

Interdigital cell death in the embryonic limb is associated with depletion of Reelin in the extracellular matrix

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Interdigital cell death is a physiological regression process responsible for sculpturing the digits in the embryonic vertebrate limb. Changes in the intensity of this degenerative process account for the different patterns of interdigital webbing among vertebrate species. Here, we show that Reelin is present in the extracellular matrix of the interdigital mesoderm of chick and mouse embryos during the developmental stages of digit formation. Reelin is a large extracellular glycoprotein which has important functions in the developing nervous system, including neuronal survival; however, the significance of Reelin in other systems has received very little attention. We show that *reelin* expression becomes intensely downregulated in both the chick and mouse interdigits preceding the establishment of the areas of interdigital cell death. Furthermore, fibroblast growth factors, which are cell survival signals for the interdigital mesoderm, intensely upregulated *reelin* expression, while BMPs, which are proapoptotic signals, downregulate its expression in the interdigit. Gene silencing experiments of *reelin* gene or its intracellular effector *Dab-1* confirmed the implication of Reelin signaling as a survival factor for the limb undifferentiated mesoderm. We found that Reelin activates canonical survival pathways in the limb mesoderm involving protein kinase B and focal adhesion kinase. Our findings support that Reelin plays a role in interdigital cell death, and suggests that anoikis (apoptosis secondary to loss of cell adhesion) may be involved in this process.

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In the developing vertebrate limb, the formation of free digits involves massive cell death within the interdigital mesoderm. These zones of cell death have been originally termed interdigital necrotic areas but constitute a classical example of physiological cell death by apoptosis.¹ Numerous studies have analyzed the molecular cascade activated in the course of the cell death process; however, the mechanisms triggering interdigital tissue regression remain controversial.^{2–8} Local activation of bone morphogenetic protein (BMP) signaling, together with local downregulation of fibroblast growth factor (FGF) signaling, is central in the course of interdigital regression.^{9–11} However, how these signals modify the interdigital tissue to initiate apoptosis is not fully understood. It has been proposed that modifications of the extracellular matrix¹² and loss of adhesion of the interdigital mesoderm to the surrounding extracellular matrix precedes the activation of intracellular apoptotic machinery.⁴ This mechanism has been termed ‘anoikis’ and it occurs in both embryonic and tumoral cell death.^{13,14} The identification of compositional changes in the interdigital matrix preceding the onset of cell death may be relevant for understanding the regulation of this degenerative process.

In a systematic analysis of a c-DNA library obtained from the undifferentiated interdigital mesoderm prior to the onset of

interdigital necrotic zones (INZ), we identified the presence of the *reelin* gene.¹⁵ Reelin is a large matrix glycoprotein thought to be expressed preferentially in the developing brain¹⁶ where it controls the migration and laminar arrangement of neurons when the cerebral cortex is formed.¹⁷ Remarkably, Reelin signaling also promotes the survival of different embryonic and adult neuronal populations.^{18–20} In the developing neural system, Reelin binds to integrins^{21–23} as well as specific receptors, including the very-low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) inducing the phosphorylation of the intracellular adaptor protein Disabled-1 (*Dab-1*),^{24–26} which in turn activates downstream effectors involved in cytoskeletal reorganization, cell migration and cell survival.²⁷

In this study, we show that Reelin and its intracellular signaling protein DAB-1 are highly expressed in the undifferentiated mesoderm during digit formation and are downregulated in the interdigits preceding the onset of apoptosis. In accordance with a pro-survival role of this signaling pathway, *reelin* gene expression is upregulated *in vivo* by local treatment with FGFs which are survival signals for the interdigital mesoderm and down-regulated by BMPs which are pro-apoptotic signals. In addition, the knockdown of *reelin* or *Dab-1* by short hairpin RNA (sh-RNA) transfection

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Abbreviations: FGFs, fibroblast growth factors; BMPs, bone morphogenetic proteins; TGFβ, transforming growth factor β; Dab-1, disabled-1; FAK, focal adhesion kinase; AKT, protein kinase B; ApoER2, apolipoprotein E receptor 2; PI3K, phosphatidylinositol-3-kinase; INZ, interdigital necrotic zones; AER, apical ectodermal ridge; PZ, progress zone; sh-RNA, short hairpin RNA; cDNA, complementary DNA; HH, Hamburger–Hamilton stages; i.d., incubation day; p.c., post-coitum; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DMEM, Dulbecco's modified Eagle's medium; PFA, paraformaldehyde; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; AP, alkaline phosphatase; PI, propidium iodide

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increases cell death in primary cultures of the undifferentiated mesoderm. Finally, we identified focal adhesion kinase (FAK) and protein kinase B (AKT) as potential mediators of the pro-survival effect of Reelin signaling.

