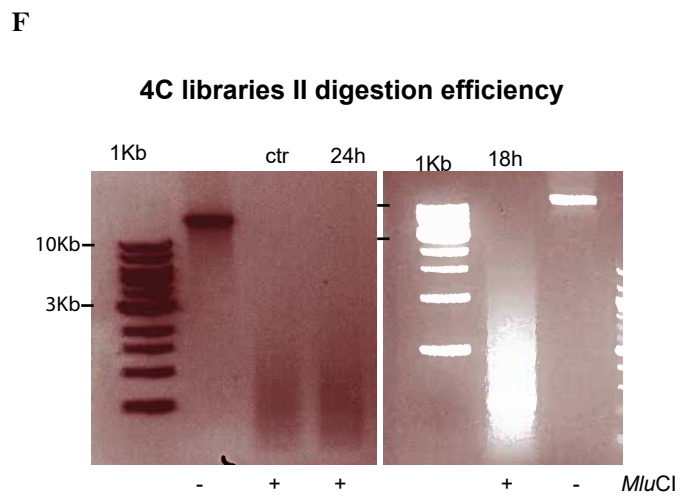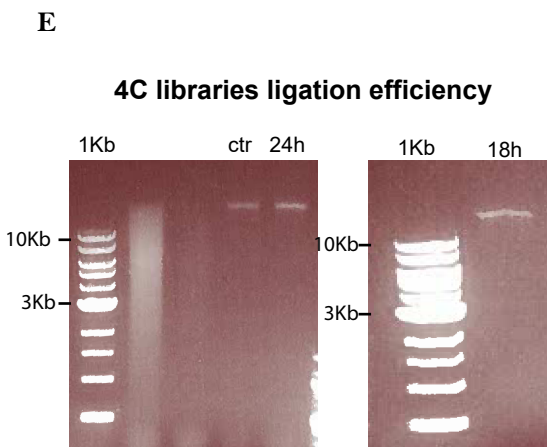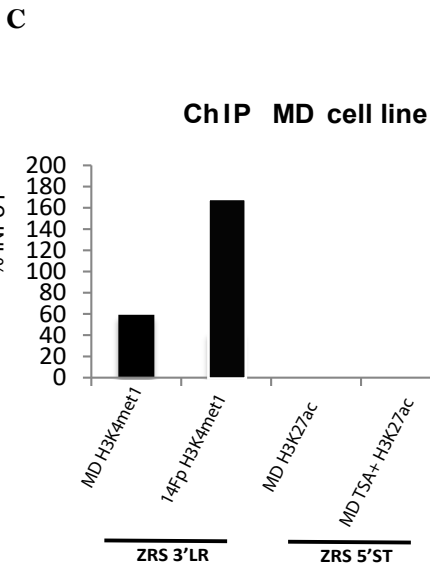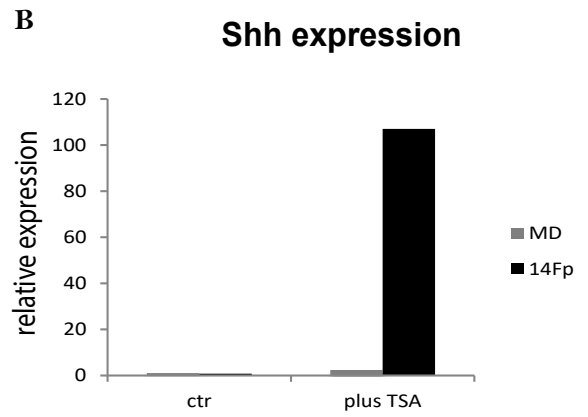