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Zeb1 links epithelial-mesenchymal transition and cellular senescence

Yongqing Liu[1], Shahenda El-Naggar[1], Douglas S. Darling[2], Yujiro Higashi[3] and Douglas C. Dean[1,*]

Overexpression of zinc finger E-box binding homeobox transcription factor 1 (Zeb1) in cancer leads to epithelial-to-mesenchymal transition (EMT) and increased metastasis. As opposed to overexpression, we show that mutation of *Zeb1* in mice causes a mesenchymal-epithelial transition in gene expression characterized by ectopic expression of epithelial genes such as E-cadherin and loss of expression of mesenchymal genes such as vimentin. In contrast to rapid proliferation in cancer cells where Zeb1 is overexpressed, this mesenchymal-epithelial transition in mutant mice is associated with diminished proliferation of progenitor cells at sites of developmental defects, including the forming palate, skeleton and CNS. *Zeb1* dosage-dependent deregulation of epithelial and mesenchymal genes extends to mouse embryonic fibroblasts (MEFs), and mutant MEFs also display diminished replicative capacity in culture, leading to premature senescence. Replicative senescence in MEFs is classically triggered by products of the *Ink4a* (*Cdkn2a*) gene. However, this Ink4a pathway is not activated during senescence of *Zeb1* mutant MEFs. Instead, there is ectopic expression of two other cell cycle inhibitory cyclin-dependent kinase inhibitors, p15Ink4b (Cdkn2b) and p21Cdkn1a (Cdkn1a). We demonstrate that this ectopic expression of p15Ink4b extends in vivo to sites of diminished progenitor cell proliferation and developmental defects in *Zeb1*-null mice.

KEY WORDS: Zeb1, Epithelial-mesenchymal transition, Senescence, Transcription

INTRODUCTION

Epithelial-to-mesenchymal transition (EMT) is an important step toward acquisition of a metastatic phenotype in cancer (reviewed by Hay and Zuk, 1995; Thiery, 2003). This transition involves repression of key epithelial genes, highlighted by the cell-cell contact modulator, E-cadherin (also known as cadherin 1). Downregulation of E-cadherin, and the resulting release of cell-cell adhesion in tumors, is thought to contribute a motile phenotype that facilitates metastasis. Conversely, mesenchymal genes including vimentin and smooth muscle actin as well as various matrix and matrix-degrading enzymes are induced during EMT. The resulting alterations in cell shape, matrix expression and ability to digest and move through different tissues are thought to work in conjunction with loss of cell-cell contact to generate the metastatic phenotype. However, EMT is not simply a cancer-driven process, it is crucial early in embryogenesis for delamination of neural crest from the neural tube, and defining an ectodermal-mesodermal boundary (Hay and Zuk, 1995; Thiery, 2003; Cheung et al., 2005). Later in gestation, EMT is important for proper kidney and heart formation. Furthermore, EMT is also a crucial feature of the pathologic fibrotic response to injury (e.g. wound healing and fibrotic organ diseases) (Hay and Zuk, 1995; Thiery, 2003; Zavadil and Bottinger, 2005). EMT during embryogenesis, in cancer metastasis and in fibrotic responses, is thought to be driven by TGF-β family members (reviewed by Zavadil and Bottinger, 2005).

[1]James Graham Brown Cancer Center, Department of Ophthalmology and Visual Sciences, University of Louisville Health Sciences Center, Louisville, KY 40202, USA. [2]Departments of Peiodontics, Endodontics and Dental Hygiene, Center for Oral Health and Systemic Disease, University of Louisville School of Dentistry, Louisville, KY 40292, USA. [3]Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan.

*Author for correspondence (e-mail: dcdean01@louisville.edu)

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Zinc finger E-box binding homeobox 1 (Zeb1; also known as Zfhx1A, δEF1, Tcf8 and Zfhep) binds a set of E-box-like elements that overlap with those bound by Zeb2 (also known as Sip1) and the Snail family (Genetta et al., 1994; Sekido et al., 1994; Postigo and Dean, 2000). Each of these E-box-binding proteins can act as a transcriptional repressor through recruitment of the co-repressor, C-terminal binding protein (CtBP; Ctbp1) (Postigo and Dean, 1999; Grooteclase and Frisch, 2000; Chinnadurai, 2002; Hemavathy et al., 2005). CtBP is a part of a larger complex including polycomb proteins and CoREST (also known as Rcor2) that causes epigenetic modification of DNA and histones leading to heterochromatin assembly and, thereby, transcriptional silencing [see Ringrose et al. (Ringrose et al., 2004) and references therein]. A CtBP-CoREST repressor complex is targeted, via interaction with Zeb1, to genes crucial for late-stage pituitary organogenesis (Wang et al., 2007).

Overexpression of Zeb1 in cancer is associated with repression of E-cadherin and EMT (Guaita 2002; Eger et al., 2005; Pena et al., 2005; Spoelstra et al., 2006; Witta et al., 2006; Peinado et al., 2007). Zeb1 can also serve as a transcriptional activator, and this seems to be directed at least in part toward mesenchymal genes such as collagens, smooth muscle actin and myosin, vimentin, and genes in the vitamin D signaling pathway, which is important in mesenchymal differentiation (Chamberlain and Sanders, 1999; Lazarova et al., 2001; Dillner and Sanders, 2002; Postigo, 2003; Postigo et al., 2003; van Grunsven et al., 2006; Nishimura et al., 2006). Heterozygous mutation of *Zeb1* leads to impaired smooth muscle actin and myosin expression and TGF-β-dependent smooth muscle cell differentiation following vascular injury (Nishimura et al., 2006). Thus, Zeb1 can contribute to repression of epithelial genes as well as to activation of mesenchymal genes.

TGF-β expression by tumors and surrounding stroma drives EMT in cancer, and TGF-β family members are also crucial for EMT during development (Zavadil and Bottinger, 2005). Accordingly, TGF-β represses epithelial genes such as E-cadherin and induces

mesenchymal genes such as vimentin. In vivo, Zeb1 is important for TGF-β-dependent smooth muscle cell differentiation, and TGF-β-dependent expression of smooth muscle actin and myosin genes (Nishimura et al., 2006). Zeb1 binds activated Smads as well as the histone acetyl transferase p300 (also known as pCaf), which is an essential Smad co-activator, and this binding facilitates assembly of a Smad-p300 complex while leading to dissociation of Zeb1 from its co-repressor, CtBP (Zhang et al., 2000; Postigo et al., 2003; Postigo, 2003; van Grunsven et al., 2006). Thus, in response to TGF-β and in the presence of activated Smads and p300, Zeb1 is switched from a repressor to a co-activator.

Zeb1 is expressed in proliferating mesenchymal and neural progenitors, and mutation of the *Zeb1* leads to cleft secondary palate, defective nasal formation and other craniofacial abnormalities (Takagi et al., 1998). Forming cartilage at these sites appears hypoplastic. In addition to craniofacial defects, the mice have skeletal abnormalities including shortened limbs and digits as well as fusion and curvatures in the skeleton and tail. A subset of the embryos exhibits severe CNS defects, including failure of neural tube closure at both cranial and caudal ends, and exencephaly (Takagi et al., 1998). The molecular basis for these various defects is unknown.

