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Direct functional consequences of ZRS enhancer mutation combine with secondary long range SHH signalling effects to cause preaxial polydactyly

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ABSTRACT

Sonic hedgehog (SHH) plays a central role in patterning numerous embryonic tissues including, classically, the developing limb bud where it controls digit number and identity. This study utilises the polydactylosus Silkie (Slk) chicken breed, which carries a mutation in the long range limb-specific regulatory element of SHH, the ZRS. Using allele specific SHH expression analysis combined with quantitative protein analysis, we measure allele specific changes in SHH mRNA and concentration of SHH protein over time. This confirms that the Slk ZRS enhancer mutation causes increased SHH expression in the posterior leg mesenchyme. Secondary consequences of this increased SHH signalling include increased FGF pathway signalling and growth as predicted by the SHH/GREM1/FGF feedback loop and the Growth/Morphogen models. Manipulation of Hedgehog, FGF signalling and growth demonstrate that anterior-ectopic expression of SHH and induction of preaxial polydactyly is induced secondary to increased SHH signalling and Hedgehog-dependent growth directed from the posterior limb. We predict that increased long range SHH signalling acts in combination with changes in activation of SHH transcription from the Slk ZRS allele. Through analysis of the temporal dynamics of anterior SHH induction we predict a gene regulatory network which may contribute to activation of anterior SHH expression from the Slk ZRS.

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Introduction

The zone of polarising activity (ZPA) is a transient area of posterior limb bud mesenchyme with the ability to induce and pattern extra digits when grafted to the anterior border of a host wing (Saunders and Gasseling, 1968). The ZPA was one of the first examples of an ‘organiser’ tissue, having the predicted morphogen-like capability of patterning the three digits of the chick wing in a time and concentration dependent manner (Tickle et al., 1975; Smith, 1980). First discovered in the chick, it is now recognised that all patterned vertebrate limbs use a ZPA mechanism to determine a specific number and identity of digits. Since then the limb bud has been the focus of intense experimentation and modelling with the aim of understanding the “universal mechanism whereby the translation of genetic information into spatial patterns of differentiation is achieved” (Wolpert, 1969).

Molecular studies have elucidated many components of this ‘universal mechanism’. The ZPA morphogen is now established as Sonic hedgehog (SHH), which is expressed in, and mediates the action of the ZPA in a time and concentration dependent manner (Smith, 1980; Tickle, 1981; Riddle et al., 1993; Yang et al., 1997; reviewed Tickle and Barker 2012). SHH co-ordinates limb growth and digit patterning simultaneously by maintaining Hedgehog-dependent growth from the posterior limb during the early digit patterning phase, resulting in digit pattern that is regulated by concentration and length of exposure to SHH directly and by the expansion of the limb field at later stages of limb development (the Growth/Morphogen model; (Harfe et al., 2004; Towers et al., 2008; Zhu et al., 2008)). This is mediated through a positive feedback loop with Fibroblast Growth Factors (FGFs) expressed in the overlying ectoderm, mediated by mesenchymal BMP-antagonist Gremlin1, promoting outgrowth of the limb (The SHH/GREMI/FGF feedback loop; Lauffer et al., 1994; Niswander et al., 1994; Lewandoski et al., 2000; Michos et al., 2004; Bénazet et al., 2009; Galli et al., 2010; Zeller, 2010). Both SHH expression and limb out-growth is terminated when high levels of FGF signalling inhibits Gremlin1 expression which disrupts the SHH/GREMI/FGF feedback loop (Verheyden and Sun, 2008).

The localisation, timing of SHH expression and strength of SHH signalling is tightly controlled to create a localised morphogen...
source, key to creating a signalling gradient in order to specify digit identity (Wolpert, 1969). The regulation of SHH expression is crucial for correct digit patterning. In the posterior limb, SHH has been shown to be autoregulatory in a negative manner as exposure to high concentrations of SHH protein induces cell death of SHH expressing cells (Sanz-Ezquerro and Tickle, 2000) while conversely inhibition of Hedgehog signalling can increase Shh expression (Schierz et al., 2007). In addition, implantation of SHH-expressing cells in the anterior of the limb can induce SHH in endogenous tissue after 48 h (Duprez et al., 1999) demonstrating that as in the neural tube, the anterior of the limb bud has the potential to express SHH (Tanaka et al., 2000) in response to SHH signalling, although the time lag suggests that this is likely to be indirect. Native SHH autoregulation in the developing limb bud, in un-manipulated circumstances has yet to be reported.

Shh expression is restricted to precise anatomical locations in the lung, larynx, pharynx, gut and limb by a number of highly conserved long range, tissue-specific, cis-regulatory elements (Lettice et al., 2003; Sagai et al., 2005, 2009). The limb specific enhancer is known as the ZPA Regulatory Sequence (ZRS; (Lettice et al., 2003); also MFCS1; (Sagai et al., 2005)). Mutations within the ZRS are associated with preaxial (anterior) polydactyly in multiple species and are thought to drive ectopic expression of Shh in the anterior portion of the limb bud, acting as a de facto ZPA. (Lettice et al., 2003, 2008; Park et al., 2008; Dunn et al., 2011). It has been proposed that cis-regulatory regions contain multiple binding sites for essential transcription factors (homotypic clustering; (Gotea et al., 2002)). This has been demonstrated in the ZRS, which contains multiple ETS factor binding sites with both repressive and activating effects on Shh expression in the limb, which when disrupted by mutations within the ZRS, cause polydactyly in humans (Lettice et al., 2012).