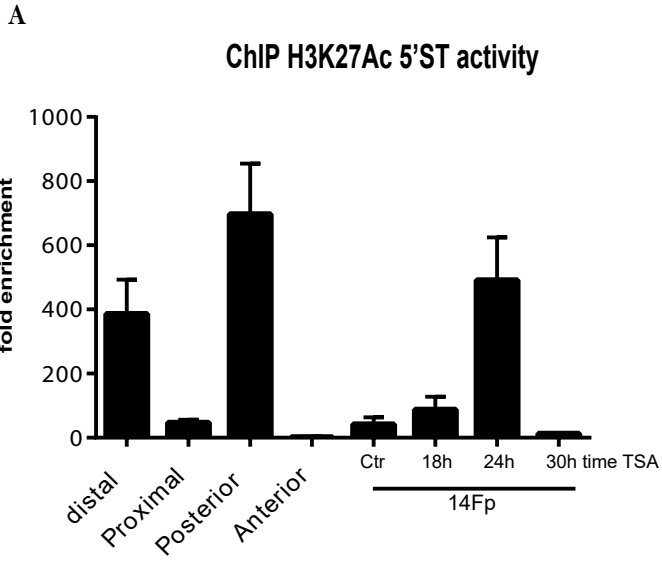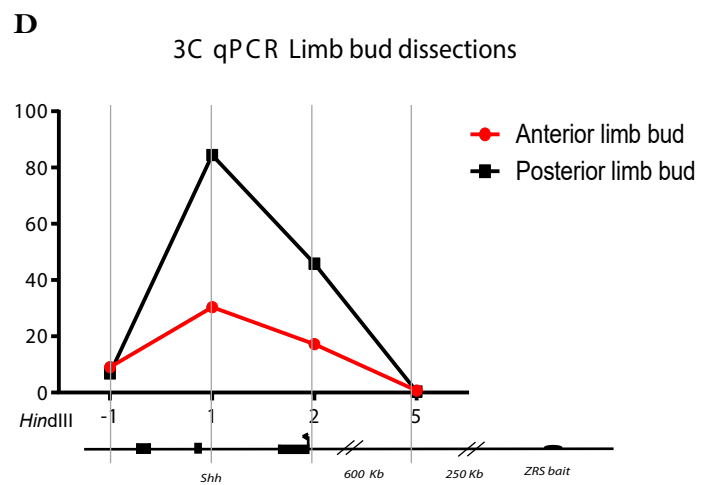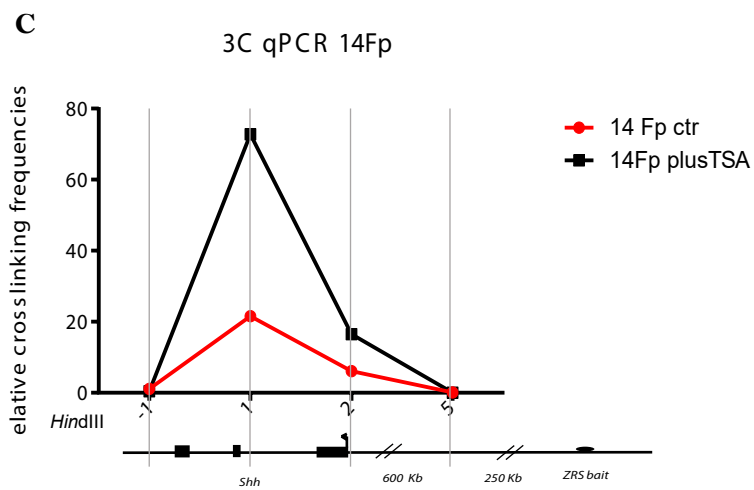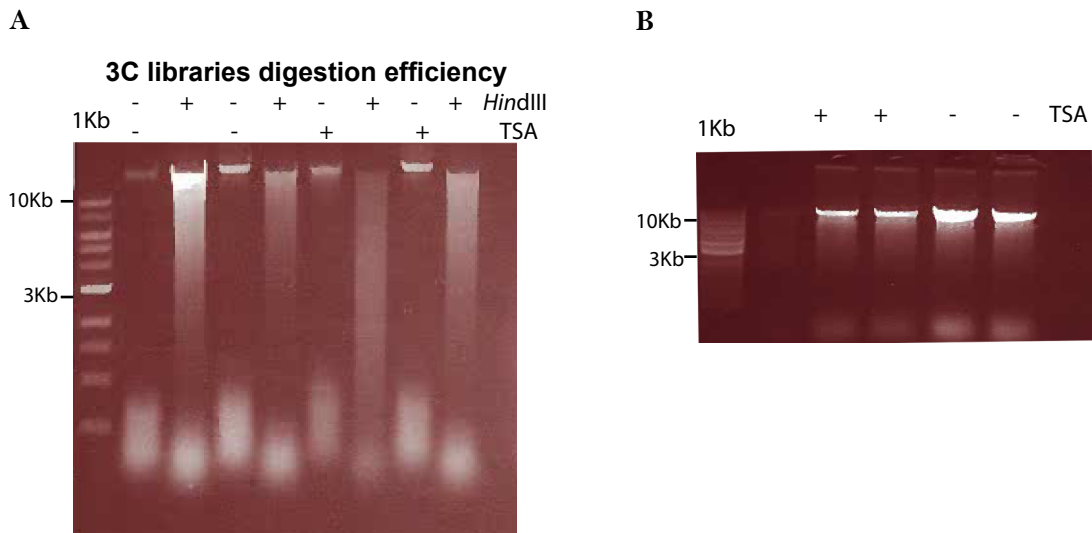
E

