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# Sim2 prevents entry into the myogenic program by repressing *MyoD* transcription during limb embryonic myogenesis

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## SUMMARY

The basic helix-loop-helix transcription factor MyoD is a central actor that triggers the skeletal myogenic program. Cell-autonomous and non-cell-autonomous regulatory pathways must tightly control *MyoD* expression to ensure correct initiation of the muscle program at different places in the embryo and at different developmental times. In the present study, we have addressed the involvement of Sim2 (single-minded 2) in limb embryonic myogenesis. Sim2 is a bHLH-PAS transcription factor that inhibits transcription by active repression and displays enhanced expression in ventral limb muscle masses during chick and mouse embryonic myogenesis. We have demonstrated that *Sim2* is expressed in muscle progenitors that have not entered the myogenic program, in different experimental conditions. *MyoD* expression is transiently upregulated in limb muscle masses of *Sim2*<sup>−/−</sup> mice. Conversely, *Sim2* gain-of-function experiments in chick and *Xenopus* embryos showed that *Sim2* represses *MyoD* expression. In addition, we show that Sim2 represses the activity of the mouse *MyoD* promoter in primary myoblasts and is recruited to the *MyoD* core enhancer in embryonic mouse limbs. *Sim2* expression is non-autonomously and negatively regulated by the dorsalising factor *Lmx1b*. We propose that Sim2 represses *MyoD* transcription in limb muscle masses, through Sim2 recruitment to the *MyoD* core enhancer, in order to prevent premature entry into the myogenic program. This *MyoD* repression is predominant in ventral limb regions and is likely to contribute to the differential increase of the global mass of ventral muscles versus dorsal muscles.

**KEY WORDS:** Sim2, MyoD (Myod1), Embryonic myogenesis, Limb, Chick, Mouse

## INTRODUCTION

The basic helix-loop-helix (bHLH) transcription factor MyoD (also known as Myod1) is central to triggering the skeletal myogenic program during the successive waves of embryonic, foetal, perinatal and adult myogenesis. A key issue is to understand the regulatory networks that drive *MyoD* expression at precise places and times in the embryo.

Embryonic myogenesis leads to the formation of the first multinucleated muscle fibres from embryonic muscle progenitors. The main role of this first wave of myogenesis is to initiate skeletal muscle differentiation at the appropriate locations in the embryo, while the second wave of foetal myogenesis uses embryonic fibres as a scaffold for muscle growth. Consequently, it is essential to understand the intrinsic and extrinsic pathways that specify the entry into the embryonic myogenic program at different places in the embryo. In addition, the embryonic myogenic program must be tightly regulated to retain a muscle progenitor pool for the following waves of myogenesis. Embryonic muscle progenitors rely on the activation of the skeletal muscle differentiation program based on the myogenic regulatory factors (MRFs). The MRFs

includes four bHLH transcription factors: Myf5, MyoD, Mrf4 and myogenin. Myf5, Mrf4 and MyoD constitute the core regulatory network for the myogenic program. In their absence, myoblasts are lacking and skeletal muscles do not form (Kassar-Duchossoy et al., 2004). Conversely, they are sufficient for skeletal muscle differentiation in ectopic contexts in vitro and in vivo (Weintraub et al., 1991; Delfini and Duprez, 2004). The epistatic relationships between *Myf5*, *Mrf4*, *MyoD* and other transcription factors modulating their expression differ according to muscle groups in different anatomical locations in the embryos (Bismuth and Relaix, 2010).

Limb embryonic myogenesis involves delamination and migration of muscle progenitor cells from the hypaxial dermomyotome (Duprez, 2002). The transcription factors *Lbx1*, *Pax3* and *Six1/4* are all involved in muscle progenitor migration into the limb buds (Bismuth and Relaix, 2010). In addition, *Pax3* and *Six1/4* positively regulate *Myf5* expression in the limbs by direct binding to different regulatory regions of the *Myf5* promoter (Bajard et al., 2006; Giordani et al., 2007). The initiation of *MyoD* expression in limb skeletal muscles is also directly and positively regulated by the paired-related homeodomain transcription factor *Pitx2*, independently of *Myf5* (L'Honore et al., 2010). The decision to enter the myogenic program or to remain undifferentiated must be tightly regulated in order to maintain the pool of muscle progenitors during development. The identified transcription factors that act autonomously in limb myogenic cells have been shown to modulate positively *Myf5* or *MyoD* expression (Bajard et al., 2006; Giordani et al., 2007; L'Honore et al., 2010). However, to date, no intrinsic repressor activity has been described to regulate the entry into the embryonic muscle program in limbs.

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The single-minded 2 (*Sim2*) transcription factor has been previously shown to be expressed in a restricted pattern in embryonic myogenic cells during chick and mouse limb development (Coumailleau and Duprez, 2009). *Sim2* expression is transiently enhanced in ventral limb muscle masses (Coumailleau and Duprez, 2009). *Sim2* contains a bHLH domain, two PAS (Per-Arnt-Sim) domains and one HST (Hif1- $\alpha$ /SIM/TRH) domain. Although most bHLH-PAS proteins are transcriptional activators, *Sim2* acts as a repressor of transcription in mammalian cells (Moffett et al., 1997; Moffett and Pelletier, 2000; Metz et al., 2006). The repressor activity has been localised into two independent repression domains in the C-terminal region of mouse *Sim2* protein (Moffett et al., 1997; Moffett and Pelletier, 2000; Metz et al., 2006). The location of the *SIM2* gene on the human chromosome 21, its expression in brain and the behaviour of transgenic mice with three copies of the *Sim2* gene, have led to the suggestion that *SIM2* is important in the mental retardation associated with Down's syndrome (Ema et al., 1999; Chrast et al., 2000). A short splice variant of the *SIM2* gene, *SIM2s*, which lacks one of the two repression domains of *SIM2*, has been alternatively identified as a tumour suppressor or activator depending on the tumour type (DeYoung et al., 2003; Aleman et al., 2005; Halvorsen et al., 2007; Kwak et al., 2007; Laffin et al., 2008). Despite the loss of one repression domain, *SIM2s* has been shown to repress directly target gene transcription in different cellular models (Kwak et al., 2007; Laffin et al., 2008; Woods et al., 2008; Farrall and Whitelaw, 2009; Wellberg et al., 2010). *Sim2* mutant mice die perinatally owing to lung atelectasis and breathing failure, but display multiple other phenotypes such as rib, vertebrae and cranio-facial abnormalities (Goshu et al., 2002; Shambloot et al., 2002). The only muscle phenotype described in the absence of *Sim2* activity in mouse mutants is a diaphragm hypoplasia at birth (Goshu et al., 2002).

In the present study, we investigated the involvement of *Sim2* in limb embryonic myogenesis. We have shown that *Sim2* is expressed in muscle progenitor cells that have not entered the myogenic program in normal or experimental conditions. *Sim2* gain- and loss-of-function experiments in mouse, chick and *Xenopus* embryos show that *Sim2* represses *MyoD* expression. In addition, *Sim2* represses the activity of the mouse *MyoD* promoter in primary myoblasts and is recruited to the *MyoD* core enhancer in embryonic mouse limbs. Moreover, *Sim2* is negatively and non-autonomously regulated by the dorsalising factor *Lmx1b*. Taken together, our results establish that *Sim2* represses *MyoD* transcription in limb muscle masses, via the recruitment of *Sim2* to the *MyoD* core enhancer, in order to prevent a premature entry into the myogenic program.

## MATERIALS AND METHODS

### Chick embryos and mouse lines

Fertilised chick eggs from commercial sources, JA 57 strain (Morizeau, Dangers) or White Leghorn (HAAS, Kaltenhouse) were incubated at 38°C. Embryos were staged according to somite number or Hamburger and Hamilton (HH) stages (Hamburger and Hamilton, 1992). *MyoD*<sup>+/−</sup> (Kablar et al., 1997), *Myf5*<sup>nlacZ</sup> (Kassar-Duchossoy et al., 2004), *Sim2*<sup>+/−</sup> (Goshu et al., 2002) or *Pax3*<sup>IRESnacZ</sup> (Relaix et al., 2004) mouse embryos were collected after natural overnight matings. For staging, fertilisation was considered to take place at midnight.

### In situ hybridisation to tissue sections and to whole-mount embryos

Chick and mouse embryos were fixed and processed for digoxigenin-labelled probe in situ hybridisation to whole mounts and to wax sections as previously described (Bonnet et al., 2010). For BrdU analyses, chick

embryos were incubated with BrdU 1 hour before fixation. For double fluorescent in situ hybridisation, the two probes were tagged with digoxigenin-11-UTP or DNP-11-UTP, and were detected using Perkin Elmer Life Sciences TSA plus Cy3/Cy5 fluorescence dual detection system. For non-fluorescent double in situ hybridisation, the two probes were tagged with digoxigenin-11-UTP or fluorescein-11-UTP and detected with NBT/BCIP and INT/BCIP reagents, respectively, as already described (Delfini and Duprez, 2000). The antisense mRNA probes were used as previously described: chick (c) *Fgfr4*, *cPax3*, *cMyoD* and *cDelta1* (Delfini et al., 2000); mouse (m) *Myf5*, *mMyoD*, *mMyog* and GFP (Bonnet et al., 2010); *Xenopus* *MyoD* (Li, H. Y. et al., 2010); *cLmx1b* (Michaud et al., 1997); and *cSim2* and *mSim2* (Coumailleau and Duprez, 2009). The *mSim2* probe used for endogenous *mSim2* expression in mouse limbs recognises exon 11 and consequently does not recognise the splice variant *mSim2s* (Metz et al., 2006). The *mSim2* probe used after chick electroporation experiments was specially designed not to crossreact with endogenous *cSim2* expression, by PCR amplification using the *Sim2N2* primers (see Table S1 in the supplementary material) from the *mSim2*(Myc)pEFIRESpuro (Woods et al., 2008). For in situ hybridisation experiments of mouse embryos, sections of mutant and wild-type embryos were mounted on the same slide and consequently were treated exactly in the same conditions to facilitate comparison. Quantification of *MyoD* expression after situ hybridisation was performed by particle counting using the Image J software. For each in situ hybridisation experiment, at least three mutants and stage-matched wild-type mouse embryos from three different litters were used.

