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Fibrosis and Hypoxia-Inducible Factor-1α–Dependent Tumors of the Soft Tissue on Loss of Von Hippel-Lindau in Mesenchymal Progenitors

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Abstract

The ability of a cell to adapt to low oxygen tension (hypoxia) is critical in normal development and differentiation and in pathologic settings, such as cancer and ischemia. The hypoxia-inducible factor (Hif)-1α and Hif-2α (Epas1) are crucial mediators of the homeostatic responses that allow hypoxic cells to survive and differentiate.[1] In normoxia, the E3 ubiquitin ligase von Hippel Lindau (Vhl) binds to Hif-1α and Epas1 and targets them to the proteasome for degradation.[2, 3, 4] Cells that lack functional Vhl are unable to degrade the Hif-αs, and this ultimately results in their progressive accumulation.[2, 3, 5, 6] However, Vhl, which is a ubiquitously expressed protein, has also a variety of biological activities, including control of the cell cycle, regulation of matrix proteins, and interaction with the cytoskeleton and the primary cilia that are HIF independent.[7, 8]

Notably, VHL is a tumor suppressor, and its deficiency is a key pathogenetic event in a variety of human familial and sporadic tumors.[9] However, to this end, it is still largely unknown whether HIF-dependent or HIF-independent VHL functions are critical for VHL-defective tumorigenesis.[8] Moreover, the phenotypic identity of the cells that give origin to VHL-deficient tumors is also the object of intense debate. Along these lines, in VHL-deficient hemangioblastomas, genetic alterations consistent with a neoplastic nature have been identified in the mesenchymal/stromal component of these tumors.[10, 11] Moreover, a mesenchymal cell origin has been provocatively suggested for VHL-associated clear cell renal carcinomas.[12]

We previously reported that Vhl is critically important for the proper development of cartilage and bone.[13, 14, 15, 16] To better understand the role of Vhl in the biology of mesenchymal cells, we analyzed mutant mice that lacked Vhl in mesenchymal progenitors that give rise to the soft tissues that form and surround

synovial joints. We thus found that Vhl prevents the development of fibrosis and mesenchymal tumors at these sites by regulating HIF activity.

Materials and Methods

All the experiments were performed using at least three independent biological replicates.

Generation of Mice

Generation and genotyping of the *Vhl* (FVB/N), *Hif1a* (FVB/N), *Epas1* (C57/B6), floxed mice, *Prx1-Cre* (FVB/N), *Col2a1-Cre* transgenic mice, and *ROSA26 mT/mG* (FVB/N) reporter mice have been previously described.[15, 17, 18, 19, 20, 21, 22] Connective tissue growth factor (*Ctgf*) (C57/B6) floxed mice were generated and characterized in the laboratory of Dr. K.M. Lyons.[23] Further details on *Ctgf* floxed mice are given elsewhere. For all studies, *cre*-positive heterozygous floxed mice and *cre*-negative homozygous floxed mice, respectively, were used as controls.

Cartilage and bone phenotype of VHL, VHL–HIF-1, and VHL–HIF-2 mice have been previously described.[16]

All procedures that involved mice were performed in accordance with the NIH guidelines for use and care of live animals and were approved by University of Michigan Committee on Use and Care of Animals (protocol PRO00005182).

Cryosections, Routine Histologic Analysis, Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling, *in Situ* Hybridization, and Immunohistochemistry Detection on Paraffin Sections

Frozen sections from p14 hindlimbs and sternum were prepared and cut as previously described.[16] Frozen sections from p21 COL2Cre-mTmG hindlimbs were prepared according to Kowamoto Tape Transfer Kit (SECTION-LAB Co. Ltd., Hiroshima, Japan).[24, 25] Briefly, freshly isolated hindlimbs were embedded in Super Cryo Embedding Medium and frozen in cold hexane. Frozen blocks were positioned in the cryostat, and a double-sided tape provided by the kit was applied on the block. Ten-micrometer sections were then cut with tungsten carbide blades and stored at −80°C.