Previously we mapped the dominant chicken Polydactyly locus (Po) in the Silkie (Silk) chicken breed, which has anterior (preaxial) polydactyly in the leg, to a novel single nucleotide polymorphism (SNP) in the chicken ZRS (Dunn et al., 2011). Chicken feet normally have four digits, labelled anterior–posterior from I to IV. Preaxial polydactyly in the Silk breed is most commonly observed as an extra digit II (II,III,IV). Unlike other ZRS mutants, the Silk ZRS SNP is not within nor creates a predicted ETS binding site. Uniquely among ZRS mutants, induction of polydactyly in the Silk leg is both time and posterior ZPA dependent. This suggests that ectopic anterior Shh expression is the consequence of intact limb bud gene expression and signalling feedback loops which are abnormally activated by aberrant posterior gene expression. Indeed, we have shown that FGF4 and GREM1 are expressed ectopically in the Silk leg (Dunn et al., 2011). Tissue recombination experiments, however, demonstrate that induction of Silk polydactyly is genotype specific, as ectopic Shh is not induced in anterior Wt tissue recombined with Silk posterior leg mesenchyme (Dunn et al., 2011). Based on these observations we have previously proposed a model, based on the Growth/Morphogen model (Towers et al., 2008) which suggests that extra Shh signalling observed in the posterior Silk leg may cause growth and long-range patterning effects which leads to preaxial polydactyly (Dunn et al., 2011). To test this hypothesis we propose that induction of anterior Shh and preaxial polydactyly in the Silk is dependent on three conditions which we test here; an increase in Shh protein from posterior mesenchyme, upregulation of normal limb responses to increased Shh signalling, such as growth and additional FGF signalling, and additional activity of the ZRS conferred by the Silk ZRS SNP in both anterior and posterior tissue. Based on our evidence we propose a model to explain the temporal regulation of polydactyly and ectopic Shh expression by the Silk ZRS.

Materials and methods

Animal maintenance

Polydactyly (Silk) and White Leghorn (WL) and talpid3 (rt3) chicken lines are maintained at the Roslin Institute under UK Home Office licence after ethical review. Birds were genotyped from gDNA using primers for ZRS SNP and the SHH promoter non-synonymous SNP as per Dunn et al. (2011). For breeding purposes and to control for breed specific traits, all experiments were undertaken using embryos produced by a SilkWt/SilkWt × WLWt/WLWt cross. For simplification, unless otherwise stated resulting embryos will be referred to in the text as the following: SilkWt/WLWt = Silk/Wt, SilkWt/WLWt = Silk/Wt

Embryo manipulations

Tungsten foil was inserted into small slits prepared between somites 29 and 30 in the leg mesenchyme using fine tungsten needles. Cyclopamine (Sigma) was prepared to a 1 μg ml⁻¹ concentration in 45% 2-hydropropyl-β-cyclodextrin (Sigma) and smoothed agonist (SAG, Calbiochem) was prepared to a concentration of 0.2 μg ml⁻¹ concentration in water. 5 μg of cyclopamine or 1 μg SAG was injected directly onto the embryo (so that the entire embryo was surrounded with compound), via a small hole made in the vitelline membranes. AG1-X2 beads were soaked in 1 mg ml⁻¹ all-trans retinoic acid (Sigma), 10 mM SU5402 (Sigma) or 1 mg ml⁻¹ trichostatin A (Sigma) for 20 min, control beads in DMSO. After washes with DMEM beads were inserted into stage 18–20HH limbs using fine tungsten needles. E10 embryos were stained with alcin green and cleared with methyl salicylate. Digit identity in the foot was assigned by phalanx number and numbered I, II, III, IV from anterior–posterior. Numbering of wing digits is 1, 2, 3 from anterior to posterior. Nile Blue staining was performed as per Dunn et al. (2011). Total limb bud area and ANZ area (post Nile Blue staining) was determined using the “Perimeter” function of Image J, with area calculated in-program.

Protein quantification

Stage 21HH and 24HH legs were homogenised in RIPA buffer (Fisher) containing protease inhibitors, centrifuged, and the supernatant is collected. Protein concentration was estimated using a DC Protein Assay kit (Bio-Rad). Recombinant mouse SHH N-terminus protein (R&D Systems) was used as a positive control. Protein samples were loaded as individual limbs per lane. Proteins were separated by electrophoresis using pre-cast 12% gels (Fisher) containing protease inhibitors, centrifuged, and the supernatant was windowed and staged (Hamburger and Hamilton, 1951) and to control for breed specific traits, all experiments were undertaken using embryos produced by a SilkWt/SilkWt × WLWt/WLWt cross. For simplification, unless otherwise stated resulting embryos will be referred to in the text as the following: SilkWt/WLWt = Silk/Wt, SilkWt/WLWt = Silk/Wt.

Quantitative real-time PCR and RFLP assays

Fertilised Silkie/White Leghorn eggs were incubated at 38 ºC, windowed and staged (Hamburger and Hamilton, 1951) and dissected between 17 and 27HH. Wings were taken whole and legs either whole (stages 17–23HH) or dissected into posterior and anterior halves (stages 24–27HH). Tissue dissociation, CDNA synthesis, RFLP–PCR and densitometry were carried out as per Dunn et al. (2011). SHH primers: Forward CCAACCTGCTTTGTTG; and
reversed AGAGCGGTGACTCAAATG. qRT-PCR was carried out using a Brilliant III Ultra-Fast SYBR Green QPCR mix (Agilent) in a Stratagene MX 3000. Standard curves of known molar concentration of PCR product were prepared in triplicate from leg cDNA. Absolute quantities of SHH and LBR were calculated using standard curves generated by MatInspector (Genomatix, (Cartharius et al., 2005)) in silico Bioinformatics. PTCH1 (Bell et al., 2004) and compared to microarray data generated by databases, and further analyses were performed using GEISHA (Marigo et al., 1996).

