Development 137, 3591-3601 (2010) doi:10.1242/dev.050070
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Cell-autonomous integrin control of Wnt and Notch signalling during somitogenesis

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SUMMARY

Integrins act at signalling crossroads, and their interactions with other signal transduction pathways are key to the regulation of normal and pathological cell cytoarchitecture and behaviour. Here, we describe a signalling cascade that acts during the formation of the defining segmental features of the vertebrate body – the somites – in which β1-integrin activity regulates epithelialisation by controlling downstream Wnt and Notch activity crucial for somite border formation. Using in vivo transcriptional inhibition in the developing chick embryo, we show that β1-integrin in the anterior presomitic mesoderm activates canonical Wnt signalling in a cell-autonomous, 'outside-inside' manner. Signalling is mediated by integrin-linked kinase (ILK), leading to modulation of glycogen synthase kinase 3β (GSK3β) phosphorylation, and activates Notch signalling in the anterior presomitic mesoderm. The two signalling pathways then cooperate to promote somite formation via cMESO1/Mesp2. Our results show that β1-integrin can regulate cell shape and tissue morphogenesis indirectly, by regulation of downstream signalling cascades.

KEY WORDS: Notch, Wnt, Integrin, Signalling, Somitogenesis, Chick

INTRODUCTION

Integrins are transmembrane proteins that are required for a variety of developmental processes, including cell adhesion, migration, gastrulation and morphogenesis, proliferation and signal transduction. Heterodimers of α and β transmembrane chains together bind to a variety of extracellular matrix molecules and ligands, including fibronectin for the β1-integrins (Juliano, 2002), thereby modulating interactions between the cell membrane and the cytoskeleton (Legate et al., 2006). Integrin-containing complexes interact with actin filaments and focal adhesion complexes, but also regulate, and are regulated by, receptor tyrosine kinases and other signalling pathways (Hannigan et al., 2005; Legate et al., 2006).

Two modes of integrin signalling have been proposed (Abram and Lowell, 2009). 'Inside-outside' signalling is well-described in lymphoid and other systems, and occurs when an intracellular stimulus (from a trans-acting signalling pathway) increases the ligand-binding affinity of integrins, leading to the recruitment of extracellular matrix that can remodel cell shape and tissue organisation (Abram and Lowell, 2009; Legate et al., 2006). 'Outside-inside' signalling results when extracellular ligand binding to integrin receptors activates integrin-linked kinase (ILK), a conserved, intracellular kinase-like protein that binds to the cytoplasmic tail of β1-integrin (Hannigan et al., 1996).

One process that appears to rely on integrin activity is segmentation, which is the sequential production of axially repeated balls of epithelial cells (somites) that give rise to vertebrae, skeletal muscles and some of the dermis. Bilateral pairs of somites form at regular intervals by budding off from the anterior of the unsegmented presomitic mesoderm (PSM) that lies on either side of the neural tube (Pourquié, 2001). Regular somite production depends on a molecular oscillator ('segmentation clock') that drives cyclic transcription in the posterior PSM (Ozbudak and Pourquié, 2008; Palmeirim et al., 1997).

α5-integrin mutations affect segmentation in both mouse and zebrafish (Jülich et al., 2005; Koshida et al., 2005; Yang et al., 1999). However, the mutations disrupt different regions in the two animals: zebrafish mutants lack only the first five to seven somites, whereas only posterior somites are affected in mutant mouse embryos. These results suggest that integrin signalling is not obligatory for somitogenesis.

The segmentation phenotype of zebrafish *α5-integrin* mutant embryos has been explained by an inability to recruit fibronectin ligand as an inside-outside response to Notch signalling, at least in anterior somites (Jülich et al., 2005). Nevertheless, it remains possible that integrins act by regulating other signalling pathways. Segmentation critically depends on both Notch and Wnt signalling, which are required in the posterior PSM for the segmentation clock and in the anterior PSM for somite differentiation (Aulehla et al., 2008; Dunty et al., 2008; Saga et al., 1997). Integrin signalling has previously been linked to Wnt signalling: in vitro, ILK can phosphorylate and inactivate glycogen synthase kinase 3β (GSK3β), a negatively acting component of the Wnt pathway (Oloumi et al., 2006; Sutherland et al., 1993). Interactions between integrin and Notch signalling have also been reported in cultured cells, although with very inconsistent outcomes that have been explained by a diversity of different mechanisms (Campos et al., 2006; Hodkinson et al., 2007).

In this paper, we use the chick embryo to investigate the mechanism of β1-integrin action during somite formation. We show that knockdown of β1-integrin in the PSM abolishes somite formation along the entire body axis, indicating that integrin activity is required for the formation of all chick somites. We

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Accepted 16 August 2010

further define an outside-inside signalling pathway in which β1-integrin signals cell-autonomously via ILK to activate Wnt signalling in the anterior PSM. Wnt signalling then activates Notch signalling, and these target pathways act in combination to drive somite boundary formation. These results define a regulatory cascade by which β1-integrin regulates cell and tissue architecture in vivo.

MATERIALS AND METHODS

Eggs

Fertilised chicken eggs (Henry Stewart, Louth, UK) were incubated at 37°C and were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Electroporation

Electroporation conditions were based on those described previously (Momose et al., 1999). In summary, DNA plasmids were diluted to 1-2 μg/μl in PBS containing 2% sucrose and mixed with Fast Green for visualisation of the injection site. For electroporations at HH9, DNA was injected in ovo over the anterior primitive streak. Electrodes were placed at either side of the embryo and five 50-msecond pulses of 35V were applied. For electroporations in the node and primitive streak at HH4/5, the negative and positive electrodes were placed above and below the embryo, respectively, and five 50-msecond pulses of 10V were applied.

Embryo culture

Chick embryos were cultured in L15 medium supplemented with 15% foetal calf serum in a roller incubator at 37°C. For pharmacological inhibition of signalling pathways, embryos were cultured for 6 hours in 10-100 μM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), 1 μg/ml recombinant mouse Dkk1 (R&D Systems), 5 mM LiCl, or 200 μM CKI-7 (Sigma-Aldrich).

In situ hybridisation, immunohistochemistry and western blot

Whole-mount in situ hybridisation was carried out as previously described (Henrique et al., 1995). Immunohistochemistry was performed using standard methods with mouse anti-chick fibronectin (B3/D6 and VA1; supernatant 1/5, Developmental Studies Hybridoma Bank), rabbit polyclonal anti-GFP (1/500, Invitrogen/Molecular Probes), and V2E9 monoclonal anti-β1-integrin (Hayashi et al., 1990) (supernatant 1/30, Developmental Studies Hybridoma Bank). The specificity of the anti-β1-integrin antibody was further confirmed using RNAi knockdown. The appropriate Alexa Fluor-conjugated secondary antibodies were used at 1/500 (Invitrogen/Molecular Probe), and final samples mounted in Slowfade Gold Anti-Fading Reagent (Invitrogen/Molecular Probes) and observed under a Zeiss LSM510 confocal microscope. For western blots, anterior PSM from three embryos of each category were dissected, homogenised and lysed in RIPA buffer (Harlow and Lane, 1988). Rabbit polyclonal pSer9-GSK3β (AbCam) and monoclonal γ-tubulin (Sigma) antibodies were used at 1/1000. Detection was performed using the ECL Plus Western Blotting Detection System (Amersham) according to the manufacturer's protocol.

Plasmids and morpholino oligonucleotide (MO) knockdown

cDNAs encoding chick *β1-integrin* and the *Notch1* intracellular domain (N^{ICD}) were cloned into the *pCAGGs-IRES-nlsGFP* expression vector (Stamataki et al., 2005). Wild-type and mutant forms of *Ilk* cDNAs and human *dnMAML* fused to *GFP* (*dnMAML-GFP*) were cloned into the *pMIWIII* expression vector (Suemori et al., 1990). For RNAi against β1-integrin, 22-nucleotide target sequences were chosen using the GenScript design tool (http://www.genscript.com/ssl-bin/app/rnai). Six sequences were cloned into *pRFPRNAi* plasmids (Das et al., 2006). Plasmids containing the target sequences 5′-GCGATCGATCAAACGGTTTGAT-3′ and 5′-TGTGAATGTAGTACAGATGAAT-3′ were the most efficient at depressing β1-integrin immunostaining in embryos and independently produced the same segmentation phenotype (the phenotype was evident in *n*=9/25 and *n*=11/25 electroporated embryos, respectively). A mix of the two plasmids was more efficient in producing the same phenotype (*n*=12/15). *Notch1* RNAi constructs were used as described (Das et al., 2006). Wild-type, S343D, E359K and S343A *Ilk* cDNAs were obtained from the Dedhar laboratory (British Columbia Cancer Research Centre, Vancouver, Canada), and additional point mutants generated using the QuikChange Mutagenesis Kit (Stratagene) according to the manufacturer's protocol.