As opposed to EMT seen when Zeb1 is overexpressed in rapidly proliferating cancer cells, we present evidence here that mutation of *Zeb1* causes mesenchymal-epithelial transition in gene expression and diminished proliferation in progenitor cells at sites of developmental defects in mouse embryos. This phenotype extends to mouse embryo fibroblasts (MEFs) derived from mutant mice. These cells ectopically express E-cadherin, which is associated with an abnormal epithelial-like morphology. Additionally, they undergo premature replicative senescence in culture, which is associated with ectopic expression of cell cycle inhibitory cyclin-dependent kinase inhibitors (CDKIs), p15Ink4b and p21Cdkn1a (also known as Cdkn2b and Cdkn1a, respectively – Mouse Genome Informatics). This ectopic expression of p15Ink4b is also seen in vivo at sites of diminished proliferation of progenitor cells (and at sites of developmental defects) in *Zeb1* mutant embryos.

Table 1. Primers used for real-time PCR

Primer	Sequence (5′ to 3′)	Tm °C	Amplicon (bp)
Mm Zeb2 LP	TAGCCGGTCCAGAAGAAATG	60.0	156
Mm Zeb2 RP	GGCCATCTCTTTCCTCCAGT	61.0	
Mm Snai1 LP	AAGATGCACATCCGAAGC	57.0	199
Mm Snai1 RP	ATCTCTTCACATCCGAGTGG	58.0	
Mm Snai2 LP	TGATGCCCAGTCTAGGAAAT	57.0	200
Mm Snai2 RP	AGTGAGGGCAAGAGAAAGG	57.0	
Mm Vim LP	CGGCTGCGAGAGAAATTGC	57.1	124
Mm Vim RP	CCACTTTCCGTTCAAGGTCAAG	56.2	
Mm PAI1 LP	TTCGGAGTAAAAGTGTTTCAGCA	54.8	176
Mm PAI1 RP	TGAGCTGTGCCCTTCTCATTG	57.5	
Mm TBX2 LP	ACCAACAACATTTCTGACAAGCA	55.6	134
Mm TBX2 RP	GGGAAGACATAGGTGCGGAAG	57.6	
Mm TBX3 LP	TGTCTCGAAAACCCTTTGCA	54.8	111
Mm TBX3 RP	GAACCTACCTGTTCCCGGAAA	56.8	
Mm Ets1 LP	CCCAGAATCCTGTTACACCTCG	57.2	236
Mm Ets1 RP	GCTTGATGGCAAAGTAGTCTGT	55.3	
Mm ARF LP	TGAGGCTAGAGAGGATCTTGAGA	56.3	91
Mm ARF RP	GCAGAAGAGCTGCTACGTGAA	57.3	
Mm p16 LP	CCCAACGCCCCGAACT	58.6	79
Mm p16 RP	GCAGAAGAGCTGCTACGTGAA	57.3	
Mm p21 LP	GTGGCCTTGTCGCTGTCTT	58.1	126
Mm p21 RP	GCGCTTGGAGTGATAGAAATCTG	55.9	
Mm Bmi1 LP	ATCCCCACTTAATGTGTGTCCT	55.9	116
Mm Bmi1 RP	CTTGCTGGTCTCCAAGTAACG	55.7	
Mm p15 LP	CCCTGCCACCCTTACCAGA	59.3	169
Mm p15 RP	CAGATACCTCGCAATGTCACG	55.7	
Mm Cdh1 LP	CAGGTCTCCTCATGGCTTTGC	58.3	175
Mm Cdh1 RP	CTTCCGAAAAGAAGGCTGTCC	56.0	
Mm GAPDH LP	AACGACCCCTTCATTGAC	56.0	191
Mm GAPDH RP	TCCACGACATACTCAGCAC	56.0	
Mm ACTB LP	GGCTGTATTCCCCTCCATCG	57.6	154
Mm ACTB RP	CCAGTTGGTAACAATGCCATGT	55.9	

MATERIALS AND METHODS

Mouse embryonic fibroblast isolation and cell culture

Embryo fibroblasts were isolated from crosses of mice heterozygous for *Zeb1* and genotyped as described (Takagi et al., 1998). Cells of different genotypes were cultured in DMEM with 10% fetal bovine serum (FBS) at 10% CO_2, and split 1:3 as soon as confluent. For experiments with TGF-β, 1 day after splitting, TGF-β1 (BioSource, Camarillo, CA) was added to the cultures at final concentrations of 5 pM, 25 pM and 75 pM, and RNA was isolated 24 hours later.

RNA extraction and real-time PCR

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). cDNA was synthesized using the Invitrogen RT Kit according to the manufacturer's protocol (Invitrogen). SYBR Green real-time quantitative PCR was performed using the Mx3000P Real-Time PCR System (Stratagene, Cedar Creek, TX) according to the manufacturer's instructions. The RT-PCR primers and annealing temperature are shown in Table 1. Three independent samples were analyzed for each condition and/or cell type, and each sample was compared in at least three independent RT-PCR amplifications.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were based on the Upstate Biotechnology protocol (http://www.millipore.com/userguides/tech1/789mrn) using formaldehyde to cross-link genomic DNA. Polyclonal antiserum for Zeb1 (Darling et al., 2003), histone H3 and histone H4 (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunoprecipitation. Equal amounts of anti-IgG or preimmune serum were used as controls. ChIP PCR reactions were similar to those described above for real-time PCR using primer sets (Table 2) to amplify promoter sequences of E-cadherin and *Gapdh* genes.

Immunohistochemistry

Mouse embryos were fixed, embedded, and sectioned at 5 μm. The primary antibody dilutions for Zeb1, vimentin (Santa Cruz Biotechnology), GFAP (Chemicon, Temecula, CA) and E-cadherin (BD-Pharmingen, San Jose, CA) were 1:100, 1:10, 1:10 and 1:50, respectively, whereas the secondary antibody dilution was 1:300 for both anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, Oregon) and anti-mouse IgG conjugated with Cy3 (Sigma, St Louis, MO). The slides were mounted with coverslips using anti-fade medium Permount (Fisher) and viewed using an Olympus confocal microscope. Western blots for antibodies are shown in Fig. S6 (see Fig. S6 in the supplementary material).

Analysis of cell proliferation in vivo

Two hours before collection of E15.5 embryos, mothers received an intraperitoneal injection of 40 mg/kg 5′-bromodeoxyuridine (BrdU) in PBS. Embryos were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 5 μm. Sections were incubated with 0.1% Tween 20, 4% goat (for Zeb1 antibody) (Darling et al., 2003) or sheep (for BrdU antibody) serum, and 2% bovine serum albumin (BSA) (Sigma) for 1 hour. Polyclonal primary antibodies against Zeb1 and BrdU (raised in rabbit and mouse,

Table 2. Primers used for ChIP assay PCR

Primer	Sequence (5′ to 3′)	Tm °C	Amplicon (bp)
Mm cdh1 PRMT LP	CATGCTGGGCTACATAGCAA	55.2	154
Mm cdh1 PRMT RP	TGGGCCTGGAATTGTCTTAG	54.6	
Mm p21 PRMT LP	CCCGAAACCCAGGATTTTAT	52.6	249
Mm p21 PRMT RP	TCCCCTCTGGGAATCTAAGC	56.0	
Mm p15 PRMT LP	CCGCCTAGAGATGAACTAGCC	56.9	198
Mm p15 PRMT RP	AAGTTGTGCCTCTGCACTCA	57.8	
Mm GAPDH PRMT LP	AGTGCCAGCCTCGTCCCGTAGACAAAATG	55.8	301
Mm GAPDH PRMT RP	AAGTGGGCCCCGGCCTTCTCCAT	53.5	

respectively) were applied to the sections at 1:50, and incubated at 4°C overnight. Slides were then incubated at 1:300 either with anti-rabbit IgG conjugated with Alexa Fluor 488 or Cy3 for 1 hour. The slides were viewed with an Olympus confocal microscope.