Routine histologic analysis, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay, and *in situ* hybridization on embryonic stages E15.5 and E17.5, newborn and postnatal ages p13-p28 forelimbs and hindlimbs were performed as previously reported.[16, 26]

For immunohistochemistry (IHC), paraffin sections from forelimbs of p21 to p28 mice were treated with sodium citrate buffer (pH 6) at 95°C for 15 minutes. Sections were then incubated with the following primary antibodies: anti–cadherin-11 at 1:100 (71-7600, Invitrogen, Life Technologies, Grand Island, NY), anti-Ctgf at 1:100 (sc-14939, Santa Cruz Biotechnology, Inc., Dallas, TX), anti-Vhl (BD556347, BD Biosciences, San Jose, CA) overnight at 4°C, anti-CD31 at a 1:25 (DIA310, Dianova, Hamburg, Germany), anti-desmin at 1:200 (AF3844, R&D Systems, Minneapolis, MN), anti-S100 at 1:2500 (Z0311, Dako North America, Inc., Carpinteria, CA), anti-cytokeratin at 1:100 (M0821, Dako North America), anti-vimentin at 1:200 (M7020, Dako North America), anti–von Willebrand factor at 1:200 (A0082, Dako North America) for 30 minutes at room temperature, and anti-CD45 at 1:200 (BD550539, BD Biosciences) for 1 hour at room temperature. After incubation with the appropriate biotinylated secondary antibody, binding was detected using the labeled streptavidin biotin and the tyramide amplification system (Perking Elmer, Waltham, MA) following the manufacturer's instructions.

Image Acquisition

Images were acquired with Eclipse E800 (Nikon, Tokyo, Japan). Additional images were captured with a Leica DM LB compound microscope (Leica Microsystems, Wetzlar, Germany). For fluorescent images, frozen sections were dehydrated at 4°C overnight, stained with DAPI (Life Technologies), and then overlayed with coverslips onto ProLong Diamond Antifade Mountant (Life Technologies). Selected photographs were taken using a Nikon A1 confocal microscope (Nikon) with filters for red fluorescent protein, fluorescein isothiocyanate, and DAPI. Representative images of each technique are shown.

Radiography

Forelimbs were analyzed by radiography, using an XPERT 80- L Cabinet X-ray System (Kevex-90Kv; Kubtec X-ray, Milford, CT) as previously reported.[16] In addition, some forelimbs were analyzed using a Faxitron X-ray cabinet (Faxitron Bioptics, Tucson, AZ) as previously reported.[27]

Cell Isolation and Culture

For tumor cell isolation, the proximal joint between the scapula and humerus of wild-type and mutant p22 mice and the adjacent soft tissues were isolated in Dulbecco's phosphate-buffered saline and subjected to a short series of 0.25% trypsin digestions at 37°C to minimize contamination with muscular tissue; after each digestion, the supernatant was discarded. The samples were placed in tissue culture–treated dishes and cut into smaller fragments to promote migration from the segments onto the surface. Cells were grown in Dulbecco's modified Eagle's medium and Glutamax supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. They were passaged twice before collection to expand the population.

All cells were incubated at 5% CO_2 under humidified atmosphere, and media were changed every 2 to 3 days before collection. Deletion of *Vhlh* was confirmed by PCR analysis of the genomic DNA extracted from tumor cells and wild-type cells in culture, as previously reported.[15]