We also knocked down gene expression using blocking MOs, fluorescent versions of which can be introduced into chick embryos by co-electroporation with carrier DNA (Voiculescu et al., 2008). In summary, 250 μM translation-blocking β1-integrin MO (5′-GGCACGCCGTG-GCCCTGTCCGCG-3′) or 750 nM splice-blocking *Ilk* fluorescein MO (5′-ACATCCAGGTGCTGCGCTCACCTCT-3′; Gene Tools) plus 250 μg/ml carrier plasmid DNA were co-electroporated into HH5 embryos as described above. The *Ilk* MO targets the exon 2-intron 2 junction, preventing excision of intron 2 (149 bases) and causing premature translational termination. We confirmed the presence of intron 2 by RT-PCR on RNA from the anterior PSM of three control-electroporated and three MO-electroporated embryos using forward (exon 2, 5′-TACATGAGGGGACAAACTTCG-3′) and reverse (exon 3, 5′-CACTGGAAGGAGAACTTCACG-3′) primers.

Single-cell qPCR

Chick embryos were harvested in PBS. Individual anterior PSM cells were isolated from anterior PSM tissue that had been lightly trypsinised into small clumps containing neighbouring RFP-positive and -negative cells. Individual cells were isolated using a glass pipette, seeded in the reverse transcription mix, and frozen in liquid nitrogen.

Single-cell RNA amplifications were performed using previously described two-stage protocols (Brady and Iscove, 1993; Tietjen et al., 2003) with modifications in primer composition and annealing temperatures to improve the reproducibility and to better preserve the relative abundance of transcripts (R. Prajapati, C.R. and D.I.-H., unpublished). In summary, cDNA copies were reverse transcribed using a 39-base primer terminating in $d(T_{24})$, d(A)-tailed using terminal transferase, and the same primer used to generate second strands. Following two sequential rounds of 25 cycles of symmetric PCR amplification, the product was used as a template for qPCR (Platinum qPCR SYBR Green Supermix, Invitrogen). For primers, see Table S1 in the supplementary material.

RESULTS

Knockdown of β1-integrin disrupts somite formation

Previous studies of ablating β1-integrin, the obligate partner of many α-integrins, have revealed novel roles of α/β1-integrins that were not predicted by studies of individual α subunits (Raghavan et al., 2000). As β1-integrin-null mouse embryos die very early owing to inner cell mass failure and peri-implantation lethality (Stephens et al., 1995), we elected to study β1-integrin function during chick somitogenesis.

Whole-mount immunostaining of chick embryos showed that β1-integrin protein accumulates around each forming and mature somite, especially at the borders (HH10, 10 somites; Fig. 1B,C), and in the anterior, but not in the posterior, PSM (Fig. 1D,E). Expression was also seen in the somitocoel, which is the core of the epithelialised somite (Fig. 1F), and in the basal lamina of the neural tube (Fig. 1B-F). Somitic β1-integrin expression patterns were similar in early and late embryos (e.g. HH19, 38 somites; Fig. 1G), implying that expression is maintained throughout somitogenesis. β1-integrin expression was also observed in the tail region, in particular in the chordoneural hinge region, within which lie the progenitors for axial extension of the embryo (Cambray and Wilson, 2002) (see Fig. S1 in the supplementary material; our unpublished results).