### Production and grafting recombinant/RCAS-expressing cells

The *Delta1*/RCAS-expressing cells were prepared for grafting as previously described (Delfini et al., 2000). The left wings were used as internal controls and compared with the grafted wings at the same proximodistal level. Six chick embryos grafted with *Delta1*/RCAS-expressing cells were analysed by in situ hybridisation.

### Neural tube and somite electroporation

Chick neural tube and somite electroporation experiments were performed as already described (Delfini and Duprez, 2004; Bonnet et al., 2010). Neural tubes were co-electroporated with *mMyf5*-pCA $\beta$  or *mSim2*-pCA $\beta$  in association with eGFP-pCA $\beta$ . Somites were co-electroporated with *mSim2*-pCA $\beta$  and eGFP-pCA $\beta$ . Control embryos were electroporated with eGFP-pCA $\beta$  alone. The *mSim2*-pCA $\beta$  plasmid was obtained by replacing the GFP *EcoRI*-*XhoI* fragment of eGFP-pCA $\beta$  by a *mSim2* fragment amplified by PCR using the primers *Sim2CS* (supplementary material Table S1) from *mSim2*(Myc)pEFIRESpuro (Woods et al., 2008). Embryos were harvested 24 hours after electroporation. The expression of each gene was analysed on at least four different electroporated embryos in each condition.

### RNA extraction and quantitative real-time PCR

Total RNAs were extracted from forelimbs of mutant and control mice using the RNeasy kit (Qiagen) and reverse-transcribed using the Superscript II Reverse Transcriptase kit (Invitrogen) according to manufacturer's instructions. Quantitative real time PCR was performed using a Lightcycler 480 Sybergreen mix (Roche). Primer sequences are listed in supplementary material Table S1. For mRNA levels, gene changes were quantified using the DDcT method. Quantitative data shown as histograms are expressed as means and S.E. Fold changes were assessed for statistical significance by Student's *t*-test. Asterisks in figures indicate the different *P* values (\*\*<0.01; \*\*\*<0.001). The experiment was performed with three independent samples for each genotype, and Q-PCR was carried out in triplicate for each gene.

### Xenopus injections

*Xenopus* eggs were artificially fertilised with minced testis. Synthetic capped mRNAs for *mSim2* and  $\beta$ -galactosidase ( $\beta$ -gal) were obtained by in vitro transcription using SP6 RNA polymerase. Microinjection and  $\beta$ -gal staining using red-gal as a substrate were performed as previously described (Li, H. Y. et al., 2010).

### Cell culture

Primary myoblasts were prepared from hindlimb muscles of 10-day-old chick embryos as described (Spitz et al., 1997; Giordani et al., 2007). Cells were transfected with 50 ng pTK-renilla, 100 ng eGFP-pCA $\beta$ , 500 ng mSim2-pCA $\beta$  or mMyoD-pCA $\beta$  or both and 850 ng of reporter plasmids, pCore-TATA-luciferase, pDRR-TATA-luciferase or TATA-luciferase according to the lipofectamin transfection kit (Invitrogen) protocol. The pDRR-TATA-luciferase was provided by Pascal Maire (Institut Cochin, Paris, France). The pCore-TATA-luciferase was obtained as followed: the core enhancer was amplified by RT-PCR using the primers MyoDcore (see supplementary material Table S1) from MyoD EnhancerpMDE-1 (provided by Shahragim Tajbakhsh, Institut Pasteur, Paris, France) and was then inserted in the TATA-luciferase vector. Cells were harvested 3 days after transfection and luciferase activity was measured according to the dual-luciferase reporter assay system protocol (Promega). All transfection experiments were performed in triplicate and repeated five times.

### Chromatin immunoprecipitation

ChIP assays were performed as already described (Havis et al., 2006). Fifty limbs from E11.5 mouse embryos were homogenised using a mechanical disruption device (Lysing Matrix A, Fast Prep MP1, 30 sec). 10  $\mu$ g of the rabbit polyclonal Sim2 antibody (Millipore), or 8  $\mu$ g of the acetylated histone H4 (ACH4) antibody (Upstate Biotechnology) as a positive control, were used to immunoprecipitate 25  $\mu$ g of sonicated chromatin. ChIP products were analysed by PCR. Three pairs of primers were used to amplify fragments associated with the core enhancer, the DRR and the PRR, respectively (supplementary material Table S1). The ChIP experiments were performed three times.

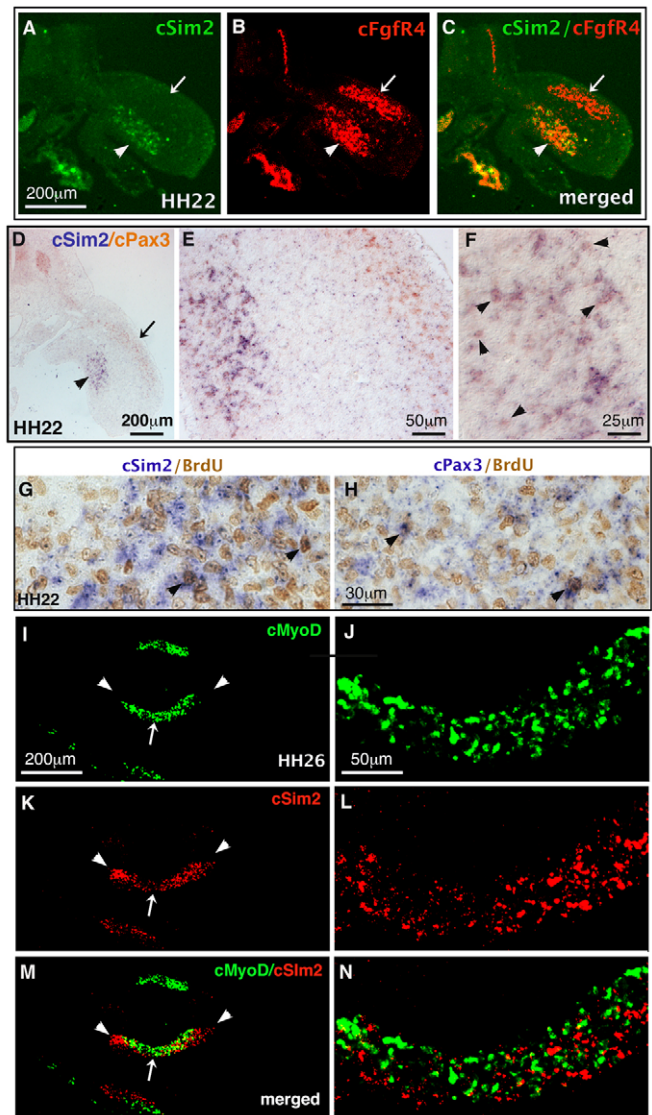
### Immunohistochemistry

Progenitor and differentiated muscle cells were detected with the monoclonal antibodies Pax7 and MF20, respectively. The Pax7 and MF20 antibodies developed by investigators A. Kawakami and D. A. Fischman, respectively, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa. GFP protein was detected using a monoclonal antibody (Roche) after in situ hybridisation. BrdU was detected using a monoclonal antibody (Amersham) after in situ hybridisation.

## RESULTS

### *Sim2* is expressed in chick limb myogenic cells not expressing *MyoD*

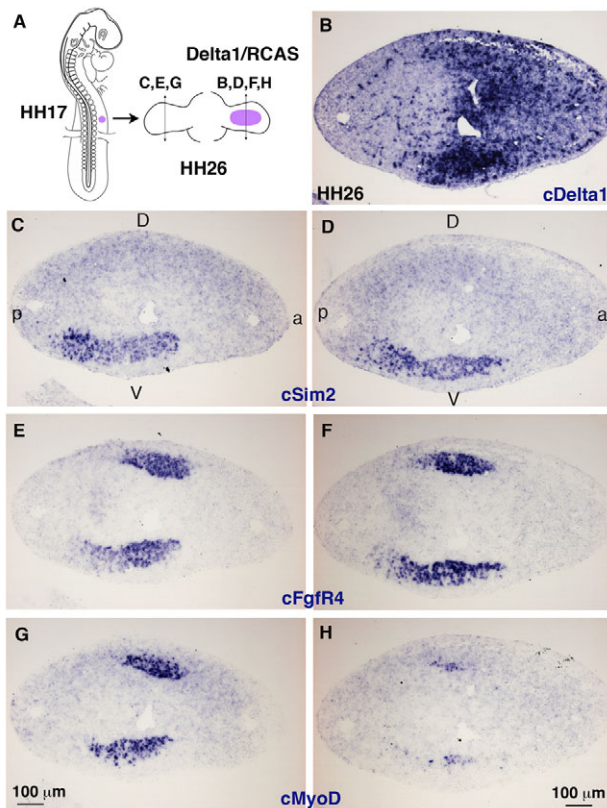
The onset of *Sim2* expression in chick limbs was observed at HH20 after the migration process but before the onset of *MyoD* expression (Coumailleau and Duprez, 2009). *Pax3* is the main marker of embryonic muscle progenitors (Relaix et al., 2004). The fibroblast growth factor receptor 4, *Fgfr4* lies genetically downstream of *Pax3* and is a direct target of *Pax3* in mouse limbs (Lagha et al., 2008). In chick limbs, *Fgfr4* transcripts were observed in replicating muscle progenitors from HH20 and were excluded from muscle fibres (Marcelle et al., 1995; Edom-Vovard et al., 2001; Bonnet et al., 2010). In order to correlate *Sim2* expression with that of muscle progenitor markers, we performed double *Sim2* in situ hybridisation with *cFgfr4* and *cPax3* probes to chick limb sections. We observed a colocalisation of *cSim2* transcripts with that of *cFgfr4* (Fig. 1A-C) and *cPax3* (Fig. 1D-F) in ventral limb muscle masses at HH22. Moreover, we identified *cSim2*-positive cells that have incorporated BrdU as *cPax3*-positive cells in limb muscle masses (Fig. 1G,H, arrowheads), suggesting that *Sim2* was associated with a proliferative state. Once limb myogenic cells have entered the differentiation program assayed by *MyoD* expression, muscle masses contain a heterogeneous population of myogenic cells, which are not at the same stage of muscle differentiation. In order to define the cellular expression of *Sim2* versus that of *MyoD*, we performed double in situ hybridisation in chick forelimbs. At HH26, *cMyoD* mRNAs were