Fold change relative cross linking frequencies

A

B

C

Dusp6 expression 14Fp

D

ChIP H3K4me1 14Fp

E

ChIP H3K4me1 14Fp

F

ChIP H3K4met1 proximal limb dissections

A

Scale
log2/input

Lmbr1

Shh

5Kb

5Kb

3.5

0

P300

-1

3.5

0

Gabpα

-1

genes

ZRS

3'   Lmbr1   5'

3'   Shh   5'

B

INPUT   P300 IP

Kda   +   -   +   +   -   +   DOX

76—

52—

GABPα

14Fp Gabpα(−)
14Fp Gabpα(+)
14Fp Gabpα(+)
14Fp Gabpα(−)
14Fp Gabpα(+)
14Fp Gabpα(+)

α−FLAG IB

C

siRNA control   siGABP-α

Kda   -   +   -   +   TSA

GABP-α

52

α− GABPα IB

17

α−H3 IB

D

14fp Gabpα Knock down

relative expression

8

6

4

2

0

■ Gabpα
■ Shh

-    +   -   +   -   +   -   +   TSA

siCTR   siCTR   siGabp-α   siGabp-α   siCTR   siCTR   siGabp-α   siGabp-α

E

Gabpα expression

relative expression

250

200

150

100

50

0

*
P 0.035

+   +   DOX

14Fp Gabpα(−)
14Fp Gabpα(+)

Shh expression

relative expression

0.25

0.20

0.15

0.10

0.05

0.00

*
P 0.001

+   +   DOX

14Fp Gabpα(−)
14Fp Gabpα(+)

F

ChIP H3K27ac

fold enrichment

8

6

4

2

0

*
P 0.021

+   +   DOX

14Fp Gabpα(−)
14Fp Gabpα(+)

ChIP ETV4

fold enrichment

5

4

3

2

1

0

*
P 0.01

+   +   DOX

14Fp Gabpα(−)
14Fp Gabpα(+)

G

ZRS 3'LR activity

% INPUT

100

80

60

40

20

0

*
P 0.047

*
P 0.044

+   +   +   +   +   DOX

14Fp Gabpα(−)
14Fp Gabpα(+)
14Fp Gabpα(−)
14Fp Gabpα(+)
14Fp Gabpα(+)

P300   GABPα   IgG

H

ZRS 5'ST activity

% INPUT

15

10

5

0

*
P 0.02

*
P 0.047

+   +   +   +   +   DOX

14Fp Gabpα(−)
14Fp Gabpα(+)
14Fp Gabpα(−)
14Fp Gabpα(+)
14Fp Gabpα(+)

P300   GABPα   IgG

A

B

C

D

Peluso_Fig. 6

A

Shh expression 14Fp

relative expression

5
4
3
2
1
0

siRNA control | siRNA HDAC1 | siRNA HDAC2 | siRNA HDAC2/HDAC1

B

Scale
log2/input

Lmbr1

Shh

5Kb

5Kb

4

HDAC2

0

-1

4

ETV4

0

-1

ZRS

genes

3' Lmbr1 5'

3' Shh 5'

C

INPUT | IP ETV4 | IP IgG | Beads

HDAC2

Kda

74

52

α–HDAC2 IB

D

Shh expression 14Fp

relative expression

8
6
4
2
0

siRNA Ctr | siRNA Etv4

ETV4 expression 14Fp

relative expression

1.5
1.0
0.5
0.0

siRNA Ctr | siRNA Etv4

E

ChIP ZRS 5'ST activity

% Input

1.5
1.0
0.5
0.0

*
P 0.04

*
P 0.02

HDAC2 | ETV4 | HDAC2 | ETV4
-          -   +   +   TSA