DEVELOPMENT

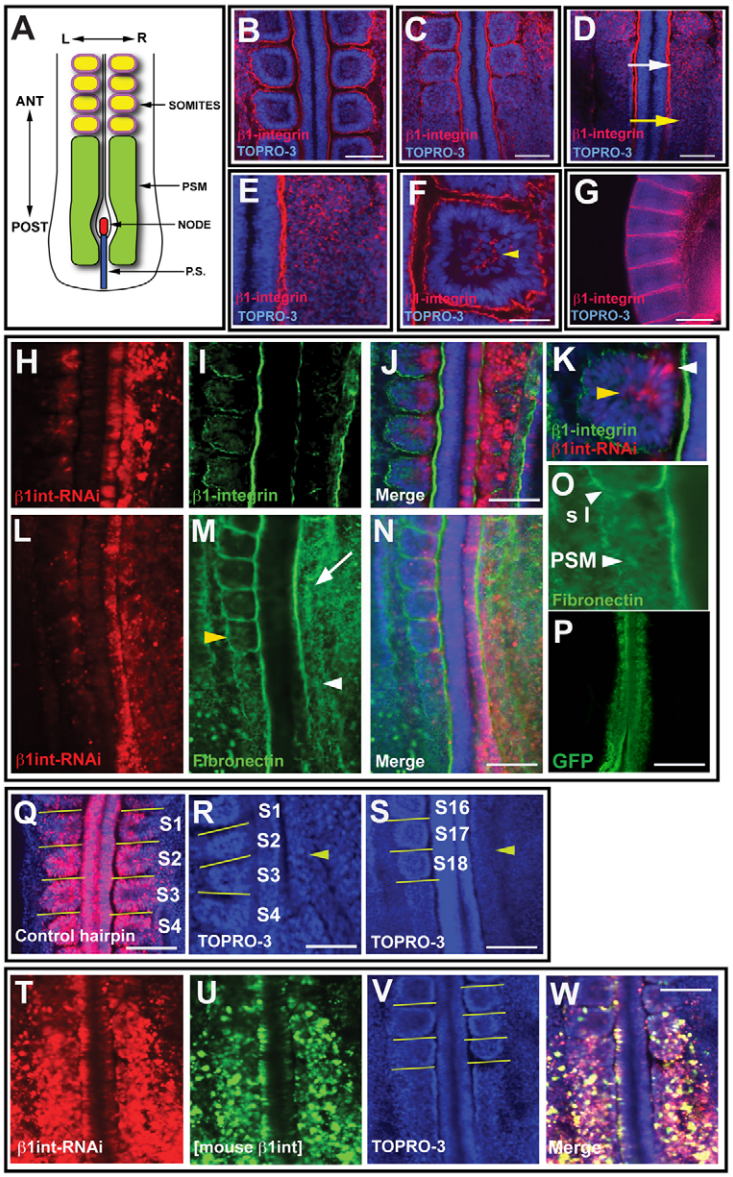

Fig. 1. Knockdown of β1-integrin expression disrupts normal somite formation. (**A**) Schematic of chicken embryo. (**B**,**C**) β1-integrin protein (red) is present in the borders of all somites, including the anterior-most (B) and newly formed (C) somites. (**D**) Expression of β1-integrin is observed in the anterior (white arrow) but not in the posterior (yellow arrow) PSM. (**E**) Higher magnification of the anterior PSM shown in D. (**F**) β1-integrin expression is also detected in the somitocoel of epithelial somites (arrowhead). (**G**) Expression in the somites persists at later stages of development. (**H-J**) Cells that have been electroporated with RNAi vector (red) do not express β1-integrin (green); *n*=15/15. (**K**) Cells electroporated with RNAi vector can be incorporated into the somite epithelium. (**L-N**) Knockdown of β1-integrin by RNAi results in defective fibronectin expression (green) in the segmental plate; *n*=10/10. Fibronectin expression where somites should have formed (M, arrow; the yellow arrowhead marks the non-electroporated side) is identical to that in the unsegmented PSM (white arrowhead). (**O**) Normal expression of fibronectin in the anterior PSM and in somite 1 in embryo electroporated with ineffective RNAi construct on both sides. (**P**) GFP expression 24 hours after electroporation of the anterior primitive streak of an HH4/5 embryo with control GFP construct (thus targeting both embryo sides), showing efficiency of electroporation. (**Q-S**) Following β1-integrin RNAi treatment, somites do not form (arrowheads). This is the case for both the anterior-most (R, *n*=10/22) and posterior-most (S, *n*=12/15) somites. An unrelated shRNA has no effect on somite formation (Q, *n*=25/25). (**T-W**) Rescue of somite formation after co-electroporation of β1-integrin RNAi with mouse β1-integrin cDNA (*n*=12/15), showing that the somitic phenotype is indeed due selectively to knockdown of β1-integrin expression. Yellow lines (Q,R,S,V) mark intersomitic space; yellow arrowheads (R,S) mark the absence of somites on the electroporated right-hand side of the embryo. Stages of embryos: (B-F) HH10, (G) HH20, (P) HH10, (H-O,Q,R,T-W) HH12, (S) HH15. In this and subsequent figures, TOPRO-3 is used to stain nuclei and show somitic organisation. Anterior is to the top in this and subsequent figures. PSM, presomitic mesoderm; P.S., primitive streak; ANT, anterior; POST, posterior; L, left; R, right. Scale bars: 100 μm in B-D; 30 μm in F; 250 μm in G; 300 μm in P; 150 μm in H-J,L-N,Q-W.

To examine whether β1-integrin is required for chick somitogenesis, we used RNA interference (RNAi) with short-hairpin RNAs (shRNAs) to target the degradation of endogenous transcripts (Das et al., 2006). We introduced plasmid DNA into HH4/5 chick embryos by electroporation (Momose et al., 1999), targeting the cells just posterior to Hensen's node that generate the PSM and somites (Fig. 1P), and used constructs that also express GFP or RFP for visualising the electroporated cells. In some cases, we targeted the right-hand side of the embryo at HH9, so that the unelectroporated left side served as a control (e.g. Fig. 1R,S; see later).

From six shRNAs tested, two reduced β1-integrin protein levels when electroporated into embryos (see Materials and methods; Fig. 1H-J). Somite formation was disrupted by expression of either construct (Fig. 1L-N), even for the anterior-most somite 1 (Fig. 1R). The phenotype was not generated by electroporation of empty vector DNA or of constructs expressing shRNAs that target unrelated transcripts (e.g. Fig. 1Q; see below). Disruption of segmentation was indeed due to selective loss of β1-integrin expression, as shown by rescue of the phenotype by co-expression of mouse β1-integrin transcripts, which are resistant to degradation by the chick-specific shRNAs (Fig. 1T-W; see Fig. S2 in the supplementary material). Somites were only lost following efficient electroporation; individual transfected cells still underwent somitogenesis (Fig. 1K), suggesting that loss of somite formation is not due merely to an inability to epithelialise.

Knocking down β1-integrin transcripts by electroporation of a fluorescent, translation-blocking, antisense morpholino oligonucleotide (MO) (Tucker, 2004) (but not of an unrelated MO) also caused loss of segmentation (see Fig. S2 in the supplementary material). The phenotype was rescued by co-expression of mouse β1-integrin transcripts, showing that it is not due to off-target effects on other transcripts.

Older embryos were equally susceptible to loss of β1-integrin signalling, at least up the stage after which electroporation of the PSM is no longer feasible (~HH13; Fig. 1S). Not only did knocking down β1-integrin disrupt somitogenesis (Fig. 1R,S), but the embryos also suffered axial truncations (see Fig. S1F in the

supplementary material). These results indicate that integrins are required for the generation of all chick somites and also for the maintenance of axial extension.

β1-integrin expression is independent of active Wnt and Notch signalling

It has been proposed that integrin signalling acts during zebrafish somitogenesis to mediate epithelialisation in response to the Notch and Wnt signalling pathways that are key in regulating and maintaining somitogenesis (Aulehla et al., 2008; Barrantes et al., 1999; Dunty et al., 2008; Jülich et al., 2005; Jülich et al., 2009; Koshida et al., 2005; Takahashi et al., 2000). Both these pathways lead to transcriptional activation of target genes. Notch acts via an activator complex that includes its proteolytically cleaved intracellular domain (N^{ICD}) (Schroeter et al., 1998). Wnt signalling occurs via nuclear β-catenin complexed with the DNA-binding factor Lef1/Tcf (Behrens et al., 1996; Huber et al., 1996).

Using DNA electroporation, we showed that β1-integrin expression in the anterior PSM is not a target of Notch signalling in the chick. β1-integrin expression was unaffected in cells expressing a dominant-negative form of mastermind-like (dnMAML), a strong inhibitor of the Notch pathway (Maillard et al., 2004) (compare the neighbouring electroporated and non-electroporated cells in Fig. 2A,B). Nor was β1-integrin expression affected by reducing NOTCH1 activity using constructs driving *Notch1* RNAi (Das et al., 2006), nor by expressing $DELTA1^{DN}$, a cell-autonomous, dominant-negative form of the DELTA1 ligand (Henrique et al., 1997) (Fig. 2C-F), and nor by hyperactivating signalling via N^{ICD} (Fig. 2G,H).

β1-integrin expression does not depend on Wnt signalling. β1-integrin levels were unaffected by inhibiting Wnt signalling [with CKI-7 (Fig. 2I) or dickkopf 1 (Dkk1; not shown)] (Glinka et al., 1998; Peters et al., 1999), or by treating embryos with LiCl, which renders Wnt signalling constitutive. These respective treatments inhibit and enhance transcription of *Lef1*, a Wnt target gene, in the anterior PSM (Gibb et al., 2009; Olivera-Martinez and Storey, 2007) (Fig. 2I; Fig. 3G,H) and other tissues (Driskell et al., 2007; Driskell et al., 2004).

β1-integrin acts upstream of canonical Wnt signalling in the anterior PSM and acts cell-autonomously

The lack of somitogenesis in β1-integrin knockdown embryos might be due to a failure of the segmentation oscillator, or because somites are not formed or maintained. The first of these alternatives seems unlikely, as β1-integrin overexpression did not alter the cycling periodicity of the clock target gene lunatic fringe (*Lfng*) (see Fig. S3J,K in the supplementary material); the effects of β1-integrin knockdown are masked by the loss of Notch signalling expression (see below).

We saw no evidence that somites are formed initially and then decay. To confirm this, we examined expression of the bHLH transcription factor cMESO1 (also known as Mesp2 in mouse) (Buchberger et al., 1998; Saga et al., 1997), which is required for somite border formation and is expressed in the prospective anterior compartment before somites are established (Morimoto et al., 2005). *cMeso1* transcription was downregulated following β1-integrin knockdown by RNAi or MO treatment (Fig. 3B,D), and restored by co-expression of knockdown-resistant β1-integrin transcripts (Fig. 3C,E). Thus, β1-integrin activity is required to establish somitic territories in the anterior PSM, the domain of β1-integrin expression.