**Fig. 1. *Sim2* is expressed in chick limb muscle cells not expressing *MyoD*.** (A-F) Transverse sections of HH22 chick embryos at forelimb level were co-hybridised with *cSim2* (green) and *cFgfr4* (red) fluorescent probes (A-C) or with *cSim2* (digoxigenin, purple) and *cPax3* (fluorescein, orange) probes (D-F). (A-D) Arrowheads indicate ventral muscle masses; arrows show dorsal muscle masses. (F) Arrowheads indicate examples of *cSim2*<sup>+</sup> and *cPax3*<sup>+</sup> cells in ventral muscle masses. (G,H) Limb sections of HH22 chick embryos, incubated with BrdU 1 hour before fixation, were hybridised with *cSim2* (E) and *cPax3* (F) digoxigenin-labelled probes (blue) and then incubated with the BrdU antibody (brown). Arrowheads indicate the *cSim2*<sup>+</sup> (E) and *cPax3*<sup>+</sup> (F) cells, which are also BrdU positive. (I-N) Transverse sections of HH26 chick forelimbs were labelled by double in situ hybridisation with *cMyoD* (green) and *cSim2* (red) fluorescent probes. (I,K,M) In ventral muscle masses, arrowheads indicate the *cSim2* expression domains not overlapping with that of *cMyoD*, whereas arrows indicate the overlapping *cSim2* and *cMyoD* expression domains. (J,L,N) High magnifications of ventral muscle masses show that in overlapping expression domains, *cSim2* mRNA and *cMyoD* mRNA are not expressed in the same cells.

expressed in myoblasts in dorsal and ventral muscle masses, whereas *cSim2* transcripts were observed mainly in ventral muscle masses. In ventral muscle masses, *cSim2* and *cMyoD* transcripts were not



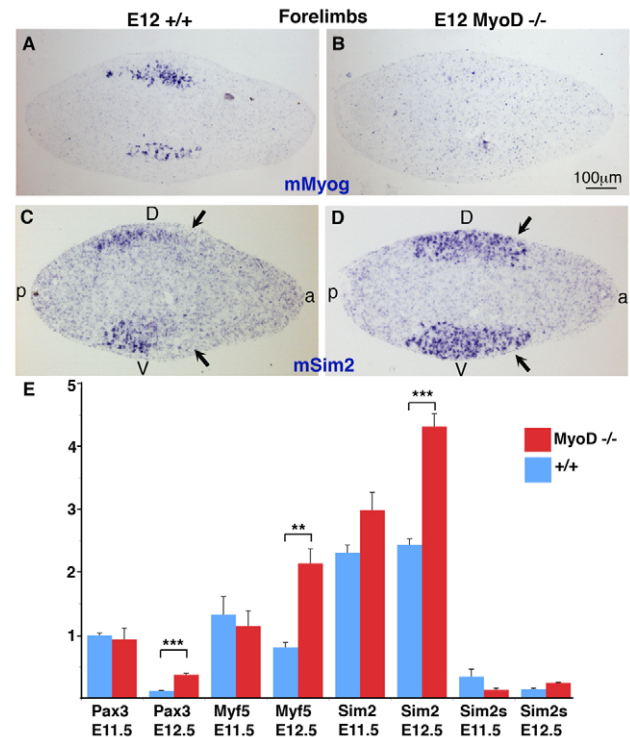


**Fig. 2. *Sim2* expression is not regulated by Notch signalling in chick limbs.** (A) Delta1/RCAS-expressing cells were grafted into the presumptive right forelimb buds of HH17 chick embryos. (B-H) Delta1-grafted right (B,D,F,H) and control left (C,E,G) forelimbs from HH26 chick embryos were cut transversely and analysed for *cDelta1*, *cSim2*, *cFgfr4* and *cMyoD* expression. Adjacent sections of experimental and control forelimbs were hybridised with *cDelta1* (B), *cSim2* (C,D), *cFgfr4* (E,F) and *cMyoD* (G,H) digoxigenin-labelled probes. *cSim2* expression and that of the progenitor marker *cFgfr4* are not affected by Notch signalling misexpression (C-F), whereas *cMyoD* expression is downregulated (G,H). a, anterior; p, posterior; D, dorsal; V, ventral.

observed in the same cells (Fig. 11-N). These results indicate that *cSim2* and *cMyoD* expression is mutually exclusive in chick limb ventral muscle masses.

### ***Sim2* is expressed in embryonic muscle progenitors that have been experimentally or genetically prevented from fully entering the myogenic program**

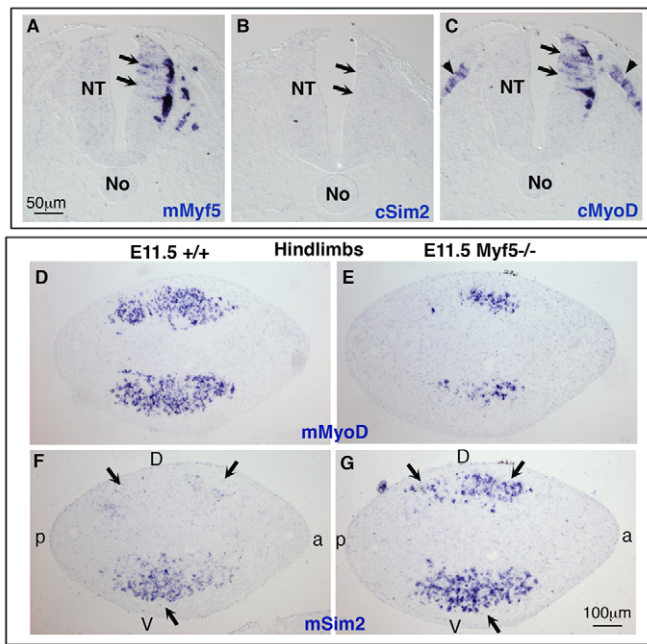
Notch signalling regulates the maintenance of embryonic muscle progenitors and inhibits muscle differentiation (Delfini et al., 2000; Schuster-Gossler et al., 2007; Vasyutina et al., 2007; Bonnet et al., 2010; Rios et al., 2011). In chick embryonic limbs, Delta1-activated Notch led to a downregulation of expression of the muscle differentiation genes (*MyoD*, myogenin, desmin, *Mef2c*) and myosins, whereas the expression of muscle progenitor markers (*Pax3*, *Six1* and *Fgfr4*) was not affected (Delfini et al., 2000; Bonnet et al., 2010). In order to determine whether *Sim2* expression was affected by Notch signalling, we analysed *cSim2* expression after *Delta1* overexpression in chick limbs. Delta1/RCAS-expressing cells were grafted into the lateral plates of HH17 embryos at limb level (Fig. 2A). Seventy-two hours later, *cDelta1* expression revealed the extent of the virus spread (Fig. 2B). In these conditions, *cSim2*



**Fig. 3. *Sim2* expression is increased in forelimbs of *MyoD*<sup>-/-</sup> mutant mice.** (A-D) Forelimb transverse sections of E12 wild-type (A,C) and *MyoD*<sup>-/-</sup> (B,D) were hybridised with the mMyog (A,B) and mSim2 (C,D) probes. A,C and B,D are adjacent sections, respectively. (C,D) Arrows indicate the extended mSim2 expression domain in *MyoD*<sup>-/-</sup> (D) and the absence of mSim2 expression in control limbs (C). a, anterior; p, posterior; D, dorsal; V, ventral. (E) Relative levels of *Pax3*, *Myf5*, *Sim2* and *Sim2s* mRNAs in forelimbs from E11.5 and E12.5 *MyoD*<sup>-/-</sup> mutant mice compared with wild-type littermates. The mRNA levels of wild-type and *MyoD*<sup>-/-</sup> mice were normalised to that of HPRT in each experiment. The error bars represent s.e.m. \*\*P<0.01; \*\*\*P<0.001.

expression was not affected, nor that of *cFgfr4*, whereas *cMyoD* expression was downregulated in ventral and dorsal muscle masses (Fig. 2C-H). We conclude that *Sim2* expression is not regulated by Notch and that *Sim2* regulation by Notch signalling is similar to that seen in muscle progenitor markers.