Results

Reelin and *Dab-1* are highly expressed in the undifferentiated mesoderm prior to the onset of INZ.

The *reelin* gene was identified in a complementary DNA (cDNA) library generated from the interdigital mesoderm of the developing chick limb in the stages preceding cell death.¹⁵ We cloned *reelin* and used *in situ* hybridization to

analyze its expression pattern during the formation of the digits in chick and mouse embryos. As shown in (Figures 1a and e), prior to INZ in the chick embryo, *reelin* exhibited intense expression domains in the undifferentiated mesoderm localized around the tip of the developing digits, as well as in the interdigital regions where cells are healthy and remain undifferentiated. In the following stages, interdigital expression was downregulated preceding the establishment of the areas of interdigital cell death that are responsible for digit freeing (Figures 1b and f). A gene expression analysis by Q-PCR was used to confirm the intense downregulation of *reelin* detected by *in situ* hybridization (Figure 1g). The presence of the Reelin protein

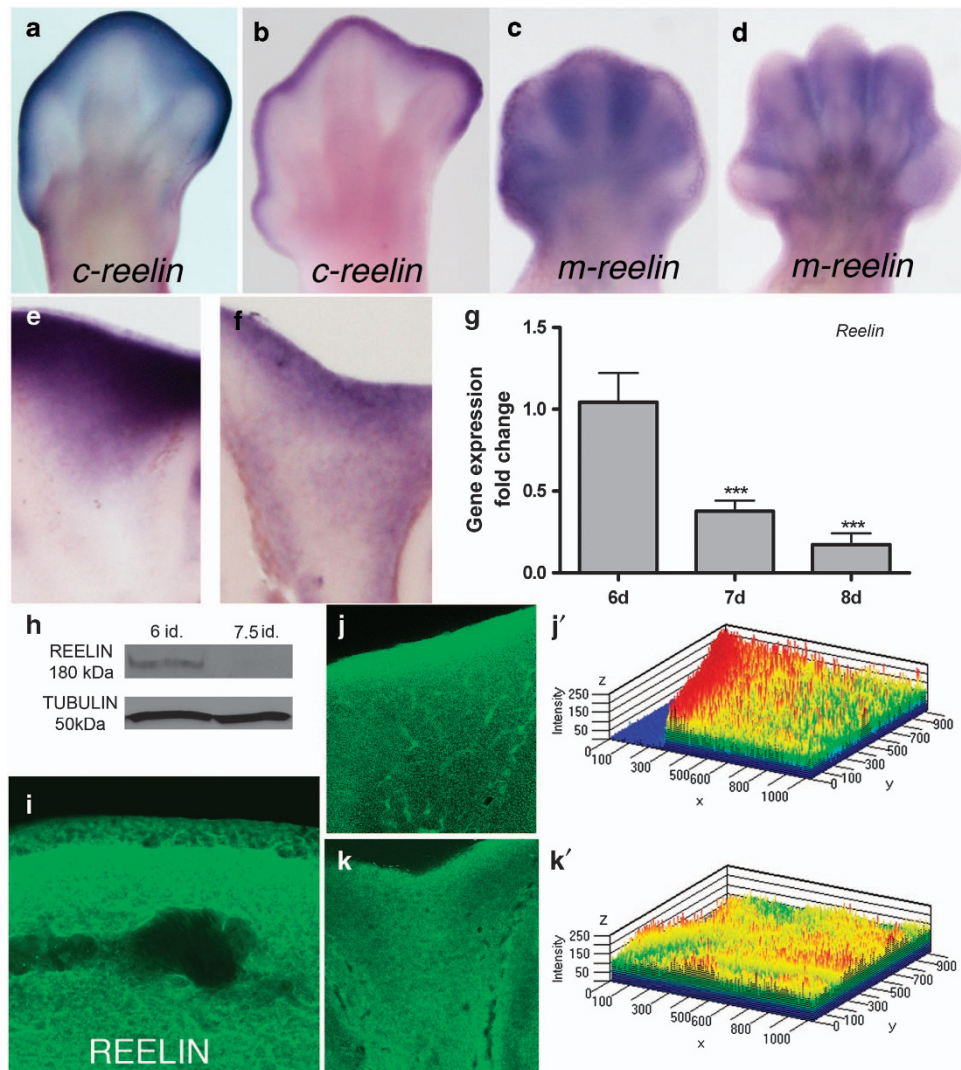


Figure 1 (a and b) *In situ* hybridization illustrating *reelin* expression in the developing chick autopod at 6 i.d. (a), and 7.5 i.d. (b). Prior to INZ (a) labeling is intense in the interdigital and distal undifferentiated mesoderm. From i.d. 7, interdigital expression is progressively downregulated (b). (c and d) *In situ* hybridization of mouse autopods prior to (p.c. day 12.5; c) and at the onset of (p.c. day 13.5; d) INZ. Note the intense labeling in the interdigital mesoderm in (c), and a weaker labeling in (d). (e and f) Detailed views of the third interdigit of chick leg buds to show differences in the intensity of *reelin* expression at 6 i.d. (e) and 7.5 i.d. (f). (g) QPCR analysis of *reelin* expression in the interdigital tissue of 6 i.d., 7 i.d. and 8 i.d. embryos. Bars represent gene expression levels in arbitrary units where values are normalized to the expression in the 6 i.d. tissue. *** $P < 0.001$. (h) Representative western blot illustrating differences in Reelin levels in 6d vs 7.5d interdigital tissues. (i) Detailed view of a section of the chick autopod at 6 i.d. showing positive Reelin immunolabeling in the distal undifferentiated mesoderm. (j and k) Low-magnification views of the third interdigit at 6 i.d. (j), and 7.5 i.d. (k) immunostained for Reelin. (j' and k') Surface plots quantifying the distribution of Reelin immunolabeling in (j and k). z axes = counts of pixel intensity; x and y axes = pixel localization in the corresponding pictures (j and k). Red and green colors represent higher and lower values, respectively