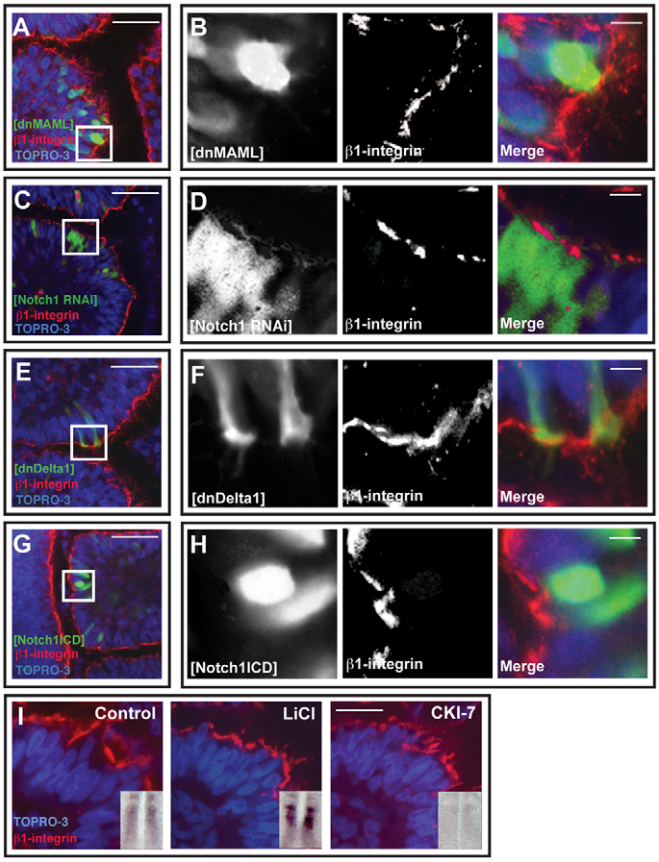

Fig. 2. β1-integrin expression in the somites is independent of Notch and Wnt signalling. (**A**,**B**) β1-integrin expression (red in all cases) following electroporation of dnMAML (green; cells are marked by GFP, which is fused to dnMAML) is indistinguishable from that in unelectroporated neighbours; *n*=10/10. (**C-F**) Similarly, β1-integrin expression is unaffected following knockdown of *Notch1* using RNAi (pseudocoloured green for RFP expressed by the same vector that encodes the shRNA for *Notch1*; *n*=12/12) (C,D), or in cells that express $Delta1^{DN}$ (green cells marked by GFP; *n*=15/15) (E,F). (**G**,**H**) Following electroporation of an N^{ICD} construct that activates Notch signalling targets, somite cells that express N^{ICD} (green) show the same levels of β1-integrin as their neighbours; *n*=10/10. The boxed areas in A,C,E,G are magnified in B,D,F,H, respectively. (**I**) β1-integrin expression is unchanged following induction (LiCl; *n*=10/10) or inhibition (CKI-7; *n*=10/10) of the Wnt signalling pathway. Insets show expression of the Wnt signalling-responsive gene *Lef1* (see also Fig. 3). Both sides of the embryo were targeted. Embryo stage is HH10. Scale bars: 30 μm in A,C,E,G; 15 μm in I; 5 μm in B,D,F,H.

Somite patterning might depend on inside-outside modulation of β1-integrin ligand-binding affinity, as previously proposed to explain genetic interactions between *notch* and *α5-integrin* during zebrafish somitogenesis (Jülich et al., 2005). According to this model, Notch signalling enhances integrin ligand-binding affinity to regulate cell adhesivity and shape during epithelialisation. Such regulation of integrin signalling by N^{ICD} has also been proposed to occur in cultured cells (ex vivo) (Hodkinson et al., 2007).

Alternatively, a ligand–β1-integrin complex might lie upstream of pathways that regulate somite differentiation and boundary formation, such as Wnt signalling. Indeed, we found that knocking down β1-integrin by RNAi or MO reduces transcription of the Wnt target *Lef1* in the anterior PSM (Fig. 3J,L), and that overexpressing

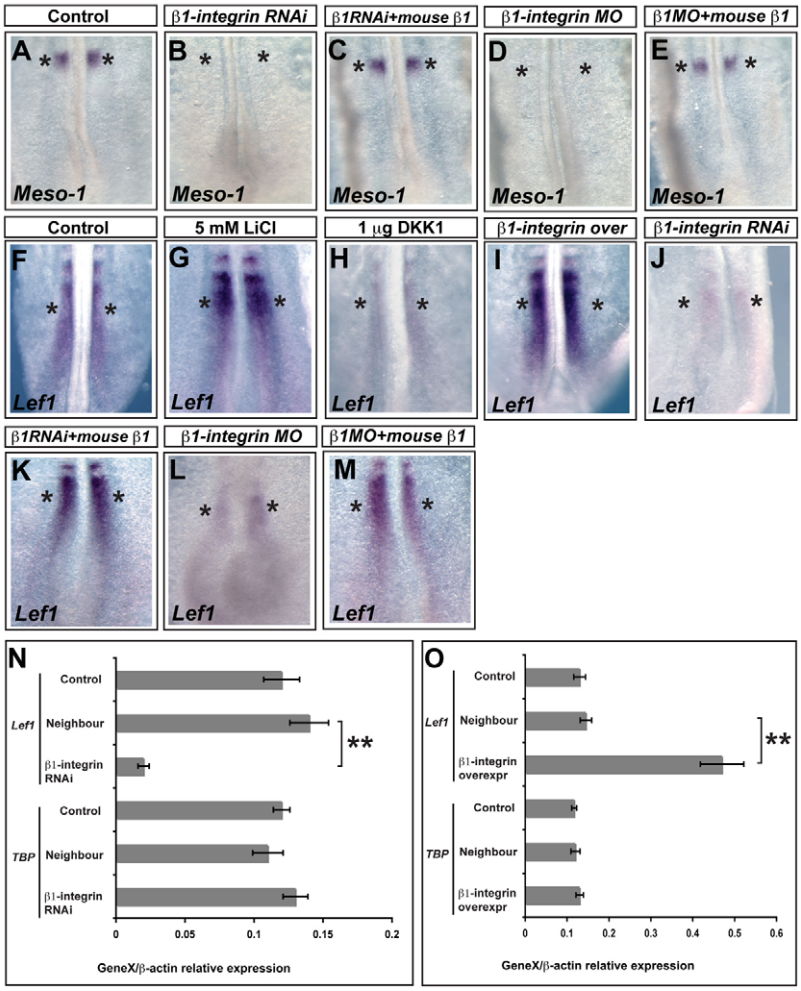

Fig. 3. β1-integrin signalling is cell-autonomously required for Wnt signalling in the anterior PSM. HH4/5 chick embryos were electroporated in the anterior primitive streak, thus targeting both sides. Twenty-six hours later, electroporated cells are located on both sides of the PSM (see Fig. 1P). (**A-E**) *cMeso1* expression depends on β1-integrin expression. *Meso1* transcription in the anterior PSM is reduced or abolished by knocking down β1-integrin via shRNA (B, *n*=10/10) or an MO (D, *n*=10/10), but not by an unrelated (A, *n*=12/12) or inactive (not shown) shRNA. *cMeso1* expression is restored by co-expressing mouse β1-integrin (C, *n*=9/9; E, *n*=7/7). (**F-H**) *Lef1* is a target of canonical Wnt signalling in the anterior PSM (asterisks). *Lef1* expression is upregulated in the PSM following LiCl treatment (G, *n*=12/15) and downregulated after culture with Dkk1 (H, *n*=12/17). (F) *Lef1* expression following control electroporation of a GFP construct (*n*=15/15). (**I**,**J**) *Lef1* expression is sensitive to β1-integrin expression (asterisks). β1-integrin overexpression and knockdown greatly increase (I, *n*=15/15) and decrease (J, *n*=20/20) *Lef1* expression, respectively. (**K**) *Lef1* expression is restored when mouse β1-integrin expression plasmid is co-electroporated with RNAi knockdown plasmid. (**L**,**M**) The same results are obtained when an MO against β1-integrin is used. (**N**,**O**) qPCR for *Lef1* on individual cells following β1-integrin RNAi (N) or overexpression (O). **, *P*<0.01; Student's *t*-test. Error bars indicate s.d. from three independent experimental repeats.

β1-integrin enhances *Lef1* expression (Fig. 3I). The specificity of these effects was demonstrated by the inactivity of unrelated shRNAs (Fig. 3A; data not shown) and by the restoration of *Lef1* expression in knockdown embryos that co-express mouse β1-integrin cDNA (Fig. 3K,M). These results show that β1-integrin signalling activates Wnt signalling in the chick anterior PSM.

Such an outside-inside signalling mechanism implies that β1-integrin acts cell-autonomously in regulating Wnt signalling activity. We assayed cell-autonomy using single-cell quantitative RT-PCR (qPCR) on individual cells to compare target gene expression between electroporated and neighbouring cells. Constructs that modulate β1-integrin expression were introduced into the right-hand side of HH9 embryos. Single electroporated (fluorescent) and neighbouring non-electroporated cells were recovered from small mixed cell clumps, and single control cells were isolated from the opposite, non-electroporated side (see later). For each class of individual cells (fluorescent cells, non-electroporated neighbours, and contralateral PSM control cells), transcripts were amplified so as to preserve their relative abundance (see Materials and methods). Levels of *Lef1* and of the control transcripts of *Tbp* and *Ymhaz* (which encode TATA-binding protein and tyrosine 3/tryptophan 5-monooxygenase activation protein, respectively) were then measured using qPCR.