We next analysed *Sim2* expression in MRF mutant mice, in which limb embryonic myogenesis is delayed. Although being crucial for muscle formation, *Myf5*, *MyoD* or *Mrf4* single mutant mice displayed no dramatic muscle phenotype, because of compensatory function between these genes (Kassar-Duchossoy et al., 2004; Bismuth and Relaix, 2010). However, *MyoD*<sup>-/-</sup> mutant mice exhibited an early delay of limb muscle differentiation by about 2.5 days (Kablar et al., 1997). This delay was assayed by the absence of mMyog expression in limbs of E12 *MyoD*<sup>-/-</sup> mutant compared with its normal expression in wild-type limbs (Fig. 3A,B). When muscle differentiation is arrested, mSim2 expression domain was extended in ventral and dorsal muscle masses compared with its restricted expression in control limbs (Fig. 3C,D, arrows). The increase of mSim2 expression was confirmed by q-RT-PCR analyses in forelimbs of mutant and wild-type mice (Fig. 3E). Transcripts for the short splice variant of *Sim2*, *sSim2*, were hardly present in embryonic wild-type and *MyoD*<sup>-/-</sup> limbs (Fig. 3E). The relative levels of *Myf5*

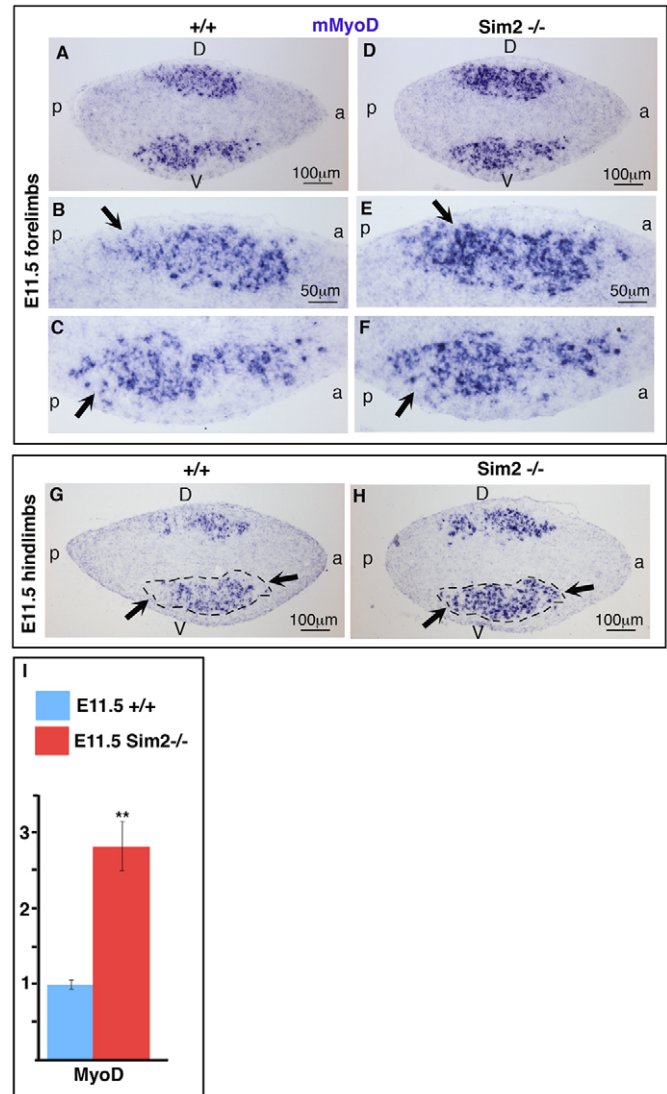


**Fig. 4. *Sim2* expression is not regulated by *Myf5*.** (A-C) Adjacent transverse sections from chick embryos electroporated with mMyf5 expression vector in neural tubes were hybridised with probes for mMyf5 (A), cSim2 (B) and cMyoD (C). cSim2 expression is not activated by mMyf5, while cMyoD is. (D-G) Adjacent and transverse sections of hindlimbs from E11.5 wild-type (D,F) and *Myf5*<sup>-/-</sup> (E,G) mice were hybridised with the mMyoD (D,E) and mSim2 (F,G) probes. mMyoD expression is reduced in hindlimbs of E11.5 *Myf5*<sup>-/-</sup> compared with control mice (D,E), indicating a delay of muscle differentiation in *Myf5*<sup>-/-</sup>. On adjacent sections, mSim2 expression is increased in dorsal and ventral muscle masses of *Myf5*<sup>-/-</sup> hindlimbs (G, arrows), compared with mSim2 in control hindlimbs (F, arrows). a, anterior; p, posterior; D, dorsal; V, ventral.

mRNAs also increased in the absence of *MyoD* activity in forelimbs (Fig. 3E). In order to determine whether *Sim2* expression was dependent on *Myf5*, we analysed *Sim2* expression in *Myf5* gain- and loss-of-function experiments. Overexpression of mMyf5 in chick neural tubes leads to ectopic myogenesis (Delfini and Duprez, 2004). In these experiments, mMyf5 does not activate cSim2 expression, whereas activating cMyoD expression (Fig. 4A-C). Conversely, in the absence of *Myf5* (*Myf4*) activity, mSim2 expression was observed in E11.5 forelimbs (data not shown) and hindlimbs (Fig. 4F,G). We also noticed an increase of mSim2 expression in hindlimbs of *Myf5*<sup>-/-</sup> compared with control hindlimbs (Fig. 4F,G, arrows), consistent with the delay of muscle differentiation observed in *Myf5*<sup>-/-</sup> hindlimbs, assayed by the reduced expression of mMyoD compared with control hindlimbs (Fig. 4D,E) (Kablar et al., 1997). The delay of muscle differentiation and the increase of mSim2 expression were only observed in hindlimbs and not in forelimbs of *Myf5*<sup>-/-</sup>. We conclude that *Sim2* can be expressed in the absence of the MRFs and is upregulated when muscle differentiation is delayed.

### ***MyoD* expression is transiently upregulated in limbs of *Sim2*<sup>-/-</sup> mutant mice**

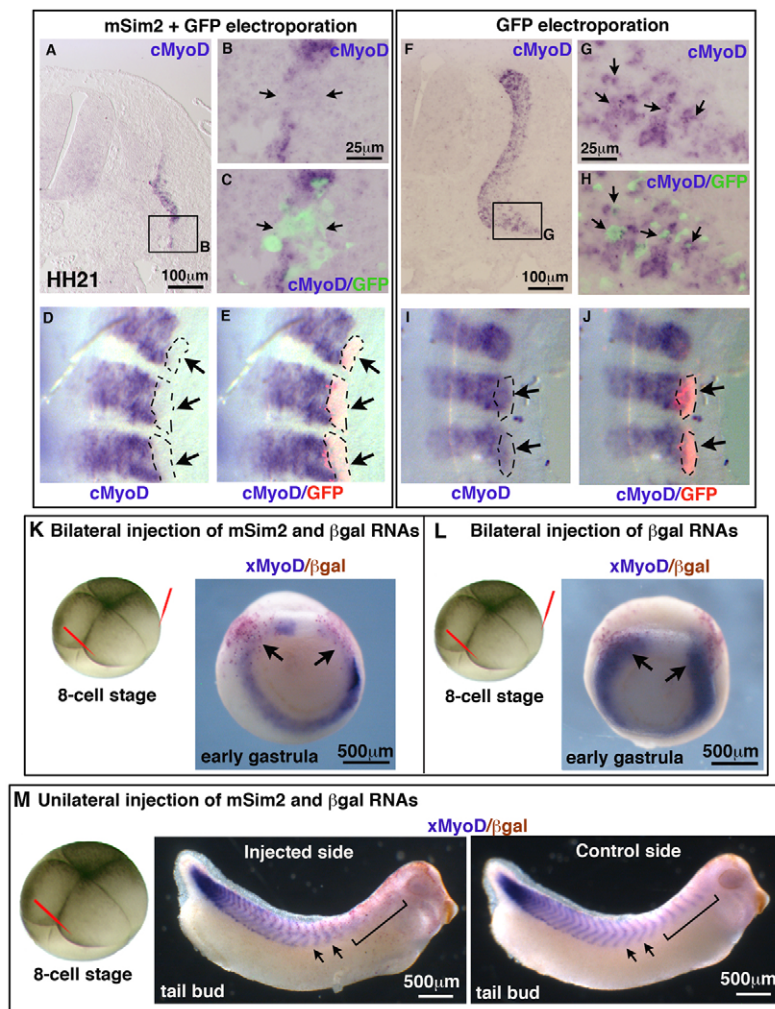
The inverse correlation between *Sim2* expression and muscle differentiation during normal and experimental conditions and the repressor activity of *Sim2* in vitro (Moffett et al., 1997; Moffett and Pelletier, 2000; Metz et al., 2006) led us to hypothesise that *Sim2*



**Fig. 5. mMyoD expression is transiently upregulated in limbs of E11.5 *Sim2*<sup>-/-</sup> mice.** (A-H) Transverse sections of forelimbs (A-F) and hindlimbs (G,H) from E11.5 wild-type (A-C,G) and *Sim2*<sup>-/-</sup> (D-F,H) mice were hybridised with the mMyoD probe. mMyoD expression appears increased in limbs of *Sim2*<sup>-/-</sup> mutant mice (D-F,H) compared with control limbs (A-C,G). (B,C,E,F) High magnifications of the dorsal and ventral muscle masses of control (A) and *Sim2*<sup>-/-</sup> mutant (D) forelimbs. Arrows indicate the increased expression of mMyoD in *Sim2*<sup>-/-</sup> forelimbs (E,F) compared with control forelimbs (B,C). (G,H) In hindlimbs, the ventral muscle mass was delineated in the *Sim2*<sup>-/-</sup> limbs (H) and copied to control limbs (G); arrows indicate the absence of mMyoD expression in control limbs compared with *Sim2*<sup>-/-</sup> limbs. a, anterior; p, posterior; D, dorsal; V, ventral. (I) Relative levels of *MyoD* mRNAs in forelimbs from E11.5 *Sim2*<sup>-/-</sup> compared with wild-type littermates. The mRNA levels were normalised to that of HPRT. Error bars indicate  $\pm$ s.e.m. \*\**P*<0.01.

could negatively regulate *MyoD* expression during limb embryonic myogenesis. We therefore analysed mMyoD expression in limb muscle masses of *Sim2*<sup>-/-</sup> mutant mice during embryonic myogenesis. In the absence of *Sim2* activity, we observed an upregulation of mMyoD expression in dorsal and ventral muscle masses of forelimbs and hindlimbs (Fig. 5). In forelimbs, the mMyoD upregulation was more pronounced in posterior regions of muscle masses (Fig. 5A-F, arrows), where endogenous mSim2





**Fig. 6. mSim2 overexpression inhibits *MyoD* expression in chick and *Xenopus* embryos.** (A–J) Chick embryos were electroporated at interlimb regions with mSim2 and GFP expression vectors (A–E) or with GFP expression vector only (F–H). Transverse sections (A–C, F–H) or whole-mount embryos (D, E, I, J) were hybridised with the *cMyoD* probe and then incubated with GFP antibody to visualise the electroporated regions. (B, C, G, H) High magnifications of the boxed regions in A, F. mSim2 overexpression in somites leads to a loss of *cMyoD* expression in electroporated regions (A–E) compared with control electroporated with GFP only (F–H), in which electroporated cells express *cMyoD*. Arrows indicate the loss of *cMyoD* expression where GFP is expressed in mSim2-electroporated somites (A–E) and *cMyoD* expression in control electroporated somites (F–J). (K–M) Eight-cell stage *Xenopus* embryos were injected bilaterally (K, L) or unilaterally (M) with mSim2 and β-gal RNAs (K, M) or with β-gal RNAs as control (L) and were processed for whole-mount in situ hybridisation to *xMyoD* expression. At early gastrula stages, *xMyoD* expression is inhibited by mSim2 overexpression in β-gal-positive regions (K, arrows), whereas normal *xMyoD* expression is observed in control embryos injected with β-gal only (L, arrows). At tail bud stages, mSim2 overexpression also leads to a loss of *xMyoD* expression in the most anterior somites on the injected sides, compared with the uninjected sides (M, arrows and brackets).