Whole-mount in situ hybridisation

RNA probe synthesis and whole-mount RNA in situ hybridisation were performed as per Nieto et al. (1996). Probes were synthesised from the following templates: SHH (Roelink et al., 1994), HOXA13 (Nelson et al., 1996), HOXD13 (ChEST414K15, Ark Genomics), and PITCH1 (Marigo et al., 1996).

Bioinformatics

Predicted transcription factor binding sites were determined in silico using MatInspector (Genomatix, (Cartharius et al., 2005)) databases, and further analyses were performed using GEISHA (Bell et al., 2004) and compared to microarray data generated by the eChickAtlas (Wong et al., 2013).

Electrophoretic mobility shift assays

5' biotin-labelled oligonucleotides (Sigma) were annealed to produce double-stranded DNA probes. Nuclear extracts were prepared from anterior and posterior halves of stage 24HH Slk/WL legs using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). 3 μg of nuclear extract was incubated with binding buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM DTT, 0.5 mM MgCl2), 1 μg poly dIdC (Thermo Scientific) and unlabelled competitor (if required) for 10 min at room temperature. 10 fmol biotin-labelled probe was added, and incubated for further 20 min. Reactions were separated by native electrophoresis at 100 V, 4°C, using equilibrated 6% polyacrylamide gels and 0.5× TBE. Nucleotides were transferred onto positively charged nylon membranes, developed using a Chemiluminescent Nucleic Acid Detection module (Thermo Scientific) and detected using autoradiography film. Oligos:

Wt ZRS 5′-3′ AATGAGCTTTCTGCACTGCTTTCAATT;
Slk ZRS 5′-3′ AATGAGCTTTATGCACTGCTTTCAATT;
Slk ZRS 3′-5′ AATAGAAGAGCATGCAATT;
Slk ZRS 5′-3′ AATGAGCTTTAATGCACTGCTTTCAATT;
Slk ZRS 3′-5′ AATAGAAGAGCATGCAATT.

Results

Increased SHH in the posterior Slk leg bud causes expression of anterior ectopic SHH and preaxial polydactyly

The anterior Slk leg develops an ectopic area of SHH expression at late stage 25HH, which leads to preaxial polydactyly (Arisawa et al., 2006; Dunn et al., 2011). Localised surgical ablation has previously demonstrated that ectopic anterior SHH expression and preaxial polydactyly in the Slk leg require posterior Slk leg bud mesenchyme (Dunn et al., 2011). To demonstrate that the prevention of polydactyly in these manipulations was due to the loss of a diffusible factor contained in the posterior tissue, rather than the

![Fig. 1](image-url). Increased SHH protein from the posterior limb is required for the formation of preaxial polydactyly. (A–D) Foil insertion between the anterior and posterior leg bud causes loss of posterior digits (B) and prevents preaxial polydactyly (D n=3/3). (E,F) Increased SHH protein in stage 21 (E) and 24HH (F) Slk/WL legs (± SEM, n=4, P<0.0005). (G–N) Cyclopamine causes loss of posterior and anterior SHH (H, n=3/3, L, n=5/5) compared to controls (G, K; arrows and asterisks) and causes loss of posterior digits (J, n=7/14) and preaxial ectopic digits (N, n=13/16) compared to controls (I, M). Scale = 0.5 mm.
ablated tissue itself we interrupted communication between the posterior and anterior mesenchyme using foil barriers inserted into stage 20HH leg buds. Control manipulations in which developing leg buds were cut but no foil barrier was inserted, caused no change in digit number (Fig. 1A, Wt/Wt digit pattern I,II,III,IV; Fig. 1C, Slk/Wt digit pattern II,II,III,IV). Insertion of a foil barrier between anterior and posterior leg bud mesenchyme, however, disrupted posterior digit patterning in both Wt/Wt and Slk/Wt legs (Fig. 1B, n=5/6) and in addition Slk/Wt legs failed to form ectopic anterior digits (Fig. 1D, n=3). By preventing communication between posterior and anterior leg mesenchyme, ectopic anterior SHH expression and preaxial polydactyly was not induced in Slk/Wt leg buds (Fig. S1A, Fig. 1D). This demonstrates that Slk/Wt posterior leg tissue contains a diffusible inductive signal which induces ectopic anterior SHH and preaxial polydactyly.

The Slk ZRS SNP alters posterior SHH expression and causes upregulation of GLI1, a gene responsive to Hedgehog signalling (Dunn et al., 2011). Thus we expect that SHH is the posteriorly localised, diffusible factor, mediating induction of preaxial polydactyly in the Slk leg. Quantitative Western blot analysis confirmed

![Fig. 2. Inhibition of FGF signalling and growth prevent polydactyly. (A-P) Localised inhibition of FGF signalling at stage 20HH prevents ectopic SHH and polydactyly. Insertion of DMSO soaked beads into the anterior (A–D) and posterior (I–L) AER-mesenchyme did not alter SHH expression or digit pattern for each genotype (A,I, arrows in C, K). SU5402 beads placed in the anterior AER-mesenchyme caused loss of digits I and II in both genotypes (F,H, n=4/4), including preaxial digits in Slk/Wt (H, n=3/3). Ectopic SHH was prevented in Slk/Wt (G, 2/2) whereas posterior SHH remained in both genotypes (E,G; asterisks, n=4/4). SU5402 administered to the posterior AER-mesenchyme caused a reduction in ZPA SHH (M,O; asterisks, n=5/6) and prevented anterior ectopic SHH in Slk/Wt (O n=2/2). Digits III and IV were lost from each genotype (N,P, n=9/11) as well as preaxial digits in the Slk/Wt (P, n=4/5). (Q–T) TSA applied to the posterior AER-mesenchyme ablated SHH expression in Wt/Wt legs (Q) and caused loss of posterior digits (R, n=9/11), whilst Slk/Wt embryos retained SHH expression (S) and retained greater numbers of posterior digits, but lost ectopic digits (T, n=11/15) when compared with DMSO controls (I–L). (U) Increased leg area, total protein (Wt/Wt n=6, Slk/Wt n=18, p < 0.05, ± SEM) and γ-tubulin protein (n=4, p < 0.00005, ± SEM) in Slk/Wt legs. Scale = 0.5 mm.