Lef1 responded cell-autonomously to modulation of β1-integrin expression: *Lef1* expression was reduced by 85% in β1-integrin knockdown cells and raised 4-fold in β1-integrin-overexpressing cells (Fig. 3N,O). These treatments did not affect *Lef1* levels in adjacent and contralateral unelectroporated cells, and levels of control *Tbp* and *Ymhaz* transcripts were also unaffected. Thus, β1-integrin acts cell-autonomously in regulating Wnt signalling activity in the chick anterior PSM.

Integrin signalling via integrin-linked kinase is required for Wnt signalling in the anterior PSM

As outside-inside β1-integrin signalling is largely mediated by ILK, we examined the effects of modulating its activity on somite formation. First, we reduced ILK activity by expressing the dominant-negative ILK mutants E359K and S343A (Novak et al., 1998; Persad et al., 2000). E359K (and S343A, not shown) disrupted segmentation (Fig. 4A,B) and *Lef1* expression (Fig. 4G,H) in a similar manner to β1-integrin knockdown.

The same results were obtained when ILK expression was reduced by electroporation of a splice-blocking MO that inhibits the production of mature *Ilk* mRNA (Fig. 4C). Segmentation and *Lef1* expression were disrupted (Fig. 4C,I) in these embryos, and restored were by co-expression of MO-insensitive human *ILK* cDNA (Fig. 4J,K), showing that endogenous ILK activity is necessary for somite formation and Wnt activity. In the converse experiment, overexpression of wild-type ILK or the constitutively active S343D ILK mutant resulted in *Lef1* upregulation (Fig. 4E,F).

We confirmed that integrin requirement in somite formation and regulation of Wnt signalling in the anterior PSM proceed via ILK, by showing that ectopic ILK activity is epistatic to loss of β1-

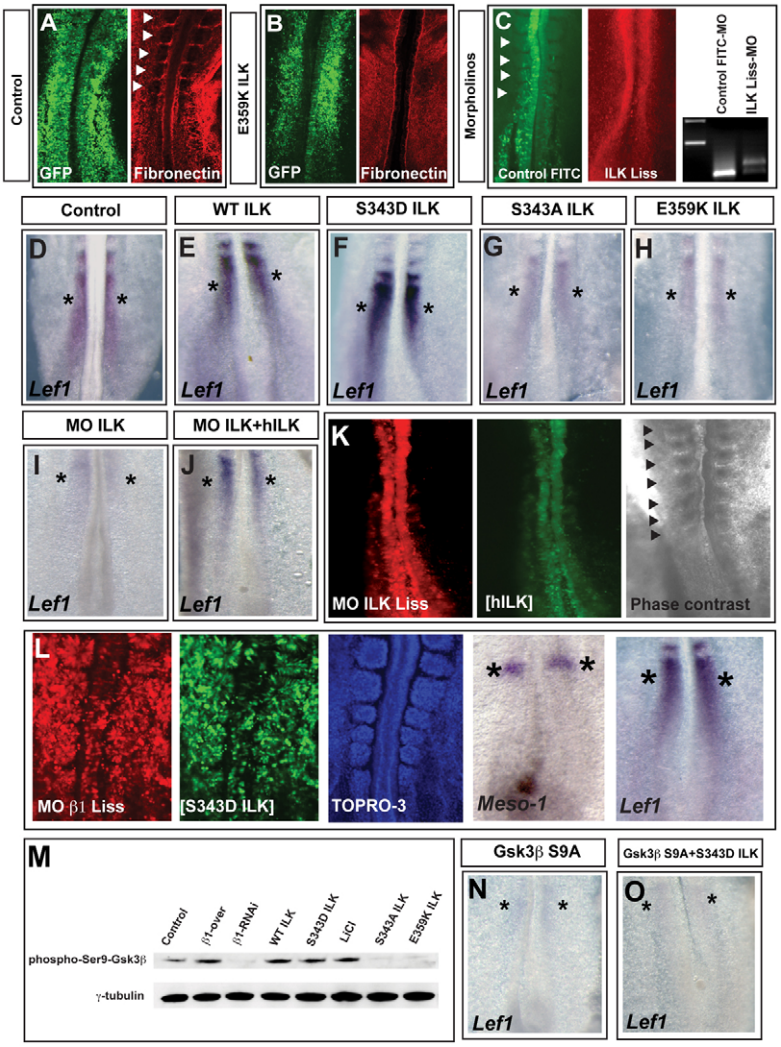

Fig. 4. Integrin signalling via ILK is required for somite formation, GSK phosphorylation and Wnt signalling. (**A-C**) Somite formation is normal following electroporation of a GFP vector (A, *n*=10/10), but is lost after electroporation of the E359K dominant-negative mutant ILK (B, *n*=27/33) or of the splice-blocking MO against *Ilk* (C; green is an unrelated FITC-conjugated MO, *n*=10/10; red is Lissamine-conjugated *Ilk* MO, *n*=11/11). The inset in C shows a PCR of the exon2-exon3 region of *Ilk* transcripts, showing that the MO efficiently inhibits splicing. Marker bands (left lane) are 0.5 and 1.0 kb. (**D**,**E**) *Lef1* expression is upregulated by ectopic ILK expression (E, *n*=10/12) as compared with a GFP control (D). (**F**) *Lef1* is upregulated by expression of constitutively active ILK^{S343D} in the PSM (*n*=15/15). (**G**,**H**) Dominant-negative ILK downregulates *Lef1* in the PSM (G, S343A, *n*=12/14; H, E359K, *n*=17/20). (**I-K**) *Ilk* MO electroporation results in downregulation of *Lef1* expression (*n*=6/6). Both the downregulation of *Lef1* (I) and somite disruption (C) are rescued by co-electroporation of human *ILK* (J, *n*=7/7; K, *n*=6/6). (**L**) ILK activity is epistatic to that of β1-integrin. Expression of S343D ILK in β1-integrin MO knockdown chick embryos restores somite formation and *cMeso1* and *Lef1* expression (*n*=9/12). (**M**) Western blot showing phosphorylation state of serine 9 of GSK3β following different embryo manipulations (see main text for details). (**N**,**O**) A constitutive, non-phosphorylatable form of GSK3β represses *Lef1* expression (N, *n*=10/10) even in the presence of the S343D constitutively active form of ILK (O, *n*=9/9). HH4/5 embryos were electroporated in the anterior primitive streak, thus targeting both sides. Arrowheads (A,C,K) indicate the bilateral somites. Asterisks, anterior PSM.

integrin activity. Thus, expressing the constitutively active mutant S343D (Legate et al., 2006) restored somite formation and *cMeso1* and *Lef1* expression to β1-integrin knockdown embryos (Fig. 4L).

Integrin signalling modulates phosphorylation of GSK3β

One mechanism by which ILK might affect Wnt signalling is via GSK3β, an inhibitor of Wnt signalling that phosphorylates and destabilises cytoplasmic β-catenin so that it is unable to activate transcription (Hart et al., 1998). ILK can phosphorylate GSK3β on Ser9 in vitro (Maydan et al., 2010; Sutherland et al., 1993) and, directly or indirectly, ex vivo (Legate et al., 2006; Marotta et al., 2001). Levels of phospho-Ser9-GSK3β, as measured on western blots, were indeed elevated in the PSM of embryos incubated in the GSK3β inhibitor LiCl (Fig. 4M).

Levels of phospho-Ser9-GSK3β in the anterior PSM were sensitive to integrin signalling and inversely correlated with Wnt signalling activity, being increased following overexpression of β1-integrin and reduced by β1-integrin knockdown or expression of dominant-negative ILK (Fig. 4M). Phosphorylation levels were also increased by expression of ectopic wild-type ILK or constitutively active S343D. Inhibition via phosphorylation of GSK3β is necessary for integrin/ILK action and Wnt signalling during segmentation, as a constitutive, non-phosphorylatable form of the enzyme, *Xenopus* S9A-GSK3β (Zhang et al., 2003), blocked segmentation (data not shown) and *Lef1* expression in both wild-type embryos and those expressing constitutive ILK (Fig. 4N,O).