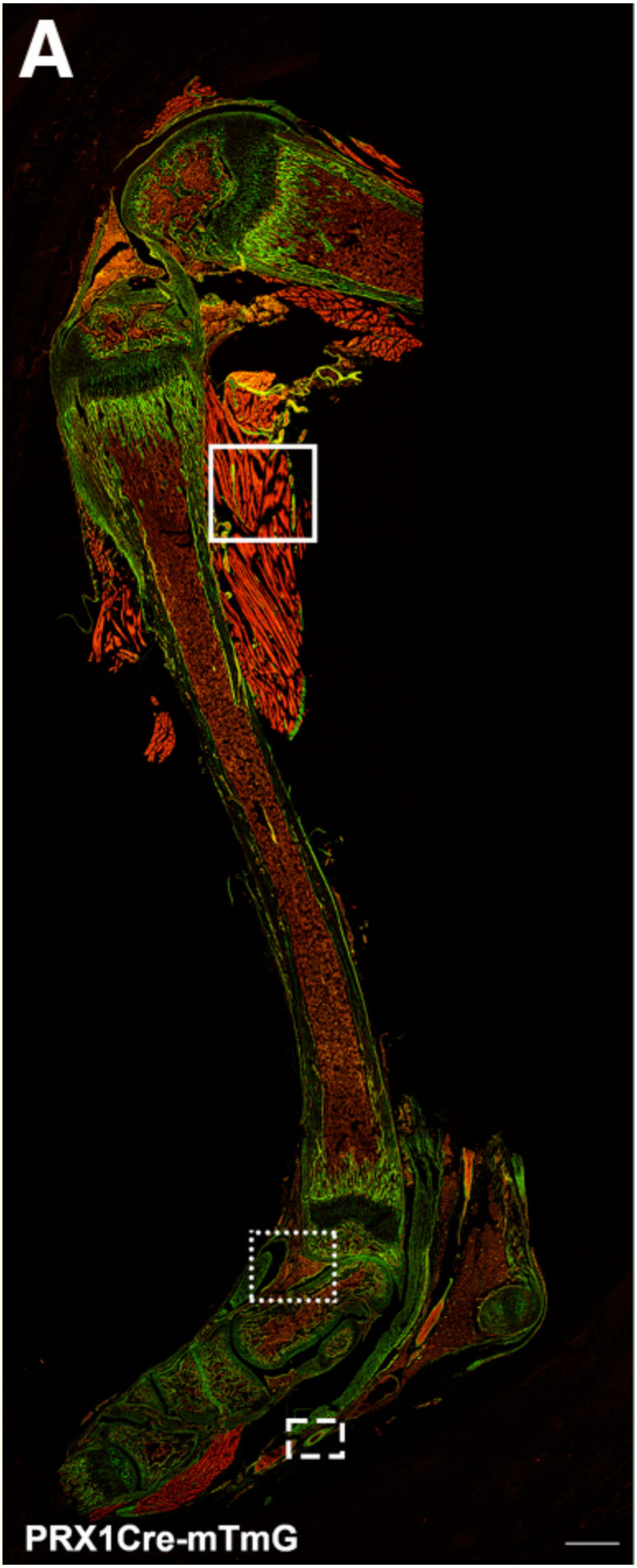
Western Blotting

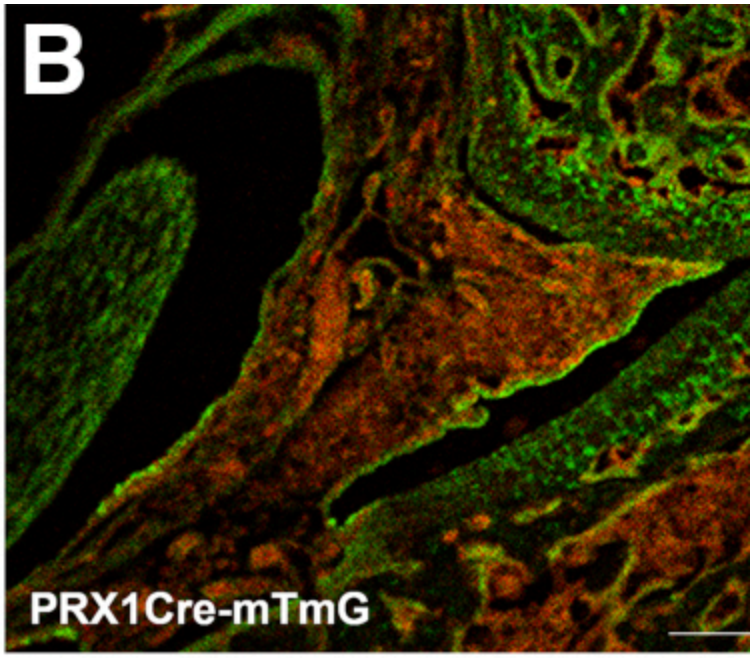
Protein extraction and Western blotting for Hif-1α and Epas1 were performed as previously reported.[16]