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that SHH protein levels were significantly increased in posterior Slk/Wt leg buds at stages 21 and 24HH compared to Wt/Wt leg buds (Fig. 1E, 1.6-fold increase in SHH protein in Slk/Wt legs stage 21HH; Fig. 1F, 1.4-fold increase in SHH protein in Slk/Wt legs stage 24HH. $n=4$ for each stage. $P>0.0005$). To confirm that the posterior factor required for the induction of anterior SHH and preaxial polydactyly was SHH, we inhibited SHH signalling at stages 17–20HH (prior to expression of ectopic anterior SHH) using cyclopamine (Chen et al., 2002a). Of the treated embryos, 7/14 Wt/Wt embryos lost digit 4 (Fig. 1J), while 13/16 Slk/Wt embryos maintained four digits but failed to develop ectopic digits (Fig. 1I). The remaining 3/16 legs were polydactylous. As the majority of the Slk/Wt legs did not form anterior-ectopic digits, this confirms that anterior Slk polydactyly is dependent on posterior SHH expression. To test if anterior SHH expression was lost in Slk/Wt legs we repeated the experiment by treating embryos with cyclopamine or carrier solution at stage 17HH, and assayed SHH expression at stage 25HH via whole mount in situ hybridisation. Whilst the carrier solution control had no effect (Fig. 1G), cyclopamine inhibited SHH expression in the posterior of Wt/Wt leg (Fig. 1H, n=3/3). SHH expression was absent in the anterior Slk/Wt leg whilst posterior SHH was maintained (Fig. 1L; n=5/5). This demonstrates that SHH signalling is required to induce anterior ectopic SHH expression in Slk/Wt legs and that there is a reduced ability of cyclopamine to downregulate posterior SHH expression or digit IV induction in Slk/Wt legs compared to Wt/Wt legs. We conclude that overexpression of SHH in the ZPA of the posterior leg bud is the primary inductive signal which induces anterior SHH expression and preaxial polydactyly in the anterior Slk leg.

Increased activation of SHH/GREML/FGF feedback network and growth, controls induction of Slk preaxial polydactyly

The inability of cyclopamine to fully repress posterior SHH and digit IV identity in Slk/Wt legs suggests that the SHH/GREML/FGF feedback loop is not fully disrupted by cyclopamine treatment. In addition, Slk legs exhibit prolonged SHH expression and extended FGF4 and Gremlin domains (Dunn et al., 2011) which may be responsible for the induction of anterior ectopic SHH. To test this possibility we inhibited FGFR signalling by inserting beads soaked in SU5402 (Mohammadi et al., 1997) between the AER and mesenchyme at stage 20HH, prior to the induction of preaxial polydactyly. Application of SU5402 between the AER and mesenchyme of the anterior limb caused a localised loss of tissue, resulting in the loss of digits I and II (Fig. 2F n=4/4), including preaxial polydactylous digits (Fig. 2H n=4/4). Digits III and IV were unaffected. Anterior expression of SHH was not observed in Slk/Wt leg buds (Fig. 2G n=2/2), even though posterior SHH expression was maintained in all leg buds (Fig. 2E,G n=4/4). It is likely that SU5402 treatment in the anterior mesenchyme prevents a response to ectopic SHH by inhibiting cell proliferation, leading to a loss of preaxial digits. We then extended this analysis by applying SU5402 to the posterior limb bud. Digits III and IV were lost in both Wt/Wt and Slk/Wt (Fig. 2N,P n=9/11). Posterior SHH expression was weakly maintained in both Wt/Wt and Slk/Wt legs (Fig. 2M,O, asterisks, n=5/6). Slk/Wt legs failed to develop preaxial polydactyly (Fig. 2P, n=4/5) or express anterior SHH (Fig. 2O, n=2/2). The loss of tissue growth and SHH/FGF feedback in the posterior limb affected not only digits IV and III but also prevented the formation of preaxial digits in the anterior limb, whilst leaving digits I and II unaffected. These findings demonstrate that inhibition of posterior FGFR can prevent anterior ectopic SHH and subsequent preaxial polydactyly by reducing posterior SHH expression and tissue growth.