We then tested whether ILK activity during segmentation depends on its kinase-like domain, in particular, on lysine 220, a conserved residue required for kinases to cleave ATP. We electroporated into the PSM constructs that express the ILK mutants K220A and K220M, which should not be able to co-ordinate ATP. Both mutants behaved as dominant negatives, disrupting segmentation (data not shown) and reducing *Lef1* transcription in the anterior PSM (see Fig. S4C-F in the supplementary material). They also dramatically reduced the levels of GSK3β phosphorylation, effects that were not reversed by double-mutant constructs that also include the S343D activating mutation (see Fig. S4G in the supplementary material). Taken together, these data indicate that β1-integrin modulation of Wnt activity is accompanied by phosphorylation of GSK3β in vivo, albeit perhaps indirectly (see Discussion).

Notch signalling also depends on β1-integrin activity

We found that β1-integrin activity also regulates Notch signalling, the other major signalling pathway associated with somite differentiation. Expression of several Notch signalling components (*Notch1*, *Delta1*) and targets (*Lfng*, *Hairy2*) was depressed

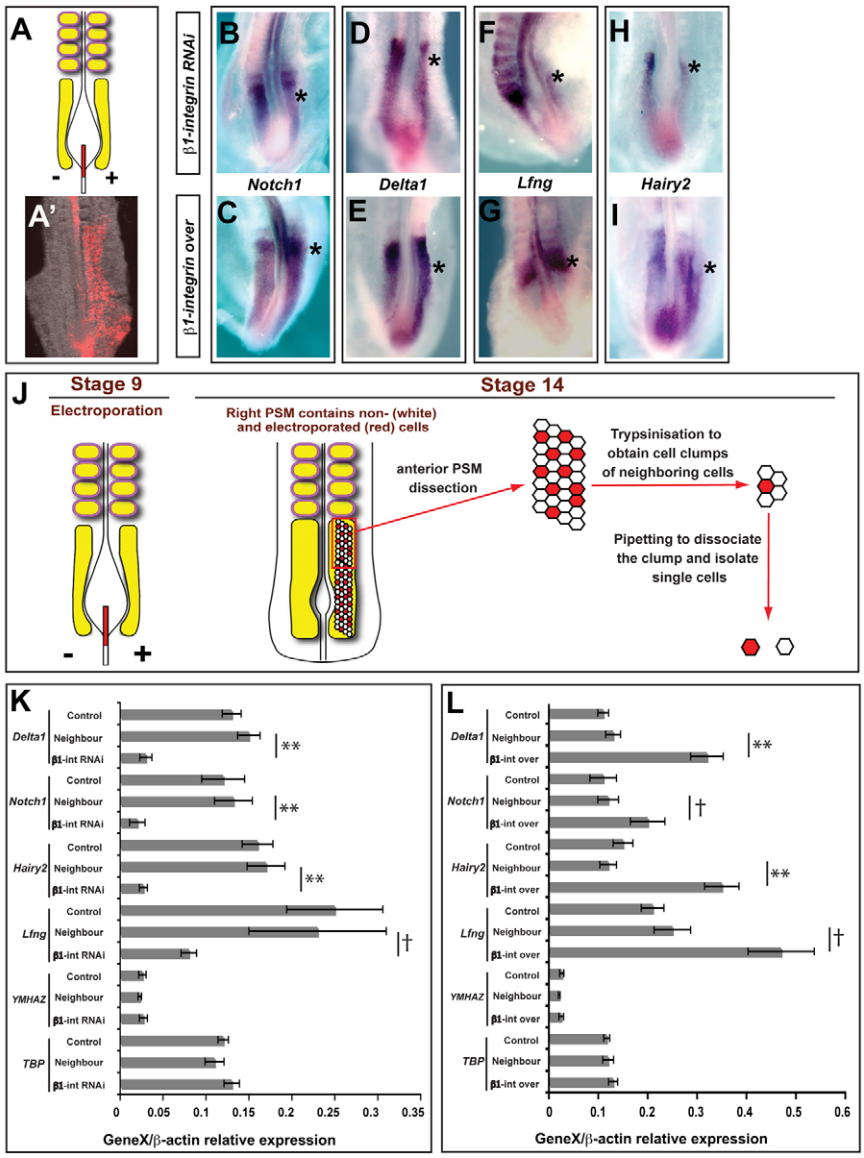

Fig. 5. Manipulation of β1-integrin expression disrupts the expression of Notch signalling components in the PSM. (**A**) Unilateral electroporation scheme. DNA (red) was injected in the anterior primitive streak at HH9. Electrodes were placed at either side of the chick embryo so that DNA is electroporated towards the right-hand side (towards the positive electrode) (marked by asterisks), the left side serving as a non-electroporated control. (**A′**) Embryo 24 hours after electroporation. Electroporated cells are located in the right-hand PSM, tailbud and neural tube. (**B-I**) In situ hybridisations for Notch signalling components. *Notch1* is downregulated following RNAi for β1-integrin (B, *n*=12/12) and upregulated when β1-integrin is overexpressed (C, *n*=15/15). The same result is obtained for *Delta1*, which encodes the ligand of NOTCH1 (D, *n*=15/15; E, *n*=14/15), and for *Lfng* (F, *n*=15/15; G, *n*=12/15) and *Hairy2* (H, *n*=15/15; I, *n*=11/15), which are targets of Notch signalling. (**J**) Schematic showing the procedure for single-cell isolation following electroporation of β1-integrin RNAi or overexpression of β1-integrin. Embryos were electroporated as described in A and were incubated until HH14 and individual cells isolated for qPCR. (**K**) Levels of *Delta1*, *Notch1*, *Hairy2*, *Lfng*, *Tbp* and *Ymhaz* expression in individual cells following RNAi for β1-integrin (see main text for details). (**L**) *Delta1*, *Notch1*, *Hairy2*, *Lfng*, *Tbp* and *Ymhaz* expression in individual cells following ectopic β1-integrin expression. (K,L) Control refers to cells from the left anterior PSM, and neighbour refers to non-electroporated neighbouring cells. **, $P<0.01$; †, $P<0.05$; Student's *t*-test. Error bars indicate s.d. from three independent experimental repeats.

following β1-integrin knockdown by either RNAi or antisense MO treatment (Fig. 5B,D,F,H; see Fig. S5A-D in the supplementary material), and β1-integrin overexpression led to enhanced *Notch1* and *Delta1* expression and increased Notch signalling (Fig. 5C,E,G,I). As expected, modulating ILK activity affected Notch signalling similarly (see Fig. S5 in the supplementary material).

As with Wnt signalling, β1-integrin regulates Notch signalling cell-autonomously. Knockdown by RNAi reduced expression of *Delta1*, *Notch1*, *Hairy2* and *Lfng* transcripts by 50-80% in electroporated cells, but had no effect on adjacent or unelectroporated cells (Fig. 5K). In the complementary experiment, overexpressing β1-integrin led to 2- to 3-fold higher expression of all five genes, but only in the electroporated cells themselves (Fig. 5L). In all cases, electroporations had no effect on the housekeeping control genes *Tbp* and *Ymhaz* (Fig. 5K,L).

ILK regulates Notch signalling via the Wnt signalling pathway

The above results could reflect independent regulation of Wnt and Notch activities by β1-integrin/ILK signalling or an upstream effect of Wnt signalling on Notch (Hofmann et al., 2004). In the latter case, one might expect that expressing a constitutively active form of β-catenin would restore Notch signalling to embryos in which ILK activity is inhibited.

Inhibition of ILK signalling resulted in downregulation of the Notch target *Lfng* in the anterior PSM (Fig. 6A; see Fig. S5G in the supplementary material). However, *Lfng* expression was restored by co-expressing an N-terminally truncated form of β-catenin (β-Cat^{Act}) that activates transcription constitutively (Fig. 6B,C) (Harada et al., 1999). This result shows that ILK acts upstream of Wnt signalling in regulating Notch signalling in the PSM.