in the autopodial tissues (Figure 1i) and its depletion preceding cell death (Figures 1 j–k) were also confirmed by western blotting (Figure 1h) and immunolabeling of autopodial vibratome sections monitored by confocal microscopy (Figures 1j–j', k–k'). *Reelin* expression was also intense in the interdigits of mouse embryos on day 11 p.c. (Figure 1c). As observed in the chick, expression diminishes in the following stages in relation with the induction of INZ (Figure 1d). Remarkably, in contrast to what was observed in the chick embryo, *reelin* expression in the distal contour of the autopod was very low. This finding is consistent with the presence of an area of cell death termed 'Sub AER Marginal Zone of cell death' in the mouse autopod that is not present in avians.²⁹

Reelin signaling is mediated by phosphorylation of the intracellular adaptor protein DAB-1 upon Reelin binding to its target receptors. Hence, to confirm that this signaling pathway is active during the stages of digit formation, we further analyzed the presence of Dab-1, both at transcriptional and protein level. As shown in Figure 2a, expression of *Dab-1* in the undifferentiated mesoderm overlaps with that of *reelin*. At protein level, DAB-1 exhibited a widespread distribution in the undifferentiated interdigital mesoderm (Figure 2b and d). Specific immunolabeling for phosphorylated DAB-1 (p-DAB-1) confirmed the presence of active signaling in a considerable number of undifferentiated mesodermal cells (Figure 2c).

Regulation of *reelin* gene expression by FGFs and BMPs. To analyze the possible functions of Reelin in the interdigital mesoderm, we studied *reelin* gene regulation by growth factors known to regulate interdigital cell death (Figure 3). As shown in Figure 3b, the interdigital expression domain of *reelin* was extended following FGF-2 overexpression in the undifferentiated limb mesoderm. This treatment has been shown to inhibit interdigital cell death by maintaining mesodermal cells in an undifferentiated state.³⁰ By contrast, interdigital overexpression of BMP7 (Figure 3c), which promotes interdigital cell death,³¹ inhibited *reelin* expression.

The regulations modulated by FGF-2 and BMP-7 treatments were confirmed by Q-PCR (Figures 3b' and c') and altogether these results demonstrate that *reelin* expression is promoted by canonical limb mesenchymal cell survival factors while it is inhibited by canonical apoptotic inducing cytokines.

Reelin/Dab1 signaling regulates mesodermal cell survival via AKT and FAK. In light of the results described above, we next explored the function of Reelin signaling in programmed cell death. For this purpose, we designed and