Cell proliferation in the brain was analyzed as described (Molofsky et al., 2006). For these experiments, sections (5 μm) from corresponding regions of wild-type and null littermate embryos were immunostained for BrdU and observed using a Zeiss LSM 510 confocal laser-scanning microscope. Optical sections (1 μm) were incorporated into a *z*-stack using a 40× objective, and LSM510 software was used to create a 3-D image. Three adjacent sections were analyzed from each embryo. The percentage of BrdU-positive cells within similar fields was determined. Similar results were also obtained simply by counting the number of BrdU-positive cells in the field. Averages from two wild-type and three null embryos (and three adjacent sections from each embryo) are presented.

Cellular senescence assays

Senescent β-galactosidase activity was analyzed using the X-Gal-based Cell Senescence Staining Kit (Sigma) following the manufacturer's protocol. Stained cells were photographed, and randomly selected fields were counted to determine the percentage of positive cells.

RESULTS

Mutation of *Zeb1* leads to ectopic expression of E-cadherin, loss of vimentin, and proliferative defects in mesenchymal progenitors

Overexpression of Zeb1 in cancer is associated with EMT. Thus, we wondered whether Zeb1 has a role normally in regulation of epithelial and mesenchymal genes during development, and whether such regulation might be linked to the developmental defects seen in *Zeb1*-null mice. A variety of developmental defects appear in *Zeb1*-null embryos late in gestation. Therefore, we began by comparing the pattern of Zeb1 expression to that of the classic epithelial and mesenchymal markers E-cadherin and vimentin, respectively, in embryonic mice late in gestation. Zeb1 was found in nasal mesenchyme and mesenchyme of the forming palate at E16.5 (Fig. 1A). By contrast, E-cadherin was confined to the epithelium and did not overlap with Zeb1 (Fig. 1B). However, vimentin expression was evident in the palatal and nasal mesenchyme along with Zeb1 (Fig. 1C). Likewise in the tongue, Zeb1 and vimentin were expressed in forming muscles, whereas E-cadherin was confined to the epithelial border (Fig. 1B; see Figs S1, S2 in the supplementary material). Next, we examined *Zeb1*-null mice to determine whether there were changes in expression of E-cadherin or vimentin in the palate and or forming nasal region (both are sites of developmental defects in the null mice). Whereas E-cadherin expression was maintained on the epithelium of the palatal and nasal region, it became ectopically expressed on palatal and nasal mesenchyme (Fig. 1D). By contrast, vimentin expression was lost from the palatal and nasal mesenchyme (Fig. 1E). Interestingly, even though Zeb1 was expressed in the forming muscles of the tongue, there was no change in expression of E-cadherin or vimentin in the tongue of *Zeb1*-null mice (see Fig. S1 in the supplementary material).

As noted above, a previous report suggested from histological evidence that at least some sites of forming cartilage are hyoplastic in *Zeb1*-null mice. We found that Zeb1 is expressed in the perichondrium surrounding forming cartilage at E15.5, which consists of mesenchymal progenitors that contribute to the forming cartilage (Fig. 2A). As in the palate and nasal region, vimentin was co-expressed with Zeb1 in the perichondrium (Fig. 2B), and E-

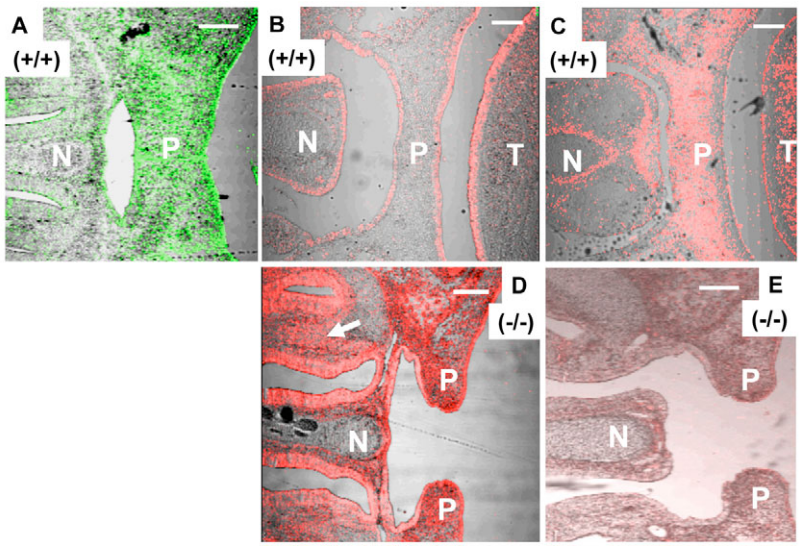

Fig. 1. E-cadherin becomes ectopically expressed, whereas vimentin is diminished in palate and nasal mesenchyme in *Zeb1* mutant mice. (**A**) Zeb1 immunostaining is shown in mesenchyme in the palate (P) and developing nasal cartilage (N). (**B**) E-cadherin immunostaining is present on the nasal (N), palatal (P) and tongue (T) epithelium. (**C**) Vimentin immunostaining is shown in palatal and nasal mesenchyme. (**D**) E-cadherin immunostaining is seen ectopically on nasal and palatal mesenchyme in *Zeb1*-null mice. Note the failure of palate closure. The arrow indicates mesenchyme in the nasal region. (**E**) Loss of vimentin immunostaining in the palatal and nasal mesenchyme of *Zeb1*-null mice. Sections of mice at E16.5 are shown. Scale bars: 100 μm.

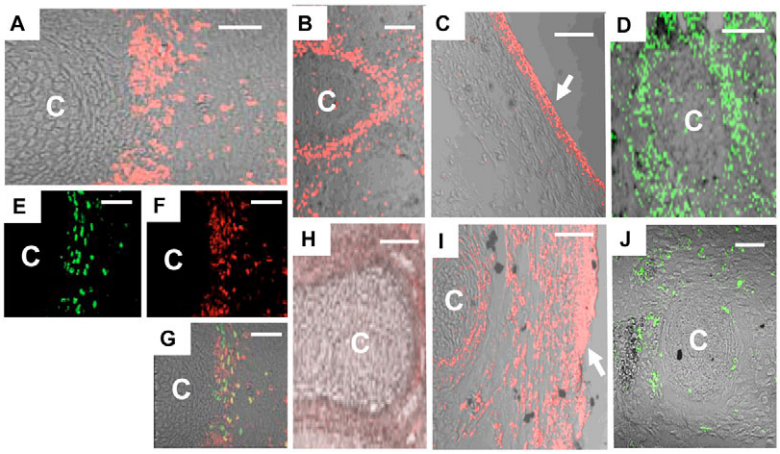

Fig. 2. Mutation of *Zeb1* leads to ectopic expression of E-cadherin, loss of vimentin, and defective proliferation in the perichondrial region of forming cartilage. (**A**) Immunostaining showing expression of Zeb1 in the perichondrium. (**B**) Immunostaining for vimentin in the perichondrium. (**C**) E-cadherin immunostaining is seen in the skin (arrow) but not in underlying mesenchymal cells. (**D**) Immunostaining for BrdU incorporation into the perichondrium. (**E**,**F**) Double immunolabeling for Zeb1 and BrdU in the perichondrium. (**G**) Overlay of E and F. (**H**) Diminished immunostaining for vimentin in the perichondrium of *Zeb1*-null mice. (**I**) Ectopic immunostaining of E-cadherin in the perichondrium of *Zeb1*-null mice. Arrow indicates skin. (**J**) Loss of BrdU immunostaining in the perichondrium of *Zeb1*-null mice. Sections of mice at E15.5 are shown. C, cartilage. Scale bars: 50 μm.