SHH signalling controls and integrates both proliferation and patterning in the limb (Growth/Morphogen model; Towers et al., 2008; Zhu et al., 2008). To identify whether additional SHH from the posterior leg mesenchyme causes increased limb growth in Slk/Wt leg buds, we measured leg bud area and protein content in Slk/Wt leg bud at stage 21HH. Leg bud area, total protein content and γ-tubulin were all increased in Slk/Wt compared to Wt/Wt (Fig. 2U). To establish if increased SHH-dependent limb bud expansion is required for the induction of anterior-ectopic SHH and preaxial polydactyly in Slk/Wt leg buds, we locally inhibited growth by implanting TSA-soaked beads into the posterior leg bud mesenchyme, proximal to the ZPA, at stage 18–19HH (Towers et al., 2008; Zhao et al., 2009; Towers et al., 2011). Unlike previous observations, where application of TSA at stage 20HH inhibits anterior digit formation while maintaining posterior digit identity (Towers et al., 2008), Wt/Wt TSA-treated legs either lost posterior digits or had posterior–anterior digit identity transformation (Fig. 2R n=9/11). Slk/Wt TSA-treated legs had posterior–anterior digit identity transformation but only one leg bud had a loss of digit IV (Fig. 2T n=13/15). Furthermore anterior-ectopic digits failed to form in 11/15 Slk/Wt TSA-treated legs (Fig. 2T). As is seen in Towers et al. (2008) (24 h after treatment with TSA) SHH expression was lost in Wt/Wt legs (Fig. 2Q), but only reduced in Slk/Wt legs (arrows, Fig. 2S). This correlates with the increased number of digits retained in Slk/Wt leg, which were also of a more posterior SHH dependent nature (digit III, although digit IV was not observed). We attribute our posterior digit loss to the timing of TSA application, during the early phase of SHH-dependent digit patterning. SU5402 and TSA treatments both exhibited the same prevention of anterior SHH and preaxial digit formation, suggesting that the effect of SU5402 was due to a localised loss of growth. However in accordance with the Growth/Morphogen model, induction of preaxial polydactyly was dependent on limb bud expansion driven by growth in the posterior leg.

Silk wings are more responsive to inductive signals activating SHH expression

Ectopic anterior SHH and leg preaxial polydactyly in Slk/Wt legs, therefore, is primarily dependent on increased SHH produced by the posterior mesenchyme. Recombination experiments between Wt and Silk leg tissue, however, have shown that posterior mesenchyme is not entirely sufficient to induce polydactyly (Dunn et al., 2011). Although allelic imbalance is also observed in the wing ZPA, and therefore the Silk ZRS SNP is not leg specific, preaxial polydactyly and ectopic anterior SHH expression is only observed in the leg in Silk birds and not the wing (Arisawa et al., 2006). We utilised the lack of ectopic SHH in the anterior Silk wing to investigate the SHH transcription response in Silk/Wt to retinoic acid (RA) which induces expression of SHH in Wt wings after implantation of a bead soaked in 1 mg ml$^{-1}$ RA after 24 h (Riddle et al., 1993). Following implantation of RA-soaked beads in to the anterior wings of Wt/Wt and Silk/Wt embryos, Wt/Wt wings did not express SHH after 21 h (Fig. 3AA, n=5) whereas a small area SHH expression was detected distal to the bead in Silk/Wt wings (arrow, Fig. 3BB, n=6). Wings of both genotypes showed ectopic expression of SHH after 26 h of incubation (Fig. 3CD; Wt/Wt n=8, Silk/Wt n=5), and both genotypes form identical mirror image digit duplications (Fig. 3EF). To further examine the ability of Silk tissue to initiate SHH expression we utilised a Smoothened agonist (SAG) to activate the Hedgehog pathway in the Silk/Wt wing (Frank-Kamenetsky et al., 2002; Chen et al., 2002b). Titration of SAG activity in Wt/Wt wings found that 5 μg SAG/embryo at 17–20HH induced an additional digit 2 (not shown), whereas treatment with 1 μg SAG/embryo did not induce SHH expression (Fig. 4AF, n=3/3) or polydactyly (Fig. 4B,F, n=6/6) in the wing or leg. Application of 1 μg SAG/embryo to Silk/Wt embryos, however, induced ectopic anterior SHH expression (arrow, Fig. 4G n=3/5) and
polydactyly in Slk/Wt wings (Fig. 4H n=4/12). Surprisingly application of 1 μg SAG/embryo prevented ectopic SHH and PITCH1 expression and polydactyly in the Slk/Wt leg (Fig. 4O; P n=9/12; Fig. 52 n=1). The ability of the Slk/Wt wing to express SHH in response to RA earlier than Wt/Wt and at sub-optimal concentrations of SAG confirms that the Slk ZRS can also act in the wing if the correct
conditions are provided. It has previously been shown that expression of SHH is controlled in the wing by apoptosis in an area of posterior mesenchyme known as the posterior necrotic zone (Sanz-Ezquerro and Tickle, 2000; Zuzarte-Luís and Hurlé, 2002) and it has been proposed that different patterns of apoptosis between the wing and leg may underlie the limb specificity ability to autoregulate SHH levels (Dunn et al., 2011). Analysis of cell death in the anterior necrotic zone (ANZ) in the wing and leg by Nile blue staining shows that the ANZ area is significantly reduced in the Slk/Wt leg compared to Wt/Wt (Fig. S3C,D). The Slk/Wt wing also exhibits reduced ANZ area (Fig. S3A,B). However, comparing ANZ area of the Slk/Wt wing and leg shows that whilst cell death is reduced, the wing still maintains robust cell death compared to the anterior leg (Fig. S3E). Therefore we propose that a lack of anterior SHH expression in the Slk wing may be a consequence of maintained apoptotic cell death.