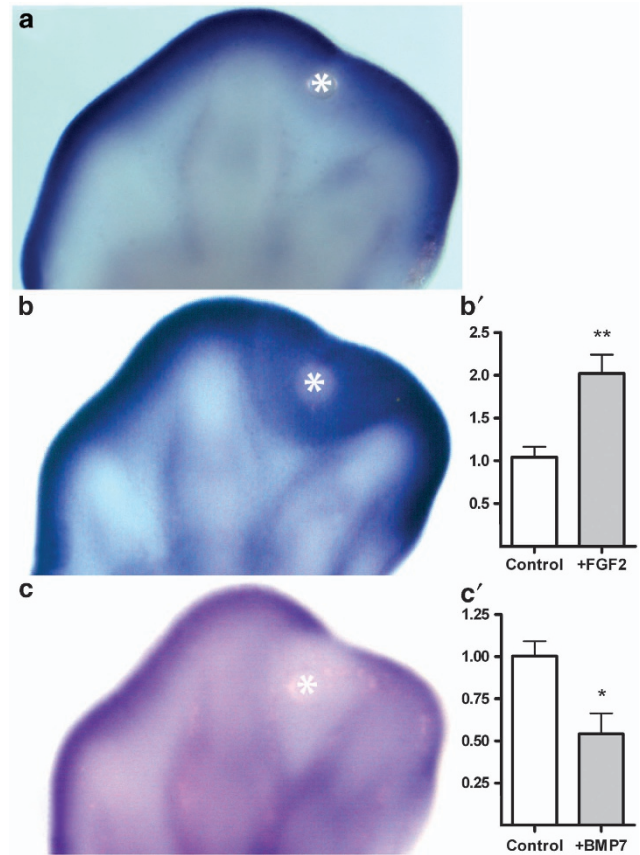


Figure 3 (a–c) Detailed view of *reelin* expression at 5.5 i.d., 6 h after interdigital implantation of PBS/BSA-soaked beads (a), FGF2-soaked beads (b), and BMP7-soaked beads (c). Note the upregulation of *reelin* expression in (b) and the downregulation in (c) in comparison to the control (a). Implanted bead is indicated by (*). (b') QPCR plot showing the positive regulation of *reelin* in samples of the third interdigit isolated from the autopod 10 h after FGF-bead implantation. (c') QPCR plots showing *reelin* downregulation in interdigit explants treated with BMP-7. ** $P < 0.01$; * $P < 0.05$

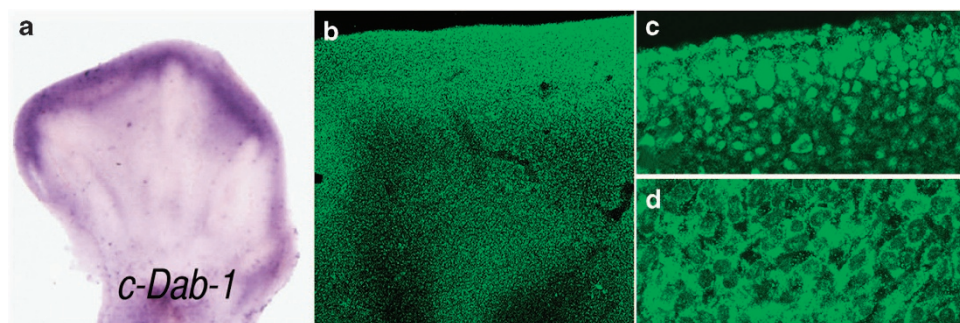


Figure 2 (a) *In situ* hybridization illustrating *Dab-1* expression in a vibratome section of the chick autopod at 7.5 i.d. Note that expression overlaps with that of *reelin* (b, in Figure 1). (b) Low-magnification view of the third interdigit at 6.5 i.d. immunolabeled with DAB-1. (c and d) Distal undifferentiated mesoderm at 6.5 i.d. showing p-DAB-1 (c) and non-phosphorylated DAB-1 (d) immunolabeling. Note that active signaling as revealed by p-DAB-1 predominates in the distal mesoderm

transfected *shReelin* or *shDab-1* constructs into undifferentiated mesodermal cells obtained from leg buds on days 4 and 5 of incubation (stage 26 HH). By Q-PCR and flow cytometry, we observed that the profile of gene regulation and cell death were similar after *reelin* or *Dab-1* silencing, although the effects of the latter were more intense. Hence, we used the *shDab-1* construct for subsequent studies. Changes in gene expression induced by *Dab-1* gene silencing (Figure 4) included downregulation of transforming growth factor $\beta 2$ (*Tgf β 2*), which is a triggering signal for digit

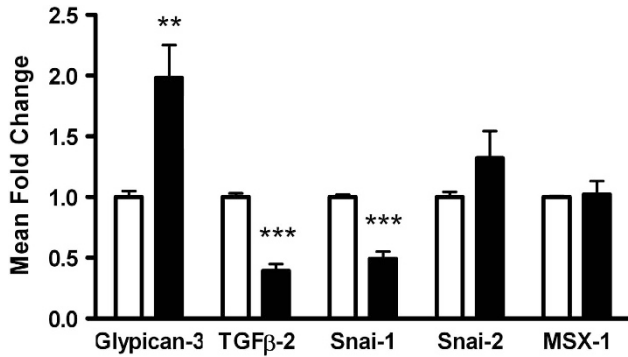


Figure 4 Chart showing the regulation of the *Glypican*, *Tgf β 2*, *Msx-1*, *Snai-1*, and *Snai-2* genes in 3-day mesodermal cell cultures transfected with *shDab-1* (black column) in comparison to control cultures (white column). *** $P < 0.001$; ** $P < 0.01$

formation;³² upregulation of *Glypican 3*, a cell surface anchored proteoglycan that inhibits cell proliferation and survival via interactions with several growth factors and adhesion and matrix molecules^{33,34} and downregulation of *Snai1*, a transcription factor that regulates cell adhesion through the integrin signaling cascade.³⁵ The expression of other transcription factors characteristic of the undifferentiated and interdigital mesoderm including *Msx1* and *Snai2/Slug* were not modified.