cadherin was not expressed in these mesenchymal cells (it was, however, expressed in the forming skin) (Fig. 2C). The perichondrium is known to be highly proliferative, and indeed these cells were labeled when pregnant mice were injected with BrdU, as were embryos harvested 2 hours later (Fig. 2D). Double immunolabeling for Zeb1 and BrdU showed that most BrdU-positive cells in the perichondrium expressed Zeb1 (Fig. 2E-G). Next, we assessed whether mutation of *Zeb1* would have an effect on expression of vimentin or E-cadherin in the perichondrium, or on proliferation of the cells. As in the palate and nasal mesenchyme, vimentin expression was lost in the perichondrium with *Zeb1* mutation (Fig. 2H), and whereas E-cadherin expression was maintained on the forming skin, it became ectopically expressed on the perichondrium (Fig. 2G). Concomitant with this change in E-cadherin and vimentin expression, the perichondrium showed diminished proliferation (Fig. 2J).

In addition to expression in mesenchymal cells, Zeb1 was also expressed in neuroectodermally derived cells, including the forming retina and optic nerve in the eye (Fig. 3A). It was also evident in forming muscles surrounding the eye (Fig. 3A). E-cadherin expression was present on the epithelium of the eyelid, cornea and lens, but it was absent from the retina, optic nerve and forming eye muscles (Fig. 3B). Vimentin was expressed on the retina and optic nerve as well as surrounding eye muscles (Fig. 3C). Whereas vimentin is expressed in neuroectodermally derived progenitor cells, glial fibrillary acidic protein (GFAP) is expressed and organized into filaments as these progenitor cells differentiate into glia (reviewed by Messing and Brenner, 2003). As with vimentin, we found that GFAP was expressed in the forming retina, optic nerve and surrounding eye muscles (Fig. 3D). Again, mutation of *Zeb1* led to ectopic expression of E-cadherin in the forming retina, optic nerve and eye muscles (Fig. 3E), whereas expression of both vimentin and GFAP in the retina, optic nerve and eye muscle was diminished (Fig. 4F).

In addition to neuroectodermally derived progenitors in the eye, Zeb1 was also evident in progenitor cells in the brain, including cells in the ventricular zone of the lateral ventricles (Fig. 4A), the olfactory bulb and other regions of the brain (see Fig. S3 in the supplementary material). Cells in these regions also expressed both vimentin and GFAP (Fig. 4B,C). And as in the retina and optic nerve, vimentin and GFAP expression was diminished in these cells in *Zeb1*-null mice (Fig. 4D). E-cadherin was not detected in the embryonic brain late in gestation (E15.5-17.5) (Fig. 4E; see Fig. S3 in the supplementary material). However, in the null mice, it appeared on cells in the ventricular zone of the lateral ventricles and in the olfactory bulb, which is populated by progenitors from the ventricular zone (Curtis et al., 2007) (Fig. 4F,G; see Fig. S3 in the supplementary material). Additionally, E-cadherin also appeared in proliferative regions of the third ventricle, the telencephalic vesicle, the thalamus and the hypothalamus (Fig. 4H; data not shown). This ectopic expression of E-cadherin was confined to sites in the brain that normally express Zeb1, and which are known to be sites of proliferating progenitor cells late in gestation.

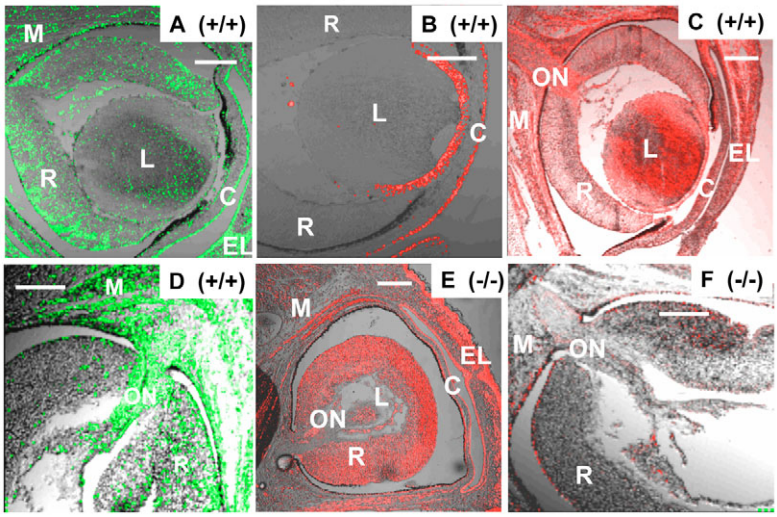

Fig. 3. Ectopic E-cadherin expression and diminished vimentin and GFAP expression in the embryonic eye of *Zeb1*-null mice. (**A**) Immunostaining for Zeb1. Retina (R); forming eye muscles (M); lens (L); cornea (C); eye lid (EL). (**B**) E-cadherin immunostaining is confined to the epithelium of the lens and cornea. (**C**) Vimentin immunostaining in the retina, lens, optic nerve (ON) and forming eyes muscles. (**D**) GFAP immunostaining in the retina, optic nerve and forming eye muscles. (**E**) Ectopic immunostaining for E-cadherin in the retina, optic nerve and forming eye muscle in *Zeb1*-null mice. (**F**) Double immunostaining for vimentin (red) and GFAP (green) shows loss of expression of both proteins in the retina, optic nerve and forming eye muscle in *Zeb1*-null mice. Sections of mice at E16.5 are shown. Scale bars: 100 μm.