**SHH controlled by Slk ZRS is expressed before SHH controlled by the Wt ZRS**

We then utilised the Slk/Wt leg to elucidate the dynamics of SHH autoregulation when SHH signalling is perturbed. To do this we constructed an allelic-specific expression profile of SHH in the posterior leg utilising a non-synonymous SNP within the SHH gene (Fig. 5A). This allowed us to examine expression of the Wt SHH allele in an abnormal SHH signalling environment (Slk/Wt leg buds). We carried out semi-quantitative RFLP assays on leg buds from stages 17 to 27HH to assess relative expression from Wt or Slk SHH alleles (Fig. 5B, Fig. S1B). As expected there was equal expression from both alleles in Wt/Wt leg buds from 17 to 27HH (Fig. 5B, green and yellow series). In contrast at stage 17HH, SHH in the Slk/Wt leg was largely expressed from the mutant Slk allele (~85%) which continued to account for ~65% of SHH expression throughout leg development (Fig. S1B, red series). As the RFLP assay is restricted to determining relative contribution of each allele, we performed quantitative RT-PCR analysis (qRT-PCR) in Wt/Wt and Slk/Wt legs to determine SHH mRNA at stages 17, 22 and 25HH. The general trend in both genotypes was an increase in SHH expression between stage 17HH and 22HH followed by a decrease between stage 22HH and ~25HH (Fig. 5B). Overall SHH expression in Slk/Wt legs was significantly increased at all three stages compared to the control Wt/Wt legs. By combining the RFLP data with qRT-PCR data and normalising to stage 17HH Wt/Wt SHH expression, we were able to accurately compare expression of each allele in Slk/Wt relative to Wt/Wt legs. In Slk/Wt legs at all stages, the Slk SHH allele is expressed at increased levels (Fig. 5B, approximately 3 fold increase at stage 22HH red series) while the Wt SHH allele level is comparable to Wt SHH alleles in Wt/Wt legs (Fig. 5B; compare blue series to yellow and green series). Analysis of relative SHH allele contribution in the anterior Slk/Wt leg, where expression is initiated at stage 25HH, confirmed that ectopic expression initially occurs only from the Slk SHH allele.

**Fig. 5. Absolute and relative quantification of allelic contribution to SHH expression in posterior and anterior legs.** (A) The Slk C > A SNP (red) is located in the 794 bp ZRS region residing within intron 5 of the LMBR1 gene, 328Kb upstream of the SHH gene in chicken chromosome 2 (Dorshorst et al., 2010). A non-synonymous SNP within the SHH promoter (C > T, arrow) was utilised to track allelic expression. (B) Combined data from absolute quantitation of SHH mRNA and relative expression from each allele in stages 17, 22 and 25HH posterior legs. In Wt/Wt posterior legs, SHH is expressed equally from both alleles (orange and green series). In Slk/Wt posterior legs the majority of SHH expression arise from the mutant Slk/Wt: ‘T’ allele (red series) compared to the wild type Slk/Wt: ‘C’ allele (blue series) (n = 5 for each stage). (C) Example of RFLP assay to determine relative allelic expression of SHH. Genomic DNA, neck cDNA and Wt/Wt posterior leg cDNA exhibit equal allelic expression, whilst Slk/Wt posterior leg cDNA shows reduced contribution from the wild type allele. (C) Onset of ectopic SHH expression occurs completely from the mutant Slk allele at stage 25HH (red series), with both alleles contributing equally from late stage 25HH onwards.

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Fig. 5C, red series), followed within half a stage (1.5–2h) by expression from the *Wt* SHH allele (Fig. 5C, blue series). This suggests that once ectopic expression is initiated from the mutant *Slk* SHH allele, the *Wt* SHH allele contributes in an autoregulative manner in order to maintain ectopic SHH expression.

Cyclopamine has previously been shown to increase SHH expression in the ZPA (Scherz et al., 2007). We sought to characterise allelic contribution to this increase in ZPA SHH upon inhibition of the Hedgehog pathway. Cyclopamine treatment at stage 17HH normalised allelic imbalance in *Slk/Wt* legs at stage 19HH, with equal expression from each allele (Fig. S1C). qRT-PCR and ISH of cyclopamine treated *Slk/Wt* legs showed a significant increase of SHH expression (Fig. S1D), suggesting that inhibition of the Hedgehog pathway drives increased SHH expression from each allele, regardless of genotype. Both the 1.5–2h transcription response of the *Wt* SHH allele during ectopic SHH and the increased posterior SHH induced by cyclopamine (~6 h) suggest that SHH transcription can be rapidly induced via autoregulation.

**Slk ZRS SNP causes changes in transcription factor binding**

The ZRS is a long-range enhancer of SHH, containing many transcription factor binding sites which mediate SHH expression through a combination of repression and activation (Lettie et al., 2012). To assess if the *Slk* ZRS mutation either creates or disrupts a transcription factor binding site, we examined the binding profiles of the *Wt* and *Slk* ZRS sequences surrounding the SNP site by EMSA. Labelled *Wt* and *Slk* ZRS probes yielded similar binding profiles when incubated with *Slk*/Wt leg nuclear extract, including a grouped set of upper bands (Band 1) and Band 2 (Fig. 6A). In addition the labelled *Slk* probe yielded an additional 'Band 3' (Fig. 6A). Nuclear extract from posterior and anterior halves of leg...
buds produced identical binding patterns (Fig. 6A). Whereas competing Slk labelled probe with unlabelled Slk probe depleted all three bands at varying concentrations (Fig. 6B), unlabelled Wt probe only competed Bands 1 and 2, and Band 3 remained (Fig. 6C). This suggests that Band 3 is due to a genuine protein: DNA interaction caused by an alteration of protein binding by Slk ZRS SNP, whereas Band 2 may represent a protein binding elsewhere on the probe, uninvolved with the Slk SNP site.