Changes in cell proliferation or cell death were analyzed by flow cytometry after propidium iodide (PI) staining. Proliferation was not modified in undifferentiated mesoderm that had been subjected to *Dab-1* silencing, but cell death increased significantly compared to control mesodermal cells transfected with the empty vector (Figures 5a–c). This increased cell death was confirmed by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Figures 5d–e). To further analyze whether cell death associated with *Dab-1* silencing recapitulates the regulatory mechanism responsible for physiological cell death in limb development, we analyzed the changes in protein kinase B (AKT). The maintenance of progress zone (PZ) mesoderm viability and proliferation is associated with the activation of AKT by phosphorylation.³⁶ We consistently detected more than 50% downregulation of AKT phosphorylation in mesodermal cells transfected with *shDab-1* (Figure 5f).

Because Reelin signaling is functionally associated with integrins,^{21–23} the modification of gene expression after *Dab-1* silencing might well reflect changes in cell adhesion. We have

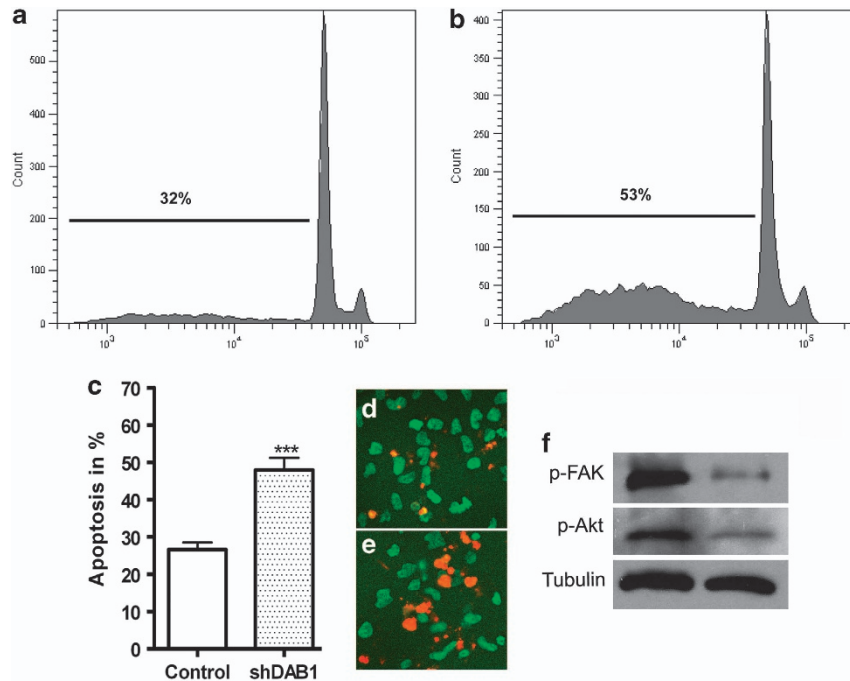


Figure 5 (a) Representative flow cytometry plots of dissociated propidium iodide-stained mesodermal cells indicating an increase in apoptosis in cells transfected with *shDab-1* (57% gene silencing), (b) in comparison to control cells transfected with the empty vector (A). (c) Average difference in the percentage of apoptotic cells in cells transfected with *sh-Dab-1* versus control cells as evaluated by flow cytometry (***) $P < 0.001$. (d and e) Cell death analysis in undifferentiated limb mesodermal cells in controls (d) and upon *Dab-1* gene silencing (e) after immunolabeling for pan-histone (green) and TUNEL (red). (f) Western blot showing the inhibition of FAK and AKT phosphorylation in *Dab-1*-deficient cultures (right lane) in comparison to control cultures transfected with the empty vector (left lane)

previously shown that different integrins that are able to bind Reelin, including $\alpha 3$, $\alpha 5$, and $\beta 1$, are expressed in the undifferentiated limb mesoderm.³⁷ Thus, we next explored changes in the integrin-dependent activation of focal adhesion kinase (FAK). As shown in Figure 5f, a $60 \pm 8\%$ reduction in phospho-FAK levels was observed by western blotting of samples obtained from *Dab-1* deficient mesodermal precursors.