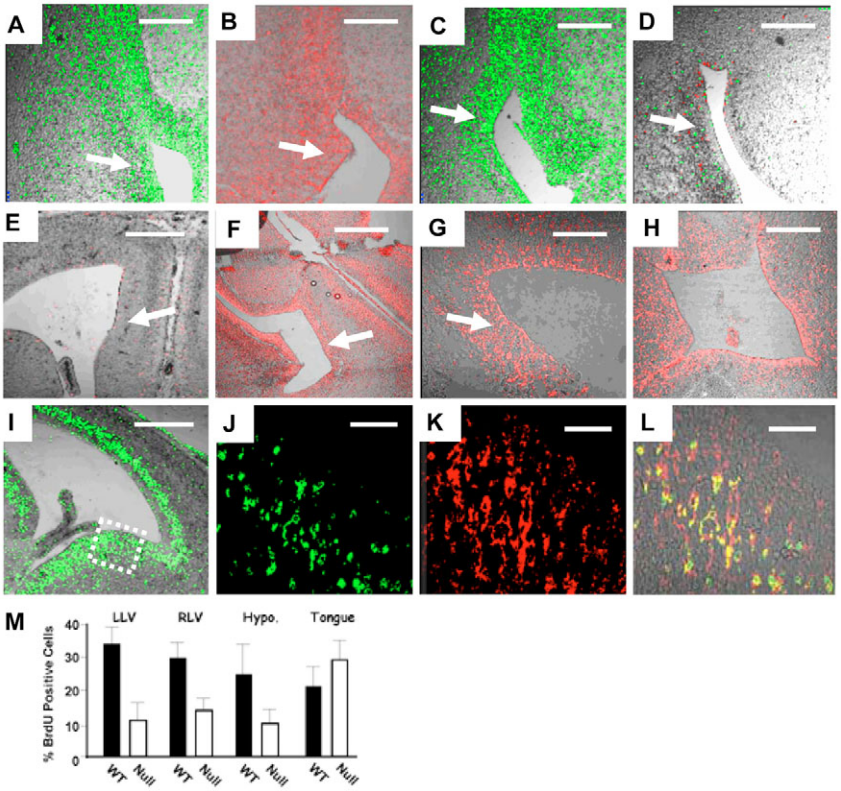

Fig. 4. Mutation of *Zeb1* leads to ectopic expression of E-cadherin, loss of vimentin and GFAP expression, and decreased proliferation in the ventricular zone of the brain. (**A**) Immunostaining for Zeb1 in the ventricular zone (arrow) of the lateral ventricle. (**B**,**C**) Immunostaining for vimentin and GFAP, respectively, in the ventricular zone. (**D**) Immunostaining for both vimentin (red) and GFAP (green) is lost in the ventricular zone of *Zeb1* mutant mice. (**E**) Immunostaining for E-cadherin is not evident in the lateral ventricle in wild-type mice. (**F**,**G**) Ectopic immunostaining for E-cadherin in the ventricular zone of the lateral ventricle in *Zeb1*-null mice. (**H**) Immunostaining for E-cadherin in the third ventricle in *Zeb1*-null mice. (**I**) BrdU immunostaining in the ventricular zone of the lateral ventricle. (**J**,**K**) Double immunolabeling of the boxed region in I for BrdU and Zeb1, respectively. (**L**) Overlay of J and K. (**M**) Quantitation of BrdU incorporation into the ventricular zone of the left lateral ventricle (LLV), right lateral ventricle (RLV), hypothalamus (Hypo.) and, as a control, the tongue. For a representative view of areas counted, see Fig. S3 in the supplementary material. Sections at E15.5 are shown. Scale bars: 100 μm in E,F,I; 50 μm in A-D,G,H; 25 μm in J-L.

A subset of *Zeb1*-null embryos show severe CNS defects, with failure of neural tube closure at both the cranial and caudal ends and exencephaly. As with proliferating mesenchymal cells, Zeb1 expression overlapped significantly with BrdU incorporation in the ventricular zone of the lateral ventricles and in other proliferative regions of the brain at E15.5 (Fig. 4I-L; data not shown). Low-magnification views of head sections of embryos immunostained for BrdU suggested that proliferation in the ventricular zone of the lateral ventricles and other regions of the brain was diminished in *Zeb1*-null mice (see Fig. S4 in the supplementary material). Therefore, we counted the percentage of BrdU-positive cells at various sites in the brain (see Materials and methods). Corresponding regions from three null and two wild-type littermates were analyzed. Three adjacent sections were counted for each embryo. A significant decrease in BrdU incorporation was seen in each of the sites in the null embryos, as compared with the wild-type embryos (Fig. 4M). This diminished proliferation in progenitors in the ventricular zone of the brain was similar to, or greater than, that seen with mutation of *Bmi1* (Molofsky et al., 2005). As a control, no significant difference in BrdU incorporation was seen in the tongue of wild-type versus *Zeb1*-null embryos (Fig. 4M; see Fig. S4 in the supplementary material). Thus, the switch in expression of the epithelial gene E-cadherin and of the mesenchymal gene vimentin in both mesenchymal progenitors in the forming cartilage and in neuroectodermally derived progenitors in the brain of *Zeb1*-null mice is linked to diminished proliferation of these populations and to developmental defects.

Ectopic expression of E-cadherin in embryonic fibroblasts from *Zeb1* mutant mice

We isolated MEFs from wild-type, heterozygous and null littermates, and asked whether *Zeb1* mutation leads to ectopic E-cadherin expression on the cells. Whereas E-cadherin was not detected by immunostaining on the wild-type cells, it was evident on the mutant cells (Fig. 5). Additionally, there was a *Zeb1* dosage-dependent induction of E-cadherin mRNA in the cells (Fig. 6A, and also Fig. 8B below). Interestingly, we also observed MEFs with abnormal epithelial-like morphology in both the null and heterozygous cell populations (Fig. 5; data not shown). These cells constituted more than 25% of the culture. It is of note, however, that E-cadherin immunostaining was evident uniformly throughout the *Zeb1*-null cell population; it was not confined to cells exhibiting epithelial-like morphology. These results link ectopic expression of E-cadherin in the mutant MEFs to an abnormal epithelial-like morphology.

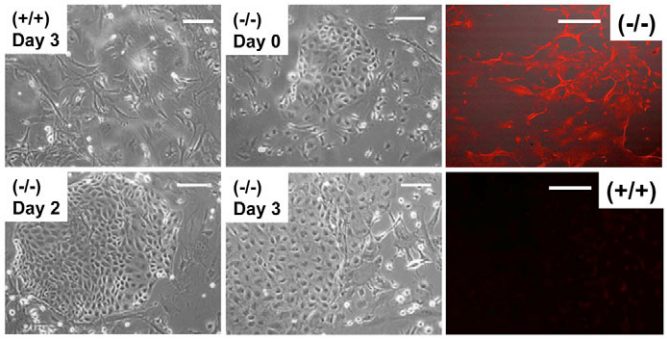

Fig. 5. E-cadherin is ectopically expressed on embryo fibroblasts from *Zeb1* mutant mice, and the cells display an abnormal epithelial-like morphology. Left-hand and middle panels show light micrographs of cells at different time points after isolation from wild-type (+/+) and Zeb1 mutant (–/–) mice. Note the increase in cells with epithelial-like morphology; such clusters of epithelial-like cells were not evident in the wild-type population. Right-hand panels show immunostaining for E-cadherin. Scale bars: 25 μm.