In order to identify candidate transcription factors which may account for the additional Slk ZRS SNP specific Band 3 we identified a number of conditions that must be met by a candidate transcription factor in order to bind to the Slk ZRS SNP. Candidate transcription factors must be co-expressed in areas of Slk limbs which express SHH and which exhibit allelic imbalance; the posterior regions of the wing and leg during stages 17–27HH and the proximal-anterior mesenchyme of the Slk leg from stage 25HH. Candidate factors may bind to the Slk ZRS in the anterior Slk wing, but ectopic SHH is prevented due a lack of cell death reduction (Fig. 53B). In addition the candidate transcription factor would have SHH dependent expression, as ectopic anterior SHH in the leg is induced by increased SHH signalling from posterior mesenchyme, the Slk wing expresses ectopic SHH in response to SAG (Fig. 4G), and anterior SHH expression is prevented by application of cyclopamine (Fig. 1L). The Slk ZRS SNP is a C > A change in a highly conserved region of the ZRS, which creates a small AT-rich region with similarity to canonical HOX binding sites (Georges et al., 2010; Hueber and Lohmann, 2008; Knosp et al., 2007). We used MatInspector to search for possible binding sites for transcription factors created by the Slk ZRS SNP. This suggested the creation of 19 potential transcription factor binding sites, of which 13 contained homeodomains (Table S1; Genomatix; Cartharius et al., 2005). While analysis of published and publically available gene expression patterns (GEISHA, Bell et al., 2004; eChickAtlas, Wong et al., 2013) with Affymetrix microarray expression analysis Wt and talpid2 chicken limbs (Bangs et al., 2010) determined that most MatInspector candidates did not fulfil the candidate gene criteria (Table S1), HOXA13, however, had appropriate spatiotemporal expression in Wt limbs. We therefore compared HOXA13 with HOXD13 in Slk/Wt, Wt/Wt embryos to confirm that it fulfilled our candidate criteria, as well as in talpid2 embryos to confirm its responsiveness to SHH signalling. We used HOXD13 as a comparison a it has been shown to bind to the Wt ZRS in complex with HAND2 (Galli et al., 2010) and has previously been shown not to be expressed in the Slk anterior leg until after initiation of SHH (Dunn et al., 2011). We confirmed that HOXA13 expression domain is anteriorly expanded in the Slk/Wt at stage 23HH (Fig. 6, compare E to F), the crucial time point for induction of preaxial polydactyly in the Slk leg (Dunn et al., 2011). Although HOXA13 is expressed in the anterior of both Slk/Wt and Wt/Wt legs at 25HH (Fig. 6, compare H to I), we have shown that at this point the Slk/Wt limb is refractory to manipulation of polydactyly at this point and therefore the action of a candidate gene must act prior to this. Expanded expression of HOXA13 in talpid2 limbs at all stages (Fig. 6G,J) demonstrates that it is a SHH-responsive gene (due to the loss of Gli repressor function in talpid2 limbs; Daviey et al., 2006). In comparison HOXD13 expression is also expanded anteriorly across the Slk/Wt legs at both stage 23HH and stage 25HH compared to Wt/Wt (Fig. 6 compare K to L and N to O) although the anterior border of expression does not reach the domain of ectopic SHH expression (Fig. 6O). As HOXA13 fulfilled the criteria we had determined to bind ectopically to the Slk ZRS, we performed supershift EMSA using an anti-HOXA13 antibody to test if the Slk specific ‘Band 3’ was due to ectopic HOXA13 binding. This, however, shifted the upper bands (Fig. 6D, Band 1), not Band 3, suggesting that although HOXA13 interacts with both the Wt and Slk ZRS in vitro, it does not account for the Slk specific Band 3.

In conclusion, HOXA13 is not responsible for ectopic SHH induction in the anterior Slk leg. The nature of the Slk exclusive Band 3 (Fig. 6A–D, Band 3), however, remains unknown.

**Discussion**

**Slk preaxial polydactyly is dependent on direct misexpression of SHH and on secondary long range patterning events**

In this study we investigate how the novel Slk ZRS SNP controls expression of ectopic SHH. In a previous analysis of the Slk leg we proposed a model, based on the Growth/Morphogen model (Towers et al., 2008) which suggested that the increased SHH signalling observed in the posterior Slk limb causes both growth and patterning effects which underlie the induction of preaxial polydactyly (Dunn et al., 2011). To test this model we examined three conditions; the expression of SHH and concentration of SHH protein sourced from the posterior mesenchyme, the subsequent changes in the limb regulatory network, and the action of the Slk ZRS in controlling SHH expression compared to Wt ZRS. Here we have shown these conditions form the basis of the induction of preaxial polydactyly and increased/ectopic SHH expression in the Slk leg, giving an insight into the dynamics and long-range patterning effects which can be induced in response to perturbed SHH signalling. In addition, our analysis has yielded important data on the autoregulation of SHH transcription in limb development, when SHH signalling is altered. These findings have relevance for all areas – normal and pathological – in which SHH is expressed. Small molecule inhibitors of Hedgehog signalling, such as Vismodegib, which has a similar mode of action to cyclopamine, have been used successfully in the treatment of basal cell carcinomas in which Hedgehog signalling is activated by mutations inSMO or PTCH1, downstream of expression of a Hedgehog ligand (Von Hoff et al., 2008). The action of Vismodegib on other tumours which are dependent on excess expression of Hedgehog ligand, such as pancreatic adenocarcinoma, has not been so successful (Sarris et al., 2013). Our work suggests that in response to a loss of Hedgehog signalling through small molecule inhibition, expression of the Hedgehog ligand can be highly and rapidly upregulated (Fig. S1C, D), which may subsequently cause an increase in Hedgehog signalling once the dose of Hedgehog inhibitor has lost efficacy.