Results

Loss of Vhl in Mesenchymal Progenitors of the Limb Bud Causes Severe Fibrosis of the Synovial Joints

To investigate the role of Vhl in the biology of mesenchymal progenitor cells, we pursued the conditional deletion of Vhl in limb bud mesenchyme by using Prx1-Cre transgenic mice. We first analyzed *cre* activity in Prx1-Cre mice by taking advantage of the mT/mG reporter.[21] The mT/mG reporter mice have *loxP* sites on either side of a membrane-targeted tandem dimer tomato (mT) cassette and express red fluorescence in all tissues. When bred to Prx1-Cre mice (PRX1Cre-mTmG), the mT cassette is deleted, allowing expression of the membrane-targeted enhanced green fluorescent protein (EGFP) (mG) cassette in the tissues where *cre* is active. In p14 limbs of PRX1Cre-mTmG mice, a positive EGFP signal could be clearly detected not only in cartilage, as previously reported,[16] but also in bone, synovium, ligaments, tendons, perimysium, epimysium, and, surprisingly, periendothelial cells within the blood vessel walls, whereas the signal was consistently negative in muscular tissue and endothelial cells (Figure 1).


A
PRX1Cre-mTmG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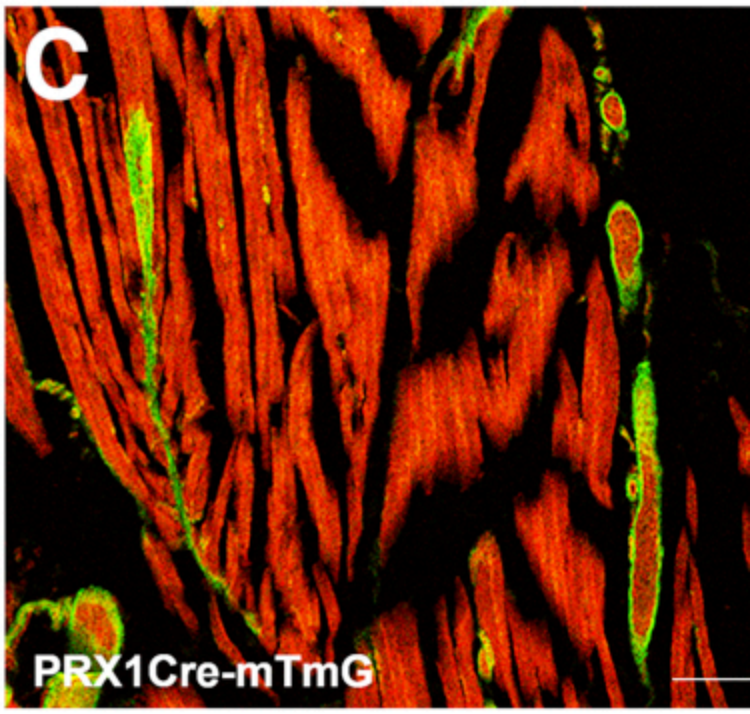