Discussion

From the pioneering study of Saunders *et al.*,³⁸ which was later confirmed by modern molecular approaches,³⁹ it is known that the population of mesodermal cells occupying the distal tip of the limb, known as the PZ, is responsible for distal limb outgrowth. Mesodermal cells within this region are connective tissue progenitors and are maintained in an undifferentiated and proliferative state due to the influence of the apical ectodermal ridge (AER) rimming the distal margin of the bud. Cells undergo differentiation as they move out of the range of AER signaling. It has been shown that progress zone progenitors require survival factors until they initiate differentiation.^{40,41} Consistent with these observations, when digit primordia are formed, undifferentiated cells that occupy the interdigital spaces suffer massive apoptosis that sculpts the digits from the hand and foot plates. FGFs produced by the AER are considered major survival signals for the undifferentiated limb mesoderm,³⁰ while BMPs exert an opposing function that promotes cell death.³¹ Here, we show that Reelin signaling is active in the undifferentiated mesoderm and is downregulated preceding the regression of interdigital tissue in chick and mouse embryos.

Reelin is a large extracellular matrix glycoprotein with putative functions in the embryonic and adult nervous systems; however, increasing information shows that Reelin is also implicated in other systems.^{42–46} Our findings suggest that Reelin contributes to the creation of an extracellular substrate required for maintaining the viability of undifferentiated limb mesodermal cells until they initiate differentiation. In chick limbs, the interdigital expression of *reelin* is intensely downregulated preceding cell death. Moreover, we observed that *reelin* is upregulated by a local application of FGFs. These treatments cause the inhibition of interdigital cell death.³⁰ In a complementary fashion, local application of BMPs, which are proapoptotic factors,⁴⁷ downregulates the expression of *reelin*. Consistent with these findings, disruption of Reelin signaling by silencing *Dab-1* increases apoptosis. Markers of the undifferentiated mesoderm such as *Msx1* and *Snai2/Slug* were not regulated. However, the zinc-finger transcription factor *Snai1*,⁴⁸ which shows the same interdigital expression pattern as *reelin* and *Dab-1* in the autopod,³⁰ was significantly downregulated. *Snai1* contributes to the maintenance of the immature phenotype of stem cells and regulates cell adhesion in the integrin signaling cascade.³⁵

Dab-1 is a central component of Reelin signaling. It is phosphorylated by Src family of non-receptor tyrosine kinases^{26,27} after binding of Reelin to its target receptors. Phosphorylated DAB-1 activates intracellular kinases, including Src, phosphatidylinositol-3-kinase (PI3K), the serine/threonine kinase AKT, and the mitogen-activated protein

kinase/extracellular signal-regulated kinase, Erk.^{25,49,50} Although the activity of phosphorylated DAB-1 has been largely associated with the stabilization of the cytoskeleton and regulation of neuronal migration (reviewed by Herz and Chen⁵¹), there is evidence that suggest that some of the neural functions of Reelin are due to the regulation of cell–matrix adhesion via binding to $\alpha 3\beta 1$ integrin^{22,23} or activation $\alpha 5\beta 1$ integrin through a biologically conserved inside-out signaling cascade.⁵² In our study, the transcriptional effects of *Dab-1* silencing are associated with reduced AKT and FAK phosphorylation, supporting a role for Reelin/*Dab-1* signaling in the promotion of cell–matrix adhesion via integrins. FAK is a major downstream effector of integrin signaling and is implicated in the formation of the prechondrogenic aggregates.^{53–55} Furthermore, the activation of AKT accounts for the pro-survival influence of FGFs in the developing limb.³⁶ Both FAK and AKT have been identified as key targets of cell death by anoikis.⁵⁶ Together, these findings suggest that the disruption of Reelin signaling in the mesodermal tissue is followed by reduced integrin-mediated cell adhesion. Many different integrins, including $\alpha 3$, $\alpha 5$, and $\beta 1$, are expressed in digit progenitors.³⁷ Integrins recognize various extracellular matrix components that are abundant in the developing limb, and deficiencies in some of these components are associated with syndactylous mice phenotypes.^{57,58} This fact makes it difficult to establish the relative importance of Reelin in the regression of the interdigital tissue. Mice deficient in *reelin* or *Dab-1* (Reeler or Yotari mutants, respectively) lack the syndactylous phenotype,⁵⁹ suggesting functional redundancy of Reelin with other matrix molecules present in the embryonic limb. In addition, it must be taken into account that the absence of Reelin in mice mutants occurs through the entire embryonic development and not in a small window of time analyzed in our *in vitro* experiments. Furthermore, predicting protein-protein interactions by a computational algorithm based on structural, functional, and evolutionary data (<http://bhapp.c2b2.columbia.edu/PrePPI>⁶⁰) identifies more than 100 matrix and regulatory molecules expressed in the developing limb that are likely to interact with Reelin. Our findings, together with these observations, suggest that Reelin, while not strictly necessary, contributes to sustain the anchoring of the undifferentiated mesoderm to the extracellular matrix to prevent anoikis.