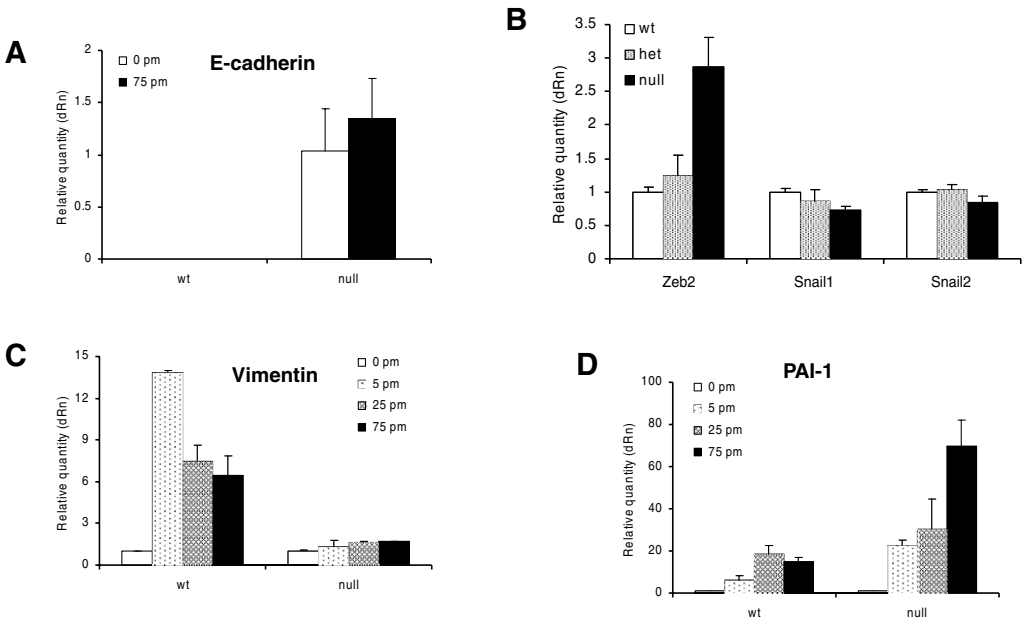

Fig. 6. Effect of *Zeb1* mutation on expression of *Zeb2* and *Snail1/2* mRNAs, and TGF-β-dependent induction of vimentin and repression of E-cadherin mRNAs. (**A**) Real-time PCR analysis shows that TGF-β is unable to repress E-cadherin mRNA levels in *Zeb1*-null MEFs. Concentrations of TGF-β are shown. No E-cadherin mRNA was detected in wild-type cells (see also Fig. 8B below). (**B**) Real-time PCR was used to assess the effect of *Zeb1* mutation on expression of other E-box-binding repressor mRNAs (*Zeb2*, *Snail1* and *Snail2*) in MEFs. Results were normalized to β-actin and *Gapdh* mRNA, with similar results. (**C**) Real-time PCR analysis shows that basal vimentin mRNA expression is unaffected by *Zeb1* mutation, but TGF-β induction is lost in *Zeb1*-null MEFs. (**D**) Real-time PCR shows that the basal plasminogen activator inhibitor 1 (PAI-1) mRNA level is unaffected by *Zeb1* mutation, and it remains inducible by TGF-β in *Zeb1-null* MEFs.

We wondered whether the ectopic expression of E-cadherin seen with *Zeb1* mutation might result indirectly from the downregulation of one of the other E-box-binding repressors. Therefore, we compared expression of *Zeb2*, *Snail1* and *Snail2* (also known as *Snai1* and *Snai2* – Mouse Genome Informatics) mRNA levels in wild-type and *Zeb1* mutant MEFs. No significant change was seen in *Snail1* or *Snail2* mRNA levels in heterozygous or null MEFs, and expression of *Zeb2* was induced with *Zeb1* mutation (Fig. 6B). This finding of *Zeb2* mRNA induction is consistent with a recent report showing increased expression of Zeb2 in smooth muscle cells of *Zeb1* mutant mice (Nishimura et al., 2006). Therefore, ectopic expression of E-cadherin in *Zeb1* mutant MEFs is not an indirect result of downregulation of *Zeb2*, *Snail1* or *Snail2* mRNA. Indeed, E-cadherin is expressed in the cells despite the fact that *Zeb2* mRNA is upregulated (and *Snail1/2* mRNA is unchanged).

Using chromatin immunoprecipitation (ChIP) assays, we determined that Zeb1 is bound to the E-cadherin gene promoter in vivo (see Fig. 8C-E below). As a negative control, we found that Zeb1 was not present at the *Gapdh* promoter. Taken together, these results are consistent with the idea that Zeb1 directly represses E-cadherin expression by binding to its promoter.

Zeb1 and TGF-β induction of vimentin and repression of E-cadherin

TGF-β drives EMT by inducing mesenchymal genes such as vimentin, and repressing epithelial genes such as E-cadherin. Given the previously documented linkage between Zeb1 and TGF-β signaling discussed above, we wondered whether the effects of Zeb1 on repression of E-cadherin and induction of vimentin might be related to TGF-β signaling. Initially, we asked whether basal vimentin mRNA expression was affected by *Zeb1* mutation in MEFs. However, we found no significant difference in the level of vimentin mRNA in wild-type versus null MEFs (Fig. 6C). Therefore, we asked whether Zeb1 might be important for TGF-β-mediated induction of vimentin. Indeed, we found that vimentin mRNA induction by TGF-β was lost in the null MEFs (Fig. 6C). However, *Zeb1* mutation did not lead to a general block in TGF-β signaling because we found that TGF-β-mediated induction of plasminogen activator inhibitor 1 (also known as *Serpine1* – Mouse Genome Informatics) mRNA was not diminished in *Zeb1*-null MEFs (Fig. 6D). We conclude that *Zeb1* is required for TGF-β-mediated induction of vimentin in the MEFs.

Our results above show that *Zeb1* is required to prevent ectopic E-cadherin expression in MEFs. We then assessed whether TGF-β would be able to repress E-cadherin expression in the null MEFs. Indeed, we found that TGF-β was unable to repress E-cadherin mRNA in the absence of *Zeb1* (Fig. 6A). Zeb1 expression is known to be induced by TGF-β (Nishimura et al., 2006). Thus, taken together, the results raise the possibility that TGF-β might repress E-cadherin through induction of Zeb1.

Mutation of *Zeb1* leads to Ink4a-independent premature replicative senescence in MEFs

We then asked whether the proliferative defects in *Zeb1*-null mice that we observed in ventricular zone and mesenchymal progenitor cells might also be reflected in MEFs from mutant mice. *Zeb1* mutant and wild-type littermate-matched MEFs were compared for proliferation. We found that *Zeb1*-null cells arrested by passage (P) 2, whereas heterozygous cells stopped proliferating by P4 (Fig. 7A). Wild-type cells continued proliferating beyond P10 (Fig. 7A; data not shown). We noticed that the arrested cells adopted a flattened morphology and they remained non-proliferative but viable for months in culture (Fig. 7B; data not shown). Because

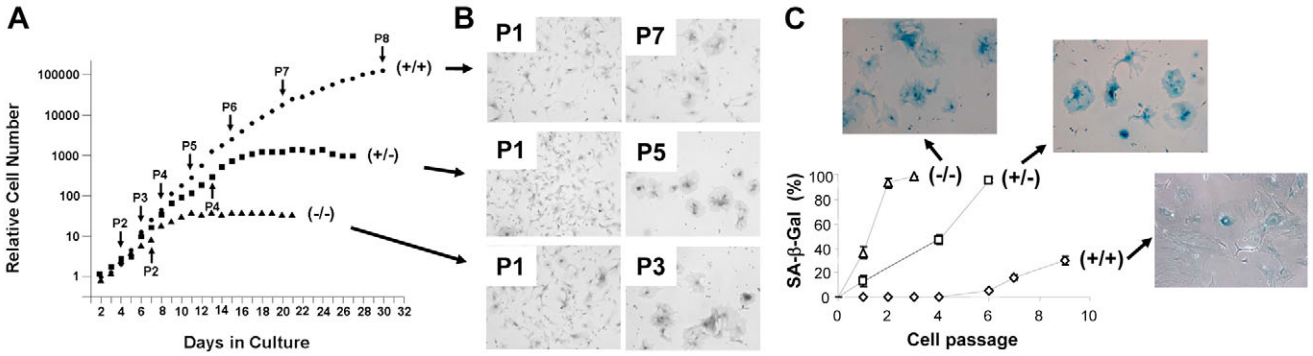

Fig. 7. MEFs from *Zeb1* mutant mice undergo gene dosage-dependent premature replicative senescence. (**A**) Effect of *Zeb1* mutation on MEF proliferation. (**B**) Micrographs of cells at early versus later passage numbers. Note the appearance of large, flat senescent-like cells associated with growth arrest. (**C**) Arrested *Zeb1* mutant cells express senescent β-galactosidase (SA-β-Gal; blue X-Gal staining).

these are properties of senescence, we stained the cells for senescent β-galactosidase activity. We found that essentially all of the arrested heterozygous and null cells expressed senescent β-galactosidase, whereas only ~30% of wild-type cells were positive at P9 (Fig. 7C). We conclude that the mutant MEFs undergo premature replicative senescence in a *Zeb1* dosage-dependent fashion.