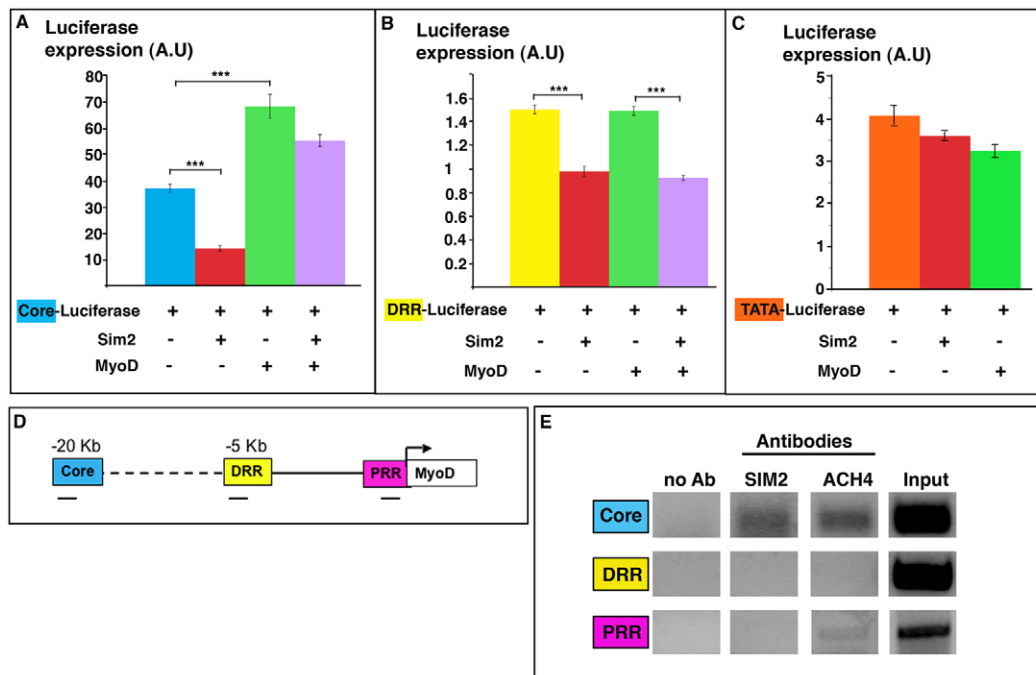
expression is enhanced (Fig. 3C; supplementary material Fig. S1). In hindlimbs, the m*MyoD* expression domain was extended in ventral regions of in *Sim2*<sup>−/−</sup> compared with control limbs (Fig. 5G, H arrows; supplementary material Fig. S3), consistent with the predominant endogenous m*Sim2* expression in ventral muscle masses (supplementary material Fig. S2). Global quantification by q-RT-PCR analyses confirmed the *MyoD* increase in limbs of E11.5 *Sim2*<sup>−/−</sup> versus *Sim2*<sup>+/+</sup>. Quantification of m*MyoD* mRNAs in muscle masses in *Sim2*<sup>−/−</sup> versus *Sim2*<sup>+/+</sup> indicates an increase of 27% in dorsal muscle masses and of 38% in ventral muscle masses in hindlimbs, and an increase of 36% in dorsal muscle masses and 41% in ventral muscle masses in forelimbs (supplementary material Fig. S3). However, the increase of m*MyoD* expression was transient, because at E14.5, *Sim2*<sup>−/−</sup> mutant mice displayed no obvious difference in m*MyoD* expression in forelimbs and hindlimbs (data not shown). We conclude that *MyoD* expression is transiently upregulated in the absence of *Sim2* activity in fore- and hindlimbs during embryonic myogenesis, suggesting a transcriptional repressor activity for m*Sim2* towards *MyoD* expression.

### Sim2 overexpression inhibits *MyoD* expression in chick and *Xenopus* embryos

Mouse *Sim2* loss-of-function experiments suggested that *Sim2* represses *MyoD* expression during embryonic myogenesis. We therefore analysed the consequences of *Sim2* gain-of-function for

*MyoD* expression, in chicken and *Xenopus* embryos. We used somite electroporation in chick embryos (Wang et al., 2011) to assess the effect of m*Sim2* overexpression on *cMyoD* expression. Interlimb somites were electroporated in HH16 chick embryos (27 somite stage) with m*Sim2* and GFP expression vectors or with a GFP expression vector only, as control. The embryos electroporated with m*Sim2* displayed a loss of *cMyoD* expression in the electroporated regions (Fig. 6A–E, arrows), whereas control embryos electroporated with GFP only displayed *cMyoD* expression in GFP-expressing somitic regions (Fig. 6F–J, arrows). *cMyf5* expression was not affected after m*Sim2* electroporation in the interlimb somites and was not activated after m*Sim2* electroporation in the neural tube, in chick embryos (supplementary material Fig. S4). We conclude that *Sim2* overexpression represses *cMyoD* expression in chick somites, but does not affect *cMyf5* expression.

In order to confirm that *Sim2* acts as a repressor of *MyoD* expression, we overexpressed m*Sim2* in *Xenopus* embryos. *Xenopus* (x) *MyoD* zygotic expression occurs at mid-blastula transition and is maintained during mesoderm induction (Rupp and Weintraub, 1991). m*Sim2* mRNAs (500 pg) were injected in the dorsal-lateral equatorial region of eight-cell stage *Xenopus* embryos. β-gal mRNAs were either co-injected with m*Sim2* mRNAs to label the targeted regions (Fig. 6K, M) or were injected alone as a control (Fig. 6L). At early gastrula stage, mesodermal cells overexpressing m*Sim2* displayed a loss of *xMyoD* expression (Fig. 6K, arrows), compared



**Fig. 7. mSim2 represses the transcriptional activity of *MyoD* promoter in vitro and is recruited to the core element in vivo in E11.5 mouse limbs.** (A–C) Primary myoblasts were co-transfected with GFP-, mMyoD- or mSim2-expression vectors and *MyoD* reporter constructs, in which the luciferase reporter gene was cloned downstream of two regulatory elements of the m*MyoD* promoter, the core (A) and the DRR (B) or a minimal promoter (C). mSim2 represses the luciferase activity of the core (A) and the DRR (B), but not that of the TATA promoter (C). Luciferase activity was measured in the same conditions for the experiments in A, B and C. The same arbitrary units (A.U.) therefore apply to all panels. (D) Schematic representation (not in scale) of the regulatory elements of the mouse *MyoD* promoter and of the position of the three amplified fragments used for ChIP experiments. (E) ChIP assays were performed from limbs of E11.5 mice with antibodies against Sim2 or Ach4, or without any antibody (no Ab) as a negative control. ChIP products were analysed by PCR to study the presence of mSim2 on regulatory regions of mouse *MyoD* promoter. We could detect the binding of mSim2 to the core element. PCR amplifications were performed on chromatin isolated before immunoprecipitation (Input) as positive control.

with control embryos, in which  $\beta$ -gal overexpression did not affect *xMyoD* expression (Fig. 6L, arrows). At tail bud stage, we also observed a loss of *xMyoD* expression in the most anterior somites, where mSim2 was overexpressed, compared with the uninjected sides (Fig. 6M). We conclude that mSim2 overexpression represses *xMyoD* expression in *Xenopus* embryos.

Collectively, these *Sim2* gain-of-function experiments show that mouse Sim2 represses *MyoD* expression in chicken and *Xenopus* embryos.