Wnt signalling in the PSM still depends on an active Wnt ligand-receptor interaction, even when integrin signalling is constitutive. Constitutively active S343D-ILK was unable to upregulate *Lfng* or *Lef1* expression when Wnt ligand binding was inhibited by treating embryos with Dkk1 (compare Fig. 4F with Fig. 6P-U).

Contrary to our model, it has recently been reported that inhibiting Notch signalling via the γ-secretase inhibitor DAPT leads to loss of Wnt signalling as marked by *Lef1* expression (Gibb et al., 2009), suggesting that Notch signalling regulates the Wnt pathway. In our hands, we found that *Lef1* expression is only inhibited by extreme doses of DAPT (100 μg/μl), at least 10-fold higher than

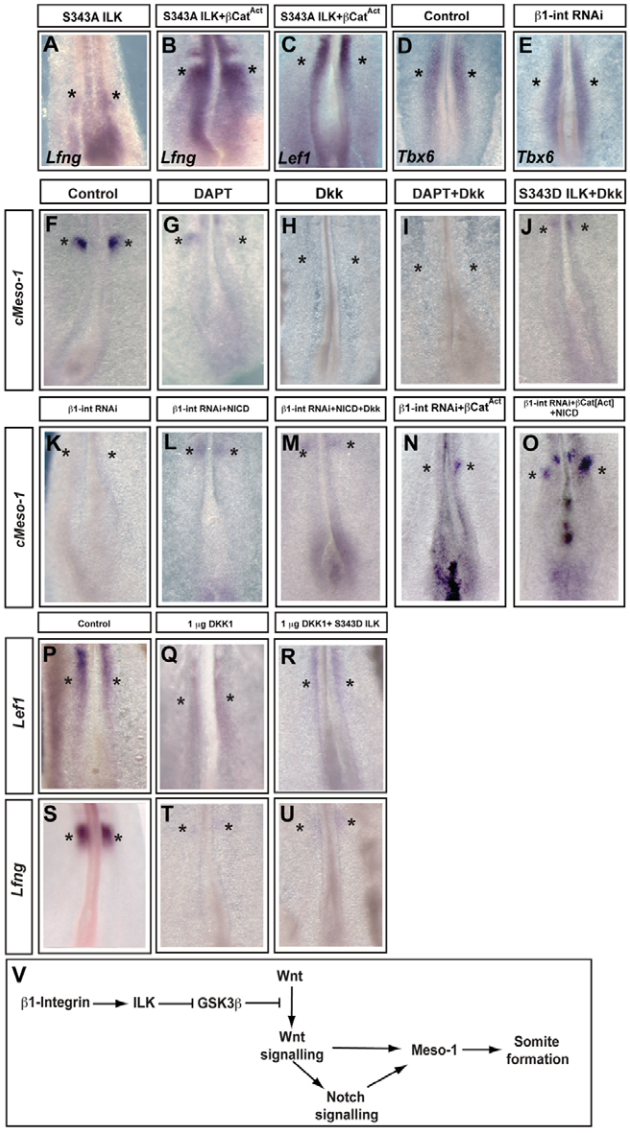

Fig. 6. Functional Wnt and Notch pathways are both required for *cMeso1* expression. (**A-C**) Dominant-active β-catenin (β-Cat^{Act}), which constitutively activates *Lef1* transcription (C, *n*=7/9), restores *Lfng* expression (B, *n*=8/8) that is otherwise reduced by dominant-negative ILK (A, *n*=8/11). (**D**,**E**) *Tbx6* expression is maintained following RNAi knockdown of β1-integrin RNAi, showing that β1-integrin is not required to maintain the mesodermal character of anterior PSM cells (E, *n*=21/21). (**F-O**) Embryo treatments and electroporations that manipulate Notch and Wnt signalling show that *cMeso1* expression is dependent on both of these pathways (see main text for details). F, *n*=5/5; G, *n*=12/12; H, *n*=11/12; I, *n*=14/15; J, *n*=10/12; K, *n*=10/10; L, *n*=8/11; M, *n*=8/10; N, *n*=11/14; O, *n*=17/23. (**P-U**) Integrin signalling via ILK promotes Wnt and Notch signalling only in the presence of a functional Wnt pathway. *Lef1* (Q) and *Lfng* (T) are downregulated in embryos cultured in Dkk1, as compared with control embryos cultured in the presence of DMSO (P,S). Electroporation of constitutively active S343D ILK does not restore *Lef1* (R) or *Lfng* (U) expression in Dkk1-cultured embryos. *n*=15 for each condition. Asterisks, anterior PSM. (**V**) Model of the hierarchical regulation by β1-integrin of Wnt and Notch signalling pathways in chick somite formation.

those required to inhibit Notch signalling as monitored by *Lfng* expression (see Fig. S6 in the supplementary material). These and our other results argue that Wnt signalling lies upstream of Notch in the chick anterior PSM, and that reduced *Lef1* expression at high drug concentrations is probably due to interference with other gene pathways.

Wnt and Notch signalling cooperate in somite border formation

β1-integrin knockdown embryos fail to express *cMeso1* (Fig. 3B,D) in the anterior compartment of presomite –1, indicating that their lack of somites is due to a failure to establish somite boundaries. However, the cells still retained the appropriate mesenchymal identity, expressing the PSM marker *Tbx6* (Fig. 6E). *Tbx6* has previously been shown to be a Wnt target in the mouse PSM (Dunty et al., 2008). Perhaps knockdown leaves sufficient residual Wnt activity to maintain *Tbx6* expression, although we cannot exclude the possibility that there are distinct modes of regulating *Tbx6* expression in mouse and chick.

cMeso1 expression depends on both Wnt and Notch activity in the anterior PSM, as expression was lost in embryos incubated in either Dkk1 or DAPT to inhibit the respective pathways (Fig. 6F-H). Wnt activation of *cMeso1* might be indirect, being mediated by Notch activity. Alternatively, the two pathways could act on *cMeso1* in combination. To distinguish between these mechanisms, we examined whether constitutive activation of Wnt or Notch signalling could restore *cMeso1* expression in β1-integrin knockdown embryos. Activating either pathway alone (via N^{ICD} or β-Cat^{Act}) caused only very weak *cMeso1* expression (Fig. 6K-N), but higher-level expression was achieved when both pathways were activated (Fig. 6O). Thus, the Wnt and Notch signalling pathways collaborate downstream of β1-integrin signalling in chick somite border formation.

DISCUSSION

Previous data have implicated integrin signalling in segmentation, one suggestion being to control cell adhesion properties that contribute to the maintenance of intersomitic borders (Jülich et al., 2005; Koshida et al., 2005; Yang et al., 1999). Our studies of chick somitogenesis reveal that β1-integrin signalling in the anterior PSM acts to establish somite boundaries by regulating other signalling pathways. Signalling via ILK directs phosphorylation of GSK3β to permit Wnt and, thereby, Notch signalling. Together, these drive somite compartmentalisation and boundary formation.

Although α5-integrin activity only appears necessary for the formation of subsets of somites in zebrafish and mouse (Jülich et al., 2005; Koshida et al., 2005; Yang et al., 1999), we find that β1-integrin is required essentially throughout chick somitogenesis. This is probably also true in the mouse in which the candidate ligand, fibronectin, is required to form somites (George et al., 1993). Perhaps a different α subunit partner substitutes in forming the α5-independent somites.

The utilisation of specific integrin subunits and ligands during somitogenesis seems to vary between species, particularly between zebrafish and higher vertebrates. Zebrafish and mice differ in their domains of α5 requirement, and also in the necessity for fibronectin activity, which is essential for somite formation in the mouse but required only for the maintenance of somite boundaries in zebrafish (Koshida et al., 2005). *loc* (*ilk*) mutant fish lack a segmentation phenotype (Postel et al., 2008). These differences might be due to compensation by paralogues or related genes that are lacking in the chick or mouse.

Wnt and Notch signalling activities are sensitive to modulation of β1-integrin expression (Figs 3, 5), but not vice versa (Fig. 2), indicating that β1-integrin signalling lies upstream of Notch and

Wnt activity in the chick anterior PSM. Wnt signalling appears to be regulated by β1-integrin/ILK, but still in a ligand-dependent manner, being blocked by Dkk1, which acts by interfering with Wnt ligand binding (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001).