Next, we asked whether the classic Ink4a (also known as Cdkn2a – Mouse Genome Informatics) senescence pathway was being activated in *Zeb1* mutant MEFs. Mutations in the transcriptional repressor Bmi1 have been shown to trigger proliferative defects and senescence in neural progenitors of the ventricular zone (Valk-Lingbeek et al., 2004). Bmi1 negatively regulates Ink4a, and in the absence of Bmi1 this locus expresses the CDKI, p16Ink4a, and the p53 regulator p19Arf (Arf) (reviewed by Gil and Peters, 2006). Induction of Ink4a in *Bmi1*-null embryo fibroblasts triggers senescence of MEFs (Valk-Lingbeek et al., 2004), and a cross of the *Bmi1*-null mice to *Ink4a*-null mice prevents senescence of the ventricular zone progenitors in vivo (Jacobs et al., 1999). It is of note that normal replicative senescence of wild-type MEFs results from time-dependent induction of the *Ink4a* locus in culture (usually leading to senescence around P10-12) (Lowe and Sherr, 2003; Gil and Peters, 2006). Mutation of Bmi1 accelerates this Ink4a activation, leading to premature replicative senescence.

To investigate whether activation of the Ink4a pathway was responsible for premature senescence of the *Zeb1* mutant MEFs, we used real-time PCR to analyze expression of mRNAs from the *Ink4a* locus, and from genes known to regulate this locus including *Bmi1* (repressor), *Ets1* (activator), *Tbx2* (repressor) and *Tbx3* (repressor) (Jacobs et al., 1999; Lessard and Sauvageau, 2003; Lingbeek et al., 2002; Ohtani et al., 2001). We failed to find upregulation of *p16Ink4a* or *Arf* (or any of the *Ink4a* regulators) in senescent *Zeb1* mutant cells (Fig. 8A). Instead, expression of both mRNAs was downregulated in the null and heterozygous cells compared with in wild-type cells. It is of note that this downregulation of *p16Ink4a* and *Arf* mRNA correlates with *Zeb1* dosage-dependent downregulation of the Ink4a inducer *Ets1* (Fig. 8A). As a positive control, we found that p16Ink4A expression was induced with passage number in the wild-type MEFs, as reported previously by a number of groups (Lowe and Sherr, 2003; Gil and Peters, 2006; Liu et al., 2007). We conclude that the premature replicative senescence seen in the *Zeb1* mutant MEFs is not the result of activation of the classic *Ink4a* locus.

p15Ink4b and p21Cdkn1a are ectopically expressed in *Zeb1* mutant MEFs

TGF-β treatment classically induces growth arrest, but this does not involve induction of Ink4a; instead, two other CDKIs, p21Cdkn1A and p15Ink4b, are induced to trigger the growth arrest (Reynisdottir et al., 1995). Because of the known linkage of Zeb1 to TGF-β signaling, we asked whether these CDKIs might be ectopically expressed in the *Zeb1* mutant MEFs. Indeed, we found a gene dosage-dependent increase in both mRNAs, with the increase in *p15Ink4b* mRNA being the most dramatic (Fig. 8B). These results demonstrate that *Zeb1* is required to prevent ectopic expression of these CDKIs in MEFs.

Next, we asked whether Zeb1 acts directly on the *p21Cdkn1a* and *p15Ink4b* gene promoters. In ChIP assays, Zeb1 was bound to both promoters, but no interaction was seen with the control *Gapdh* promoter (Fig. 8C-E). These results are consistent with the notion that Zeb1 is represses these CDKIs directly by binding to their promoters in vivo.

p15Ink4b is ectopically expressed in mesenchymal and ventricular zone progenitor cells in *Zeb1*-null embryos

p15Ink4b expression was dramatically induced in *Zeb1* mutant MEFs (Fig. 8B), and expression of p15Ink4b is known to block cell proliferation (Reynisdottir et al., 1995). Therefore, we asked whether p15Ink4b might become ectopically expressed in *Zeb1*-null mice at sites of diminished proliferation. We did not detect p15Ink4a expression in mesenchymal cells or in the ventricular zone in wild-type mice (Fig. 9A,B). Indeed, the developing skin was the only tissue where we observed significant expression (see Fig. S5 in the supplementary material). However, as in the MEFs, p15Ink4b became ectopically expressed in the perichondrium in *Zeb1*-null mice (Fig. 9C,D; see Fig. S5 in the supplementary material), and these cells showed diminished proliferation (Fig. 2D,J).

p15Ink4b expression was also ectopically expressed in the ventricular zone of *Zeb1*-null mice (Fig. 9E,F). Although there was overlap between p15Ink4b and BrdU incorporation, it is of note that there were a number of additional p15Ink4b-positive cells in this region that were BrdU-negative (Fig. 9G-I), and there was overall diminished proliferation of cells in this region. These results are consistent with the notion that ectopic expression of p15Ink4b in *Zeb1*-null mice in both mesenchymal and ventricular zone progenitors leads to diminished proliferation. Previous studies have

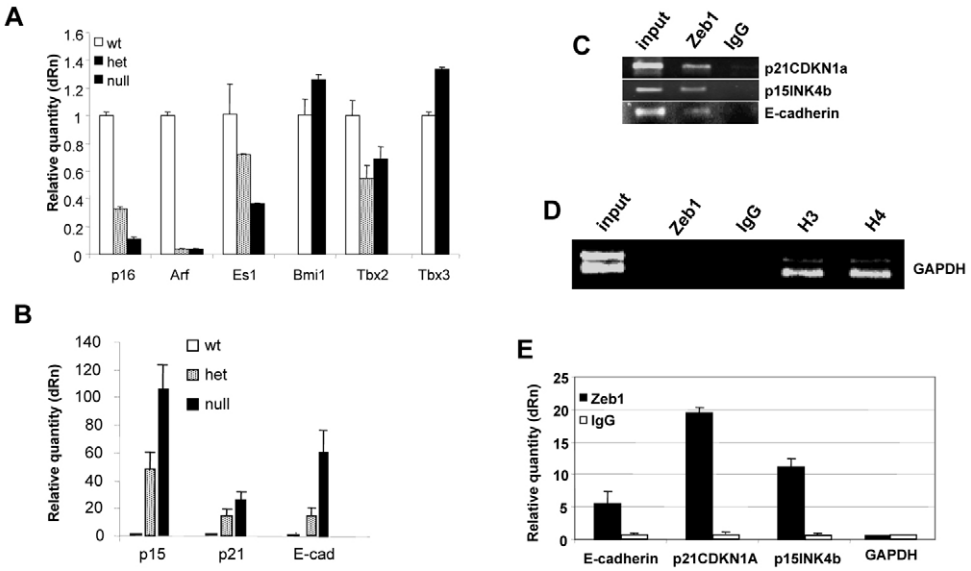

Fig. 8. Mutation of *Zeb1* does not trigger the classic Ink4A replicative senescence pathway in MEFs; instead, it is associated with induction of *p15Ink4b* and *p21Cdkn1a*. (**A**) Real-time PCR was used to compare mRNA expression from the *Ink4a* locus (*p16Ink4a* and *Arf*), and genes known to regulate the *Ink4a* locus (*Ets1*, *Bmi1*, *Tbx2*, *Tbx3*), in proliferating wild-type MEFs (P3) and senescent *Zeb1* heterozygous (P5) and null (P2) cells. (**B**) *p15Ink4b*, *p21Cdkn1a* and E-cadherin mRNAs are induced in a gene dosage-dependent fashion in *Zeb1* mutant MEFs. Real-time PCR results using the same samples as in A are shown. (**C**) ChIP assays. Input, starting chromatin used for the immunoprecipitations; IgG, pre-immune serum. (**D**) Control ChIP assay showing that Zeb1 does not bind to the *Gapdh* promoter. Histone H3 and H4 are positive controls for binding to the *Gapdh* promoter. (**E**) Real-time PCR quantification of the results in C and D. The same input DNA was used for each ChIP assay, and the relative input value is set at 100. Primers for the promoters are shown in Table 2.

found expression of p15Ink4b in the chick embryo hindbrain, consistent with a role in controlling cell proliferation in this region (Kim et al., 2006).