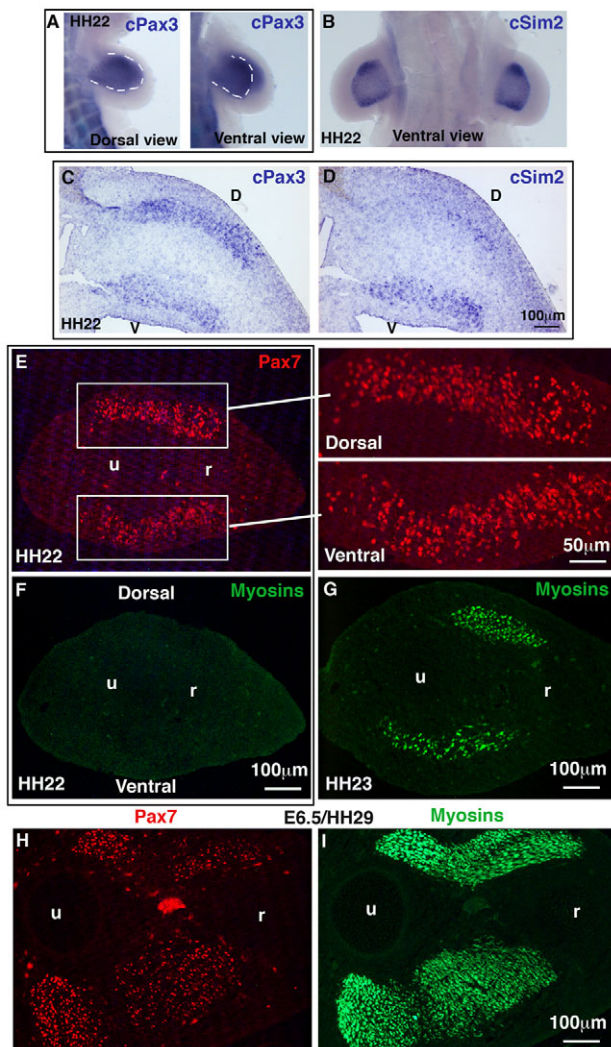
### Sim2 represses *MyoD* transcription via Sim2 recruitment to the core enhancer of the mouse *MyoD* promoter

*Sim2* expression, regulation and loss- and gain-of-function experiments indicated that Sim2 represses *MyoD* transcription. We next determined whether Sim2 could repress *MyoD* expression via the regulatory elements of the mouse *MyoD* promoter. We took advantage of the characterisation of two muscle-specific enhancers in mammals, the core enhancer and the distal regulatory region (DRR) located respectively -20 kb and -5 kb upstream from the *MyoD* transcription start site (Goldhamer et al., 1992; Tapscott et al., 1992; Asakura et al., 1995; Goldhamer et al., 1995). Mouse mutagenesis experiments have shown that the core enhancer regulates the initiation of *MyoD* in limb buds during embryonic development (Kablar et al., 1999; Chen et al., 2001; Chen and Goldhamer, 2004), whereas the DRR is associated with the onset of skeletal muscle differentiation (Asakura et al., 1995; Chen et al.,

2001) and is essential for normal *MyoD* expression in adult muscles (Chen et al., 2002). The core and DRR regulatory elements were cloned upstream the luciferase reporter gene and transfected into primary myoblasts with mSim2 and mMyoD expression vectors. mSim2 was able to significantly repress the reporter gene expression via the core (Fig. 7A) and DRR (Fig. 7B) elements but not via a minimal promoter (Fig. 7C). We also observed that mMyoD activates its own transcription via the core element and that this activation was not significantly prevented by mSim2 (Fig. 7A). We conclude that mSim2 represses the transcriptional activity of both the core and DRR elements of the *MyoD* promoter in vitro.

We next analysed the in vivo recruitment of Sim2 to the *MyoD* regulatory elements, in limbs. We performed chromatin immunoprecipitation (ChIP) assays on E11.5 mouse limbs to analyse the Sim2 occupancy to the core, the DRR or the Proximal Regulatory Region (PRR) (Fig. 7D). We observed that mSim2 was recruited to the core of the m*MyoD* promoter and not to the DRR nor the PRR (Fig. 7E). The acetylation of Histone4 (ACH4) in the core and the PRR testifies to an open state of the chromatin of these regions at this stage (Fig. 7E). Interestingly, the DRR was not acetylated in E11.5 mouse limbs (Fig. 7E), reflecting an absence of DRR activity in E11.5 limbs, which is consistent with transgenic analyses (Asakura et al., 1995; Chen et al., 2001; Chen et al., 2002). These results established that mSim2 was recruited to the core element of the *MyoD* promoter in vivo in E11.5 limbs. We conclude that Sim2 represses *MyoD* expression via the core element in vivo during limb embryonic myogenesis.

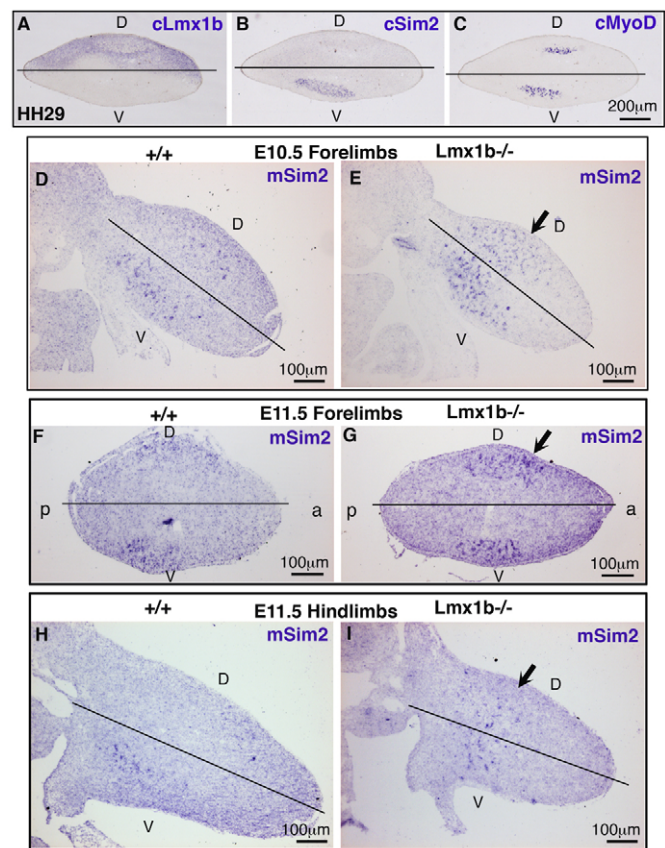




**Fig. 8. Differential increase of dorsal versus ventral muscle masses in chick forelimbs.** In situ hybridisation to whole-mount (A,B) or to tissue sections (C,D) of forelimbs of HH22 embryos using the *cPax3* (A,C) or *cSim2* (B,D) probes. (A) Dorsal and ventral views of the same forelimbs hybridised with the *cPax3* probe. (B) Ventral view of forelimbs hybridised with the *cSim2* probe. (C,D) Transverse and adjacent sections of HH22 forelimbs were hybridised with the *cPax3* (C) and *cSim2* (D) probes. (E-I) Double immunohistochemistry using the Pax7 and MF20 antibodies on transverse sections of forelimbs at the level of the mid-forearm, at different stages of development: HH22 (E,F), HH23 (G) and HH29 (H,I). D, dorsal; u, ulna; r, radius; V, ventral.

### Enhanced *Sim2* expression in ventral muscle masses is correlated with a greater global muscle size in ventral regions compared to that of dorsal limb regions

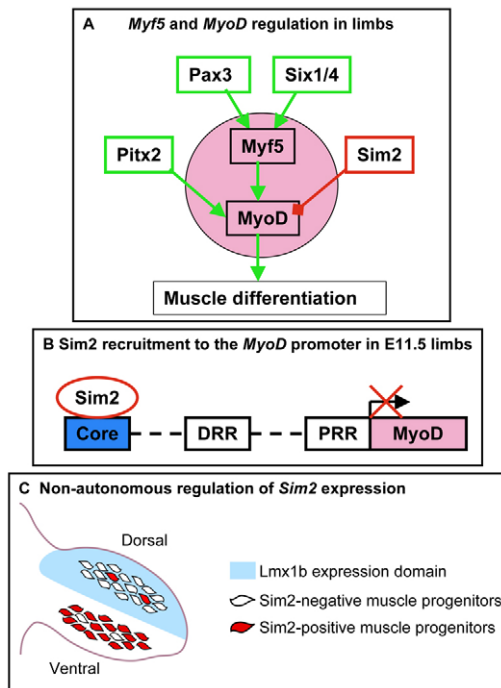
Our results indicate that *Sim2* prevents entry into the myogenic program by repressing *MyoD* transcription. We next asked why this delay of myogenic progression would be predominant in ventral limb muscle masses, where *Sim2* expression is enhanced. In chick forelimbs, at the onset of embryonic myogenesis, the global mass of muscle progenitors, visualised with *Pax3* transcripts (Fig. 8A,C) or with Pax7-positive cells (Fig. 8E), appeared to be equivalent in dorsal and ventral regions. We did not observe any obvious delay in myosin expression in dorsal versus ventral masses (Fig. 8F, no



**Fig. 9. *mSim2* expression is upregulated in dorsal regions of embryonic limbs deficient for *Lmx1b* activity.** (A-C) In situ hybridisation experiments on transverse HH29 chick limb sections with the *cLmx1b* (A), *cSim2* (B) and *cMyoD* (C) probes show that *cLmx1b* expression in dorsal limb regions is excluded from the *cMyoD* expression domain and from the *cSim2* expression domain in ventral limb muscles. (D-I) E10.5 forelimbs (D,E) and E11.5 hindlimbs (F-I) from wild-type (D,F,H) and *Lmx1b* mutant mice (E,G,I) were hybridised with the *mSim2* probe (D-I). *mSim2* expression is upregulated in dorsal limb regions of *Lmx1b*-deficient limbs (E,G,I, arrows), compared with wild-type limbs (D,F,H). In all pictures, the dorsal and ventral limb regions are separated by a line. Arrows indicate the extension of the *mSim2* expression domain in dorsal limb regions in *Lmx1b* mutants. a, anterior; p, posterior; D dorsal; V, ventral.

expression in muscle masses at HH22; Fig. 8G expression in both masses at HH23), indicating that muscle differentiation occurs simultaneously in both muscle masses in chick forelimbs. However, the global mass of forming muscles (including muscle progenitors and differentiated cells) is increased in ventral regions at the end of embryonic myogenesis (Fig. 8H,I). During foetal myogenesis, when forelimb muscles are individualised, the global mass of ventral muscles is also greater than that of dorsal muscles (Duprez et al., 1999). We observed a similar situation in chick hindlimbs, where the global mass of ventral musculature is greater than that of dorsal musculature (see supplementary material Fig. S5). Based on the Mouse Limb Atlas Anatomy (<http://www.nimr.mrc.ac.uk/3dlimb/>), the ventral mouse limb regions also display a greater muscle size, in forelimbs and hindlimbs (see supplementary material Figs S6, S7). Interestingly, in mouse forelimbs, where *mSim2* expression is also enhanced in posterior regions (Fig. 3C; supplementary material Fig. S1), the global muscle size is also greater in posterior regions compared with anterior regions (supplementary material Fig. S5).