Materials and Methods

In this work, we employed Rhode Island chicken embryos from 4.5 to 8 days of incubation (*i.d.*), which were equivalent to stages 25 to 36 HH and C57BL6 mouse embryos ranging from 11 to 14 days *post coitum* (*p.c.*).

In situ hybridization. *In situ* hybridization was performed in whole-mount specimens and vibratome tissue sections. Samples fixed in 4% paraformaldehyde (PFA) were treated with 10 $\mu\text{g}/\text{ml}$ of proteinase K for 20–30 min at 20 °C. Hybridization with digoxigenin-labeled antisense RNA probes was performed at 68 °C. Alkaline phosphatase-conjugated anti-digoxigenin antibody (dilution 1:2000) was used (Roche Applied Science, Indianapolis, IN, USA). Reactions were developed with the BM Purple AP Substrate precipitating solution (Roche Applied Science).

The probes for *reelin* and *Dab-1* were obtained by PCR: chick Reelin 5'-CCTAACAAATCGCAACCAAGG-3' and 5'-AGCCTTCTGTTGGAGTCAGG-3'; for mouse *reelin* 5'-AACACAGTCCATGCAGATCG-3' and 5'-CTTCTGCCA GTCTGATTCC-3'; for chick *Dab-1* 5'-AACCTGTGATTCTGGACTTGC-3' and 5'-TGAACAAAGTGGTTGCTGTGCC-3'.

In vivo gene regulation analysis was performed in the embryonic chick by whole-mount *in situ* hybridization following interdigital implantation at 5.5 i.d. of heparin beads (Sigma-Aldrich, St Louis, MO, USA) incubated for 1 h in 0.5 mg/ml FGF2 (PeproTech, Rocky Hill, NJ, USA) or 0.5 mg/ml BMP-7 (R&D Systems, Minneapolis, MN, USA). The contralateral left limb or limbs treated with beads incubated in phosphate-buffered saline (PBS) were used as controls. After manipulation, the eggs were sealed and further incubated until processing.

Mesodermal cultures. Undifferentiated mesodermal cells obtained from the region located under the AER of chick leg buds at 4.5 i.d. (25 HH) were suspended in medium DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were seeded on fibronectin-coated coverglasses and were grown as monolayers or cultured at a high density as micromass cultures.

Micromass cultures were made by pipetting 10-µl drops of cell suspension at a density of 2.0×10^7 cells/ml into each well of a 24-well plate. The cells were left to attach for 2 h and then 200 µl serum-free medium was added.

Micromasses were used to analyze cell proliferation and cell death in mesodermal cells subjected to *reelin* signaling knockdown. Monolayer cultures were used for TUNEL assay to detect cell death.

Quantitative analysis of *reelin* regulation by BMPs was performed in interdigital explants cultured for 5 h in presence of 0.6 µg/ml of BMP7.

Immunolabeling and TUNEL assay. Samples were fixed with 4% PFA O/N at 4 °C, washed with 0.1% Triton/PBS and incubated O/N at 4 °C with the primary antibody. Specimens were next washed with PBS, incubated O/N with the secondary antibody, washed for 2 h with PBS, dehydrated and cleared. Anti-Reelin (SC-5578, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-phospho-DAB-1 (SC-133292, Santa Cruz Biotechnologies) and anti-Dab1 (SC-7827, Santa Cruz Biotechnologies) polyclonal antibodies were used.

Samples were monitored by confocal microscopy, and analysis of immunolabeling intensity was performed measuring pixel intensity distribution using the LSM 5 Image Examiner software (LSM Software Zeiss, Oberkochen, Germany).

TUNEL was performed using the *in situ* cell death detection kit (Roche Applied Science) according to the manufacturer's instructions.

Cell nucleofection and target gene silencing. Functional studies were performed in chick embryos by a loss-of-function approach. For this purpose, limb mesodermal cells were transfected with a short hairpin RNAi for *reelin* (*shreelin*) or *Dab-1* (*shDab-1*) and cloned into the pcU6-1-shRNA (a generous gift of Dr Tim J Doran) as described by Wise *et al.*²⁸ The level of gene silencing was evaluated by Q-PCR and samples with a reduction in gene expression ranging between 45% and 60% were selected for further studies. Control transfections using a chU6-1v irrelevant control plasmid were performed in all experiments.