DISCUSSION

Zeb1 is important for regulating the balance between epithelial and mesenchymal gene expression in vivo. Overexpression in cancer leads to EMT, whereas mutation of *Zeb1* causes the opposite: mesenchymal-epithelial transition. This mesenchymal-epithelial transition in *Zeb1* mutant mice is accompanied by diminished proliferation of progenitor cells. The alteration in mesenchymal and epithelial gene expression and proliferative defects are evident in MEFs from *Zeb1* mutant mice, allowing us to examine these alterations in more detail. The proliferative defects in the MEFs result in cellular senescence. However, this is not mediated through the classic Ink4a pathway, which can trigger senescence in response to oncogenic mutation and aging. Instead, this senescence is associated with induction of other CDKIs, p15Ink4b and p21Cdkn1a, and we demonstrate that *Zeb1* mutation causes ectopic expression of p15Ink4b at sites of proliferative defects in vivo. In a recent study, we found that expression of Zeb1 is linked to the Rb/E2F cell cycle control pathway, which appears to be the mechanism responsible for restricting Zeb1 expression to proliferating cells (Liu et al., 2007).

The epithelial and mesenchymal genes as well as the CDKIs that are deregulated in response to *Zeb1* mutation, have in common their regulation by TGF-β, effecting EMT and cell cycle arrest, respectively. The linkage of each of these genes to TGF-β, together with the fact that Zeb1 participates in TGF-β signaling via binding to activated Smads, suggests that the function of Zeb1 in maintaining the balance between mesenchymal and epithelial gene expression and in cell proliferation might be associated with TGF-β superfamily signaling. In this regard, it is of note that Zeb1 was required for TGF-β regulation of key epithelial and

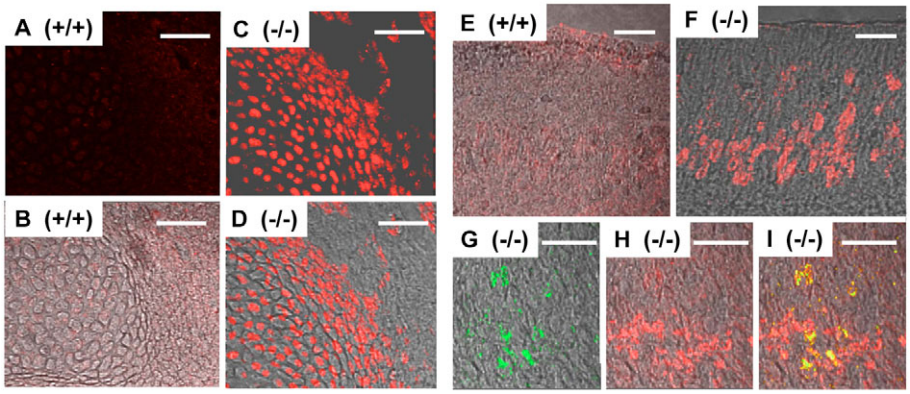

Fig. 9. p15Ink4b is ectopically expressed in the perichondrium, on forming cartilage, and in the ventricular zone of the lateral ventricle in *Zeb1*-null mice. (**A**,**C**) Immunostaining for p15Ink4b on forming cartilage. (**B**,**D**) Overlay of the immunostaining in A and C with a Nomarski image. (**E**,**F**) Immunostaining for p15Ink4b in the ventricular zone of the lateral ventricle. (**G**,**H**) Immunostaining for BrdU and p15Ink4b, respectively, in the ventricular zone of the lateral ventricle. (**I**) Overlay of G and H. Immunostaining of E15.5 embryos is shown. Scale bars: 50 μm.

mesenchymal genes (e.g. E-cadherin and vimentin). Furthermore, Zeb1 had opposing effects on the two genes, in keeping with the mesenchymal-epithelial gene expression transition seen in *Zeb1* mutant mice. Mutation of *Zeb1* led to ectopic expression of E-cadherin, and TGF-β was no longer able to repress the gene. Because TGF-β induces Zeb1 (Nishimura et al., 2006), we suggest that induction of Zeb1 is a mechanism for TGF-β repression of E-cadherin, and this is likely to occur via recruitment of a Zeb1-CtBP repressor complex to the E-cadherin promoter. In contrast to E-cadherin, vimentin expression is induced by TGF-β. Its expression was diminished in *Zeb1* mutants, and Zeb1 was required for TGF-β-mediated induction of vimentin in MEFs. Gene induction by TGF-β is mediated by activation of Smad transcription factors, and we suggest that Zeb1-dependent TGF-β-mediated induction of genes such as vimentin and smooth muscle actin and myosin might be the result of Zeb1 being required to mediate efficient assembly of a Smad-p300 transcription complex at their promoters (a complex which excludes CtBP) (Postigo, 2003).

It has been shown that overexpression of Zeb1 facilitates TGF-β induction of the *p15Ink4b* promoter in transfection assays (Postigo, 2003). Yet, here we found that p15Ink4b is ectopically expressed in *Zeb1* mutant cells. These findings are not necessarily contradictory. Taken together, they suggest that *p15Ink4b* is under repression by Zeb1. Because the *p15Ink4b* gene is a known target of activated Smads, we propose that recruitment of a Smad-Zeb1-p300 complex to the promoter in response to TGF-β might serve to displace CtBP from Zeb1, leading to derepression of the gene.

We suggest that Zeb1 is important for regulating the balance between mesenchymal and epithelial gene expression and for maintaining the proliferation of a subset of progenitor cells late in gestation. But, it is interesting that this phenotype extends to premature replicative senescence in cultured MEFs. Mutations in other genes such as *Bmi1* show a similar premature senescence phenotype in MEFs. Such a phenotype in MEFs is closely linked to diminished proliferation and senescence of progenitor cells in the CNS and bone marrow. Although these other mutations involve Ink4a regulators and p16Ink4a itself, it is of note that we observe diminished CNS progenitor proliferation in *Zeb1*-null mice, and this effect was similar to, or even greater than, that observed with *Bmi1* mutation (Molofsky et al., 2005). Interestingly, TGF-β and p15Ink4b have key roles in restricting proliferation of T-cell progenitors, and mutation or epigenetic silencing of p15Ink4b leads to lymphoproliferative disease (Latres et al., 2000; Wolff et al., 2003; Lessard and Sauvageau, 2003; Mishra et al., 2005), and is common in leukemia (reviewed by Claus and Lubbert, 2003). It is of note that *Zeb1*-null mice have a diminished number of T-cell progenitors, which fail to populate the thymus (Higashi et al., 1997; Takagi et al., 1998). These results raise the possibility of a proliferative defect in a subset of bone marrow-derived progenitors with *Zeb1* mutation.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/3/579/DC1

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