**Fig. 10. *MyoD* regulation during limb embryonic myogenesis.**

(A) Gene network involved in the initiation of *MyoD* expression in limbs during embryonic myogenesis. *Pax3*, *Myf5* and *Pitx2* cell-autonomously activate the expression of *Myf5* and *MyoD*, whereas *Sim2* cell-autonomously represses *MyoD* expression. (B) *Sim2* recruitment to the core element of *MyoD* promoter in E11.5 mouse limbs. (C) *Sim2* expression is enhanced in ventral limb muscle progenitors. *Lmx1b* expressed in dorsal limb regions and excluded from myogenic cells is involved in the non cell-autonomous repression of *Sim2* expression in dorsal limb regions.

We conclude that the delay in the myogenic progression by *Sim2* is enhanced in restricted areas of limb muscle masses, where the muscle size is increased at later stages. This delay is predominant in ventral limb muscle masses and correlates with the greater size of ventral musculature compared with dorsal musculature.

### **m*Sim2* expression is negatively and non-autonomously regulated by the dorsalis limb signal *Lmx1b***

As *Sim2* expression was enhanced in ventral limb muscle masses during embryonic myogenesis, we next analysed whether the *Sim2* ventral expression could be linked to dorsoventral limb patterning. The LIM-homeodomain transcription factor *Lmx1b* is known to be necessary and sufficient for specifying dorsal fates in limbs (Riddle et al., 1995; Vogel et al., 1995; Chen et al., 1998). In chick limbs, *cLmx1b* is expressed in dorsal limb regions, whereas *cSim2* expression is increased in ventral muscle masses (Fig. 9A-C). In the absence of *Lmx1b* activity, mouse limbs displayed a biventral limb pattern, mainly in distal regions (Chen et al., 1998; Li, Y. et al., 2010). In the absence of *Lmx1b* activity, m*Sim2* expression was increased in dorsal fore- and hindlimb regions, compared with wild-type limbs (Fig. 9D-I). As *Lmx1b* is not expressed in myogenic cells (Li, Y. et al., 2010), we conclude that *Lmx1b* in dorsal connective tissue cells non-autonomously represses m*Sim2* expression in dorsal limb muscle cells.

## **DISCUSSION**

In this manuscript, we have demonstrated for the first time the functional involvement of the bHLH-PAS transcription factor *Sim2* in limb embryonic myogenesis. Only four intrinsic transcription factors (*Six1/4*, *Pax3* and *Pitx2*) have been identified as directly regulating the expression of the MRFs during limb embryonic myogenesis (Bajard et al., 2006; Giordani et al., 2007; L'Honore et al., 2010). With the present work, we identified *Sim2* as a new regulator of limb embryonic myogenesis. In contrast to the already identified MRF regulators, which have been shown to promote myogenesis, *Sim2* acts as a negative regulator of myogenesis by repressing *MyoD* transcription in limb muscle progenitors (Fig. 10A). *Sim2* does not seem to be downstream of *Myf5*, as *Sim2* expression is observed in limbs of *Myf5* mouse mutants and is not activated upon overexpression of *Myf5* in chick embryos. Cell-autonomous regulators of *Sim2* expression in muscle progenitors remain to be characterised. We have shown that *Sim2* is recruited to the core element of the *MyoD* promoter in vivo in mouse limbs at E11.5 and represses *MyoD* transcription via this element in vitro (Fig. 10B). The specific *Sim2* recruitment to the core element of the *MyoD* promoter in vivo is consistent with the idea that *Sim2* negatively regulates the initiation of *MyoD* expression in limbs, as the core enhancer is essential for the timely initiation of *MyoD* expression in limb buds (Kablar et al., 1999; Chen and Goldhamer, 2004). We have identified *Sim2* as a new and negative regulator of the *MyoD* core enhancer activity. The transient upregulation of *MyoD* expression in limbs of *Sim2* mutant mice is consistent with the 2-day delays of limb *MyoD* expression in mice lacking the *MyoD* core enhancer (Chen and Goldhamer, 2004). *Pitx2* has recently been shown to directly bind and to positively regulate the activity of the *MyoD* core enhancer in mouse limbs at E12 (L'Honore et al., 2010). Further studies are required to analyse putative interactions between *Pitx2* and *Sim2* in regulating the core element activity during limb embryonic myogenesis.

*Sim2* expression is enhanced in ventral limb muscle progenitors during chick and mouse embryonic myogenesis (Coumailleau and Duprez, 2009). One obvious difference between dorsal and ventral limb muscles is the greater global volume of ventral muscles compared with dorsal muscles at the autopod and zeugopod levels of chick and mouse limbs (Fig. 8; see supplementary material Figs S5-S7). Our hypothesis is that the presence of *Sim2* in muscle progenitors will prevent them from prematurely entering the myogenic program. The enhanced expression of *Sim2* in ventral progenitors will lead to a progressive increase of the muscle progenitor pool in ventral limb regions. The *Sim2* repressor activity on muscle differentiation provides a mechanism that allows differential muscle growth between dorsal and ventral limb regions. In chick and mouse hindlimbs, the mass of muscle progenitors is smaller in ventral regions than that in dorsal regions (supplementary material Figs S5, S6), indicating that the delay of myogenic progression by *Sim2* must be capable of overcoming this initial difference, in addition to increasing the pool of ventral muscle progenitors. The *Sim2* repressor activity in muscle progenitors is obviously not the only mechanism involved in the differential muscle growth between dorsal and ventral limb regions, because *Sim2* mutant mice display a normal limb muscle pattern at E14.5. Embryological experiments and genetic analyses have shown that the position, shape and size of limb muscles are driven by signals provided by non-myogenic limb cells (Kieny, 1982; Chen et al., 1998; Kardon, 1998; Duprez et al., 1999; Duprez,

2002). However, it is interesting to note that *Sim2*, although it is not the master gene for regulating the muscle size, is negatively regulated by the factor specifying the dorsal limb pattern, *Lmx1b* (Fig. 10C).

In conclusion, these results identify *Sim2* as a new intrinsic regulator of limb embryonic myogenesis by negatively regulating *MyoD* expression through the recruitment of *Sim2* to the *MyoD* core enhancer. This prevention of entry into the embryonic myogenic program is predominant in ventral limb regions and is likely to contribute to the differential increase of the global mass of ventral muscles versus dorsal muscles.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