Previously we had shown that the Slk ZRS causes a number of changes in SHH expression; SHH is expressed both in a larger posterior domain and for a longer period. We also demonstrated, indirectly, that the formation of preaxial polydactyly is dependent on posterior leg tissue (Dunn et al., 2011). In this study we have shown that more SHH is produced by posterior cells, and importantly, that there is a proportional increase in SHH protein to leg volume (Fig. 1E,F). Inhibition of Hedgehog signalling in the posterior leg (Fig. 1G–N) confirmed that the dependence of preaxial polydactyly induction is due to increased SHH protein originating in posterior leg mesenchyme.

The final requirement of our model was that alterations in SHH and growth in the leg must still be able act upon anterior tissue of the correct genotype. This was based on recombination experiments which demonstrated that additional SHH produced in the posterior Slk mesenchyme alone was not sufficient to induce preaxial polydactyly (Dunn et al., 2011). Perturbation of SHH signalling via application of RA or SAG to the wing bud suggests that the Slk ZRS SNP increases tissue responsiveness to exogenous treatment, causing endogenous tissue to express SHH at an earlier time point than the Wt equivalent (Fig. 3B,B, Fig. 4G). Thus we propose that in Slk legs additional signalling from the posterior mesenchyme, which is not sufficient to induce SHH expression in
Wt anterior leg tissue, is sufficient in Slk anterior mesenchyme. Surprisingly application of SAG inhibited preaxial polydactyly formation in the Slk leg (Fig. 4P). SAG application has been shown to upregulate Ptch1 in mouse embryos (Frank-Kamenetsky et al., 2002) which we believe would change the diffusion dynamics of the additional SHH protein in the posterior Slk leg bud, limiting its action to the posterior mesenchyme. SAG is also known to be an inhibitor of Hedgehog signalling if added in excess or in combination with high levels of endogenous Hedgehog signalling (Frank-Kamenetsky et al., 2002). Therefore the posterior Slk leg may be more likely to reach the SAG concentration threshold for Hedgehog pathway inhibition than the Wt leg.

The striking absence of preaxial digits in the Slk wing may be explained by the environment of anterior wing tissue. Recent work has shown that temporal differences in the onset of ANZ formation, coupled with AER regression from the presumptive anterior digit forming mesenchyme, might be responsible for the reduced number of digits in the chicken wing (Nomura et al., 2014). We had previously observed reduced cell death in the anterior Slk leg (Dunn et al., 2011). The retention of significant ANZ size in the wing compared to the highly reduced ANZ of the Slk leg (Fig. S3) could explain why ectopic SHH and preaxial digits are never observed in the Slk wing. The requirement of anterior AER-mesenchyme interaction to induce SHH in the anterior Slk leg (Fig. 2G,H) may not be achieved in the Slk wing due to the time-dependent regression of the AER away from proximal-anterior mesenchyme. Our data suggests that even with increased SHH-dependent growth in posterior tissue, anterior tissue must be responsive (reduced ANZ cell death and necessary genotype) in order to form preaxial digits.

We have demonstrated that the Slk ZRS exhibits a different binding profiles compared to the Wt ZRS (Fig. 6), although the identity of the binding complex exclusive to the Slk ZRS (Fig. 6A, Band 3) remains unknown. HOXA13 binds both the Wt and Slk ZRS in vitro, and does not appear to be responsible for the additional band (Band 3) binding to the Slk ZRS. However, a caveat in our approach is that the in vitro binding affinity of HOX proteins may not represent the greater specificity of in vivo DNA:HOX protein interactions (Georges et al., 2010). We are further investigating the binding dynamics at this locus.

The time-dependent element to the induction of anterior preaxial polydactyly by posterior SHH expressing tissue, exemplified by the delay between posterior SHH expression at stage 17HH and the expression of SHH in the anterior limb at late stage 25HH (Fig. 7), is not observed in other polydactylous models such as the AUS and AC human mutations (Lettice et al., 2012). In AUS and AC ZRS mutants a combination of ETS genes are expressed throughout the mouse limb in a manner that is neither SHH, nor time dependent (Lettice et al., 2012; Ristevski et al., 2002) and therefore activation of the transcription of SHH in the anterior of the limb bud is not time dependent as it is in the Slk ZRS mutant. This highlights that even closely associated SNPs may have widely varying mechanisms by which their action is precipitated.

**Dynamics of autocrine regulation of SHH expression in the limb**

Our work suggests that mutations within the ZRS, combined with ectopic growth and increased feedback loops cause preaxial polydactyly (Fig. 7A,B). SHH expression is upregulated in Slk/Wt posterior legs from stage 17HH (Fig. 5B). Subsequent increase in SHH protein causes an increase in Hedgehog-dependent leg growth (Fig. 7, green arrows) and feeds into the SHH/FGF/GREM feedback loop by driving increased FGF4 expression (Fig. 7B; Dunn et al., 2012).
et al., 2011). By stage 25HH, ectopic Fgf4 is in the AER and expressed in the anterior Shh (Dunn et al., 2011). At this point ectopic Shh expression is initiated, mediated by altered transcription factor binding to the Shh SNP site (Fig. 6A, Band 3). Initial expression is driven solely from the Shh ZRS (Fig. 5C) and triggers a de novo, ectopic Shh/Fgf/Grem feedback loop. Autoregulation of Shh expression occurs, with both alleles contributing to the ectopic Shh that results in preaxial polydactyly. The temporal and inductive aspects of this study propose a novel model for the mechanics and timing of preaxial polydactyly induction. As an example of a common human developmental disorder in a highly conserved regulatory element it illustrates how closely related mutations can have diverse outcomes in the developmental biology underpinning the phenotype.

Contributions

EJJ undertook experimental work, analysis and manuscript preparation. DMM contributed cyclopamine manipulations. ICD developed experimental approaches. MGD conceived and supervised the study and undertook manuscript preparation and intellectual contribution.

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Appendix A. Supporting information

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