Inhibiting Notch signalling does not affect Wnt signalling (see Fig. S6B in the supplementary material). Rather, Notch is a target of Wnt: reduced β-catenin function in the PSM of β1-integrin knockdown embyos leads to severe downregulation of the Notch targets *Hairy2* and *Lfng* (Fig. 5H,F; see Fig. S5B,D in the supplementary material), which is due, at least in part, to reduced transcription of *Notch1* and *Delta1* (Fig. 5B,D). Evidence for direct regulation of mouse *Delta1* expression by Wnt has been described previously, including the presence of multiple Lef1 binding sites in its promoter and the defects in mouse somitogenesis caused by mutating *Lef1* (Galceran et al., 2004). Although β1-integrin could also regulate N^{ICD} activity directly, as proposed for neural stem cells (Campos et al., 2006), such a mechanism would have to be secondary to regulation via Wnt/β-catenin.

A series of epistasis experiments support the above model. *Lef1* expression is restored in β1-integrin knockdown embryos that express constitutive ILK (Fig. 4L). $\beta\text{-Cat}^{Act}$ expression restores *Lfng* expression in embryos expressing dominant-negative ILK (Fig. 6B).

Levels of phospho-Ser9-GSK3β in the anterior PSM correlate with Wnt signalling activity during segmentation, being reduced by knocking down β1-integrin or by expression of dominant-negative forms of ILK, and being enhanced by expression of wild-type or constitutive ILK (Fig. 4M; see Fig. S4 in the supplementary material). These phosphorylation changes are linked with effects on Wnt signalling: *Lef1* expression is lost in $Ilk^{constit}$ embryos expressing the constitutive, non-phosphorylatable S9A-GSK3β mutant (Fig. 4O).

One interpretation of our results is that ILK directly phosphorylates and inactivates GSK3β during segmentation. Indeed, we find that the kinase-like domain is important for ILK activity in vivo; mutating K220 in ILK so that it can no longer bind ATP efficiently renders the protein dominant negative (see Fig. S4 in the supplementary material). This explanation is also consistent with a recent study showing that ILK is a bona fide serine/threonine protein kinase that phosphorylates GSK3β in vitro (Maydan et al., 2010).

However, the ILK kinase domain also behaves as a protein-protein binding interface (Fukuda et al., 2009; Legate et al., 2006; Wickström et al., 2010), and several lines of evidence argue for this being its major mode of action. Mutation of the putative ATP-binding site of ILK in *Drosophila*, mice and worms supports almost normal development (Lange et al., 2009; Mackinnon et al., 2002; Zervas et al., 2001). Also, although ILK can phosphorylate GSK3β on Ser9 in vitro, this modification does not necessarily inactivate ILK in vivo; S9A-GSK3β transgenic mice appear to develop normally (McManus et al., 2005).

Thus, we cannot exclude the possibility that ILK acts indirectly on GSK3β during segmentation, e.g. as a protein adapter. Our results also leave open the possibility that ILK affects Wnt signalling in additional ways (e.g. by directly modulating the nuclear localisation of β-catenin) (Oloumi et al., 2006). Indeed, ILK might have different modes of action in different tissue contexts.

The posterior PSM differs from the anterior in several respects. Levels of β-catenin are higher (Aulehla et al., 2008), although levels in the anterior must still be sufficient to support the anterior expression of *Lef1* (Gibb et al., 2009; Olivera-Martinez and Storey, 2007) and *Ripply2* (Biris et al., 2007). Genetic regulatory interactions also differ between the two domains (Morales et al., 2002; Morimoto et al., 2005), presumably due in part to combinatorial interactions between signalling pathways and regionalised accessory factors that together determine regionally restricted outcomes such as the Wnt responsiveness of *Lef1* transcription (Fig. 3G,H). Thus, our studies make no predictions about how β1-integrin, Wnt and Notch signalling interact in the posterior PSM.

Jülich and co-workers have proposed a contrasting model for somite formation in which Notch signalling enhances the affinity of α5/β1 receptors for binding their fibronectin ligand (Jülich et al., 2005; Jülich et al., 2009). Such an inside-outside signalling model might predict that modulating Notch signalling would alter the efficiency of ligand accumulation and integrin signalling, and should thereby affect Wnt signalling. However, blocking Notch signalling with low doses of DAPT does not affect fibronectin accumulation or *Lef1* transcription (see Fig. S6B,E,F in the supplementary material), and N^{ICD} electroporation has no effect on the levels of phospho-Ser9-GSK3β (see Fig. S6D in the supplementary material), indicating that signalling by Wnt, and hence by β1-integrin, lies upstream of that by Notch.

Somite compartmentalisation and boundary formation appear to rely on Wnt and Notch acting together. Blocking either pathway inhibits somite formation and the *cMeso1*/*Mesp2* expression that prefigures somite compartmentalisation and boundary formation (Fig. 6G,H). *cMeso1* expression is efficiently restored in β1-integrin knockdown embryos only by co-activation of both Wnt and Notch pathways (Fig. 6O). This result is consistent with studies showing that mouse *Mesp2* is regulated by Wnt activity (Dunty et al., 2008). As the role of Mesp2 during mouse somitogenesis is, at least in part, to negatively regulate Notch signalling (Morimoto et al., 2005), our results indicate that Mesp2 acts both upstream and downstream of Notch activity in establishing somitic boundaries.

Integrin may act by modulating Wnt and Notch activities in other contexts, e.g. in the axial extension that accompanies segmentation. Interfering with β1-integrin activity leads to truncated embryos (see Fig. S1F,H,J in the supplementary material) that resemble those caused by reduced Wnt and Notch activity (Dunty et al., 2008; Huppert et al., 2005; Takada et al., 1994). Integrin signalling might also target these pathways to promote axial cell proliferation.

Somitogenesis provides an example of outside-inside integrin signalling regulating cell epithelialisation via a signal cascade. Nevertheless, we do not exclude a second, later role for inside-outside integrin signalling, e.g. in somite maintenance and in regulating Eph/ephrin signalling (Durbin et al., 2000; Watanabe et al., 2009). Our results showing that β1-integrin activity lies upstream of Wnt and Notch in primitive presomites do not preclude integrins acting downstream of Notch in forming or formed somites (Nakajima et al., 2006).

Integrin regulation of Wnt signalling is likely to be a recurrent theme during animal development; tissues in which Notch is a Wnt target might recapitulate the signalling network that we have defined for the anterior PSM. Other tissues might use part or variants of the cascade. Genetic studies have demonstrated related Notch and integrin mutant phenotypes in vascular development (Karsan, 2008; Leong et al., 2002), suggesting that these signalling pathways cross-regulate each other. Elevated ILK expression and activity are associated with a variety of cancers in humans (Ahmed et al., 2003; Bravou et al., 2003; Graff et al., 2001), and ILK levels

and activity correlate with β-catenin-dependent transcription and with human colon tumour grade progression (Bravou et al., 2006; McDonald et al., 2008). In leukaemic cells, ILK activity stimulates *Notch1* and *Hes1* expression and promotes phosphorylation of GSK3β (Tabe et al., 2007). Thus, Notch and Wnt dysregulation in cancer might be linked to excessive integrin signalling.

Acknowledgements
We thank Julian Lewis, Holger Gerhardt, Michael Stauber, Krzysztof Wicher and Annalisa Vezzaro for helpful discussions and input on the manuscript; Shoukat Dedhar for communicating data prior to publication and for providing wild-type, S343D, E359K and S343A ILK cDNAs; Ravindra Prajapati for providing and testing PCR primers for chicken *Tbp* and *Ymhaz*; Astrid Buchberger, Julian Lewis, Despina Stamataki and Peter Klein for providing the in situ hybridisation probe for *Meso1* and activated β-catenin expression, *pCAGGS-IRES-nlsGFP* and S9A-GSK3β constructs, respectively; and Richard P. Tucker for the β1-integrin antisense MO. The V2E9 anti-β1-integrin and B3/D6 and VA1 anti-fibronectin monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank of the University of Iowa. This work was supported by Cancer Research UK.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.050070/-/DC1

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