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#### References

- Aleman, M. J., DeYoung, M. P., Tress, M., Keating, P., Perry, G. W. and Narayanan, R. (2005). Inhibition of Single Minded 2 gene expression mediates tumor-selective apoptosis and differentiation in human colon cancer cells. *Proc. Natl. Acad. Sci. USA* **102**, 12765-12770.
- Asakura, A., Lyons, G. E. and Tapscott, S. J. (1995). The regulation of MyoD gene expression: conserved elements mediate expression in embryonic axial muscle. *Dev. Biol.* **171**, 386-398.
- Bajard, L., Relaix, F., Lagha, M., Rocancourt, D., Daubas, P. and Buckingham, M. E. (2006). A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev.* **20**, 2450-2464.
- Bismuth, K. and Relaix, F. (2010). Genetic regulation of skeletal muscle development. *Exp. Cell Res.* **316**, 3081-3086.
- Bonnet, A., Dai, F., Brand-Saberi, B. and Duprez, D. (2010). Vestigial-like 2 acts downstream of MyoD activation and is associated with skeletal muscle differentiation in chick myogenesis. *Mech. Dev.* **127**, 120-136.
- Chen, H., Lun, Y., Ovchinnikov, D., Kokubo, H., Oberg, K. C., Pepicelli, C. V., Gan, L., Lee, B. and Johnson, R. L. (1998). Limb and kidney defects in *Lmx1b* mutant mice suggest an involvement of LMX1B in human nail patella syndrome. *Nat. Genet.* **19**, 51-55.
- Chen, J. C. and Goldhamer, D. J. (2004). The core enhancer is essential for proper timing of MyoD activation in limb buds and branchial arches. *Dev. Biol.* **265**, 502-512.
- Chen, J. C., Love, C. M. and Goldhamer, D. J. (2001). Two upstream enhancers collaborate to regulate the spatial patterning and timing of MyoD transcription during mouse development. *Dev. Dyn.* **221**, 274-288.
- Chen, J. C., Ramachandran, R. and Goldhamer, D. J. (2002). Essential and redundant functions of the MyoD distal regulatory region revealed by targeted mutagenesis. *Dev. Biol.* **245**, 213-223.
- Chrast, R., Scott, H. S., Madani, R., Huber, L., Wolfer, D. P., Prinz, M., Aguzzi, A., Lipp, H. P. and Antonarakis, S. E. (2000). Mice trisomic for a bacterial artificial chromosome with the single-minded 2 gene (*Sim2*) show phenotypes similar to some of those present in the partial trisomy 16 mouse models of Down syndrome. *Hum. Mol. Genet.* **9**, 1853-1864.
- Coumilleau, P. and Duprez, D. (2009). *Sim1* and *Sim2* expression during chick and mouse limb development. *Int. J. Dev. Biol.* **53**, 149-157.
- Delfini, M. C. and Duprez, D. (2000). Paraxis is expressed in myoblasts during their migration and proliferation in the chick limb bud. *Mech. Dev.* **96**, 247-251.
- Delfini, M. C. and Duprez, D. (2004). Ectopic Myf5 or MyoD prevents the neuronal differentiation program in addition to inducing skeletal muscle differentiation, in the chick neural tube. *Development* **131**, 713-723.
- Delfini, M. C., Hirsinger, E., Pourquie, O. and Duprez, D. (2000). Delta 1-activated notch inhibits muscle differentiation without affecting Myf5 and Pax3 expression in chick limb myogenesis. *Development* **127**, 5213-5224.
- DeYoung, M. P., Tress, M. and Narayanan, R. (2003). Identification of Down's syndrome critical locus gene *SIM2-s* as a drug therapy target for solid tumors. *Proc. Natl. Acad. Sci. USA* **100**, 4760-4765.
- Duprez, D. (2002). Signals regulating muscle formation in the limb during embryonic development. *Int. J. Dev. Biol.* **46**, 915-925.
- Duprez, D., Lapointe, F., Edom-Vovard, F., Kostakopoulou, K. and Robson, L. (1999). Sonic hedgehog (SHH) specifies muscle pattern at tissue and cellular chick level, in the chick limb bud. *Mech. Dev.* **82**, 151-163.
- Edom-Vovard, F., Bonnin, M. A. and Duprez, D. (2001). Misexpression of Fgf-4 in the chick limb inhibits myogenesis by down-regulating *Frek* expression. *Dev. Biol.* **233**, 56-71.
- Ema, M., Ikegami, S., Hosoya, T., Mimura, J., Ohtani, H., Nakao, K., Inokuchi, K., Katsuki, M. and Fujii-Kuriyama, Y. (1999). Mild impairment of learning and memory in mice overexpressing the *mSim2* gene located on chromosome 16, an animal model of Down's syndrome. *Hum. Mol. Genet.* **8**, 1409-1415.
- Farrall, A. L. and Whitelaw, M. L. (2009). The HIF1 $\alpha$ -inducible pro-cell death gene *BNIP3* is a novel target of *SIM2s* repression through cross-talk on the hypoxia response element. *Oncogene* **28**, 3671-3680.
- Giordani, J., Bajard, L., Demignon, J., Daubas, P., Buckingham, M. and Maire, P. (2007). Six proteins regulate the activation of Myf5 expression in embryonic mouse limbs. *Proc. Natl. Acad. Sci. USA* **104**, 11310-11315.
- Goldhamer, D. J., Faerman, A., Shani, M. and Emerson, C. P., Jr (1992). Regulatory elements that control the lineage-specific expression of *myoD*. *Science* **256**, 538-542.
- Goldhamer, D. J., Brunk, B. P., Faerman, A., King, A., Shani, M. and Emerson, C. P., Jr (1995). Embryonic activation of the *myoD* gene is regulated by a highly conserved distal control element. *Development* **121**, 637-649.
- Goshu, E., Jin, H., Fasnacht, R., Sepenski, M., Michaud, J. L. and Fan, C. M. (2002). *Sim2* mutants have developmental defects not overlapping with those of *Sim1* mutants. *Mol. Cell. Biol.* **22**, 4147-4157.
- Halvorsen, O. J., Rostad, K., Oyan, A. M., Puntervoll, H., Bo, T. H., Stordrange, L., Olsen, S., Haukaas, S. A., Hood, L., Jonassen, I. et al. (2007). Increased expression of *SIM2-s* protein is a novel marker of aggressive prostate cancer. *Clin. Cancer Res.* **13**, 892-897.
- Hamburger, V. and Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo. *Dev. Dyn.* **195**, 231-272.
- Havis, E., Anselme, I. and Schneider-Maunoury, S. (2006). Whole embryo chromatin immunoprecipitation protocol for the in vivo study of zebrafish development. *Biotechniques* **40**, 34, 36, 38 passim.
- Kablar, B., Krastel, K., Ying, C., Asakura, A., Tapscott, S. J. and Rudnicki, M. A. (1997). MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. *Development* **124**, 4729-4738.
- Kablar, B., Krastel, K., Ying, C., Tapscott, S. J., Goldhamer, D. J. and Rudnicki, M. A. (1999). Myogenic determination occurs independently in somites and limb buds. *Dev. Biol.* **206**, 219-231.
- Kardon, G. (1998). Muscle and tendon morphogenesis in the avian hind limb. *Development* **125**, 4019-4032.
- Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V. and Tajbakhsh, S. (2004). *Mrf4* determines skeletal muscle identity in Myf5:MyoD double-mutant mice. *Nature* **431**, 466-471.
- Kieny, M. A. (1982). Cell and tissue interactions in the organogenesis of the avian limb musculature. *Prog. Clin. Biol. Res.* **110**, 293-302.
- Kwak, H. I., Gustafson, T., Metz, R. P., Laffin, B., Schedin, P. and Porter, W. W. (2007). Inhibition of breast cancer growth and invasion by single-minded 2s. *Carcinogenesis* **28**, 259-266.
- L'Honore, A., Ouimette, J. F., Lavertu-Jolin, M. and Drouin, J. (2010). *Pitx2* defines alternate pathways acting through MyoD during limb and somitic myogenesis. *Development* **137**, 3847-3856.
- Laffin, B., Wellberg, E., Kwak, H. I., Burghardt, R. C., Metz, R. P., Gustafson, T., Schedin, P. and Porter, W. W. (2008). Loss of single-minded-2s in the mouse mammary gland induces an epithelial-mesenchymal transition associated with up-regulation of slug and matrix metalloprotease 2. *Mol. Cell. Biol.* **28**, 1936-1946.
- Lagha, M., Kormish, J. D., Rocancourt, D., Manceau, M., Epstein, J. A., Zaret, K. S., Relaix, F. and Buckingham, M. E. (2008). Pax3 regulation of FGF signaling affects the progression of embryonic progenitor cells into the myogenic program. *Genes Dev.* **22**, 1828-1837.
- Li, H. Y., Bourdela, A., Carron, C. and Shi, D. L. (2010). The RNA-binding protein *Seb4/RBM24* is a direct target of MyoD and is required for myogenesis during *Xenopus* early development. *Mech. Dev.* **127**, 281-291.
- Li, Y., Qiu, Q., Watson, S. S., Schweitzer, R. and Johnson, R. L. (2010). Uncoupling skeletal and connective tissue patterning: conditional deletion in cartilage progenitors reveals cell-autonomous requirements for *Lmx1b* in dorsal-ventral limb patterning. *Development* **137**, 1181-1188.



- Marcelle, C., Wolf, J. and Bronner-Fraser, M. (1995). The in vivo expression of the FGF receptor FREK mRNA in avian myoblasts suggests a role in muscle growth and differentiation. *Dev. Biol.* **172**, 100-114.
- Metz, R. P., Kwak, H. I., Gustafson, T., Laffin, B. and Porter, W. W. (2006). Differential transcriptional regulation by mouse single-minded 2s. *J. Biol. Chem.* **281**, 10839-10848.
- Michaud, J. L., Lapointe, F. and Le Douarin, N. M. (1997). The dorsoventral polarity of the presumptive limb is determined by signals produced by the somites and by the lateral somatopleure. *Development* **124**, 1453-1463.
- Moffett, P. and Pelletier, J. (2000). Different transcriptional properties of mSim-1 and mSim-2. *FEBS Lett.* **466**, 80-86.
- Moffett, P., Reece, M. and Pelletier, J. (1997). The murine Sim-2 gene product inhibits transcription by active repression and functional interference. *Mol. Cell. Biol.* **17**, 4933-4947.
- Relaix, F., Rocancourt, D., Mansouri, A. and Buckingham, M. (2004). Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev.* **18**, 1088-1105.
- Riddle, R. D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, T. M. and Tabin, C. (1995). Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell* **83**, 631-640.
- Rios, A. C., Serralbo, O., Salgado, D. and Marcelle, C. (2011). Neural crest regulates myogenesis through the transient activation of NOTCH. *Nature* **473**, 532-535.
- Rupp, R. A. and Weintraub, H. (1991). Ubiquitous MyoD transcription at the midblastula transition precedes induction-dependent MyoD expression in presumptive mesoderm of *X. laevis*. *Cell* **65**, 927-937.
- Schuster-Gossler, K., Cordes, R. and Gossler, A. (2007). Premature myogenic differentiation and depletion of progenitor cells cause severe muscle hypotrophy in Delta1 mutants. *Proc. Natl. Acad. Sci. USA* **104**, 537-542.
- Shamblott, M. J., Bugg, E. M., Lawler, A. M. and Gearhart, J. D. (2002). Craniofacial abnormalities resulting from targeted disruption of the murine Sim2 gene. *Dev. Dyn.* **224**, 373-380.
- Spitz, F., Salminen, M., Demignon, J., Kahn, A., Daegelen, D. and Maire, P. (1997). A combination of MEF3 and NF1 proteins activates transcription in a subset of fast-twitch muscles. *Mol. Cell. Biol.* **17**, 656-666.
- Tapscott, S. J., Lassar, A. B. and Weintraub, H. (1992). A novel myoblast enhancer element mediates MyoD transcription. *Mol. Cell. Biol.* **12**, 4994-5003.
- Vasyutina, E., Lenhard, D. C., Wende, H., Erdmann, B., Epstein, J. A. and Birchmeier, C. (2007). RBP-J (Rbpsi) is essential to maintain muscle progenitor cells and to generate satellite cells. *Proc. Natl. Acad. Sci. USA* **104**, 4443-4448.
- Vogel, A., Rodriguez, C., Warnken, W. and Izpisua Belmonte, J. C. (1995). Dorsal cell fate specified by chick Lmx1 during vertebrate limb development. *Nature* **378**, 716-720.
- Wang, H., Bonnet, A., Delfini, M. C., Kawakami, K., Takahashi, Y. and Duprez, D. (2011). Stable, conditional, and muscle-fiber-specific expression of electroporated transgenes in chick limb muscle cells. *Dev. Dyn.* **240**, 1223-1232.
- Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benzeira, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S. et al. (1991). The myoD gene family: nodal point during specification of the muscle cell lineage. *Science* **251**, 761-766.
- Wellberg, E., Metz, R. P., Parker, C. and Porter, W. W. (2010). The bHLH/PAS transcription factor single-minded 2s promotes mammary gland lactogenic differentiation. *Development* **137**, 945-952.
- Woods, S., Farrell, A., Procko, C. and Whitelaw, M. L. (2008). The bHLH/Per-Arnt-Sim transcription factor SIM2 regulates muscle transcript myomesin2 via a novel, non-canonical E-box sequence. *Nucleic Acids Res.* **36**, 3716-3727.