Western blot. For western blot analysis, total protein extracts were obtained from control and *shDab-1* silenced mesodermal cultures or interdigital tissues at different stages. Cell lysis was performed with RIPA buffer (150 mM NaCl, 1.5 mM MgCl₂, 10 mM NaF, 10% glycerol, 4 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholate and 50 mM Hepes, pH 7.4) supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 1 mM), leupeptin (10 µg/ml) and aprotinin (10 µg/ml), for 15 min on ice. The cell lysates were clarified of cellular debris by centrifugation (13200 × g) for 10 min at 4 °C. Proteins were separated by 8–10% polyacrylamide gel electrophoresis containing 0.1% SDS and transferred to nitrocellulose membrane (Protan; Whatman, Dassel, Germany). The membranes were incubated for 1 h at room temperature in bovine serum albumin and incubated overnight with the following antibodies: rabbit polyclonal antibody anti-phospho-Akt (SC-1011629, Santa Cruz Biotechnologies), anti-Reelin (SC-5578, Santa Cruz Biotechnologies) and anti-phospho-FAK Y576 (Abcam, Cambridge, UK; ab-4815), and mouse polyclonal antibody anti-tubulin (Sigma-Aldrich, T5168). Protein bands were detected in the membranes with an ODYSSEY infrared-imaging system (LI-COR Biosciences, Lincoln, NE, USA) according to ODYSSEY western blot protocol. Immunoblots were monitored using anti-mouse IRDye 800DX or anti-rabbit IRDye 680DX (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA). The public domain image analysis program 'ImageJ' was employed to perform quantitative analysis.

Flow cytometry. Cell proliferation and cell death was deduced from measurement of DNA content by flow cytometry. Control and *shreelin* or

shDab-1 silenced cultures were dissociated to single-cell level by treatment with Trypsin EDTA (Lonza, Basel, Switzerland). One million cells were used in each test. For PI staining, the cells were washed twice in PBS and centrifuged at 405 × g for 5 min at 4 °C. The samples were then incubated overnight at 4 °C with 0.1% sodium citrate, 0.01% Triton X-100 and 0.1 mg/ml PI. Cell suspension was subjected to flow cytometry analysis in a Becton Dickinson FACSanto cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with Cell Quest software (BD Biosciences). This technique allows the quantification of apoptotic (hypodiploid) and proliferating (hyperdiploid) cells according to their DNA content deduced from PI staining.

Real time quantitative PCR (Q-PCR) for gene expression analysis. Total RNA was extracted and cleaned from specimens using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA samples were quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was synthesized by reverse transcription-PCR (RT-PCR) using random hexamers, and M-MuLV reverse transcriptase (Fermentas GmbH, St Leon-Rot, Germany). The cDNA concentration was measured in a spectrophotometer (NanoDrop Technologies) and adjusted to 0.5 µg/µl. Q-PCR was performed using the Mx3005P system (Stratagene Inc., La Jolla, CA, USA) with automation attachment. In this work, we have used SYBRGreen (TaKaRa Inc., Otsu, Japan) based Q-PCR. *Gapdh* had no significant variation in expression across the sample set and therefore was chosen as the normalizer in our experiments. Mean values for fold changes were calculated for each gene. Expression level was evaluated relative to a calibrator according to the $2^{-(\Delta\Delta Ct)}$ equation. Each value in this work represents the mean ± standard error of the mean of at least three independent samples obtained under the same conditions. Samples consisted of 4 micromass cultures or 20 interdigital spaces. Data were analyzed using one-way analysis of variance followed by Bonferroni tests for post-hoc comparisons for gene expression levels in treated micromass cultures and Student's *t* test for gene expression levels in overexpression and silencing experiments. Statistical significance was set at $P < 0.05$. All the analyses were done using SPSS for Windows version 18.0. Q-PCR specific primers for chick genes were: for *Glypican 3* 5'-GGAGAAGTACCAGGCAGTGG-3' and 5'-CAGCATTCTGGATGACAAGG-3'; for *Tgfβ2* 5'-TGCACTGCTATCTCCTGAGC-3' and 5'-GCATGAACTGATCCA TGTCG-3'; for *Msx1* 5'-CAAGCACAAAGACCAACAGGA-3' and 5'-TACTGTCTCTGGCGGAATTT-3'; for *Snai2/Slug* 5'-ATACCGCAGCCAGAGATCC-3' and 5'-AGCGGAGAGAGGTCATTGG-3'; for *Snai1/Snai1* 5'-TGCAGAGAAGGAGTA TGTGAGC-3' and 5'-GCACATCTTGACAGACAGG-3'; for *reelin* 5'-GCACAGT GACAGCATATCC-3' and 5'-GCTCTCCAATCTCACAGTTGC-3'; for *Dab1* 5'-AACCTGTGATTCTGGACTTGC-3' and 5'-ATACCGCCTGTTCCACTGC-3'.

Conflict of Interest

The authors declare no conflict of interest.

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