C
PRX1Cre-mTmG

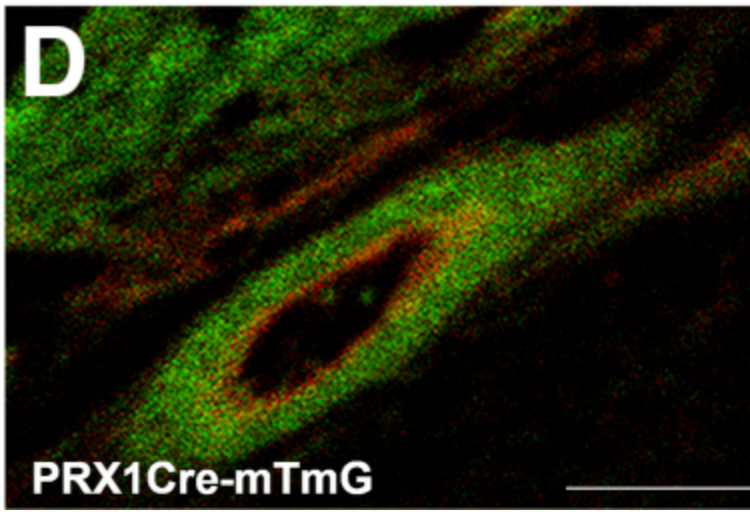
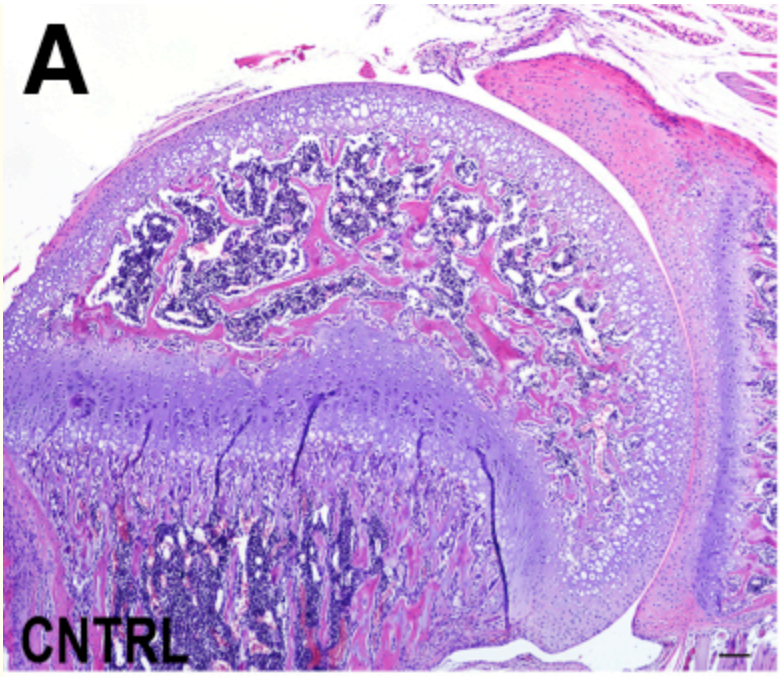

D
PRX1Cre-mTmG

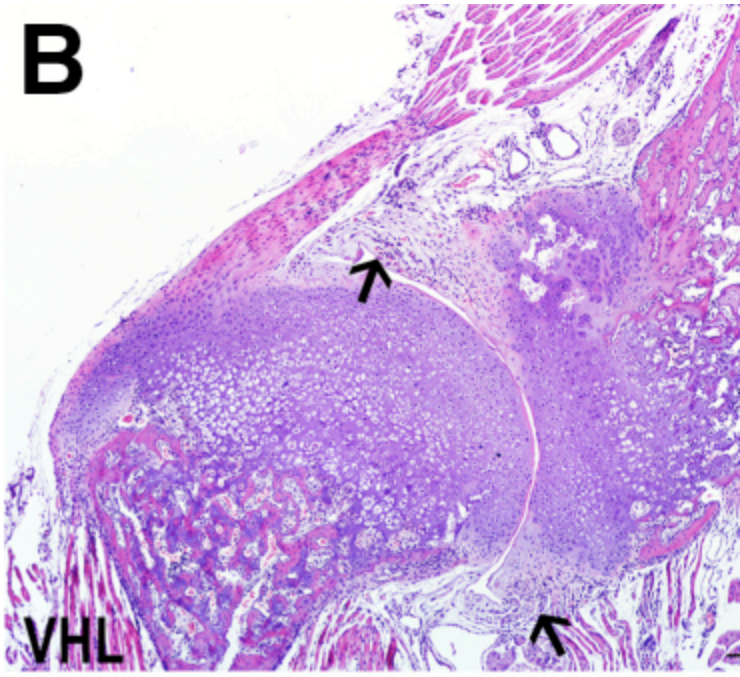
Figure 1

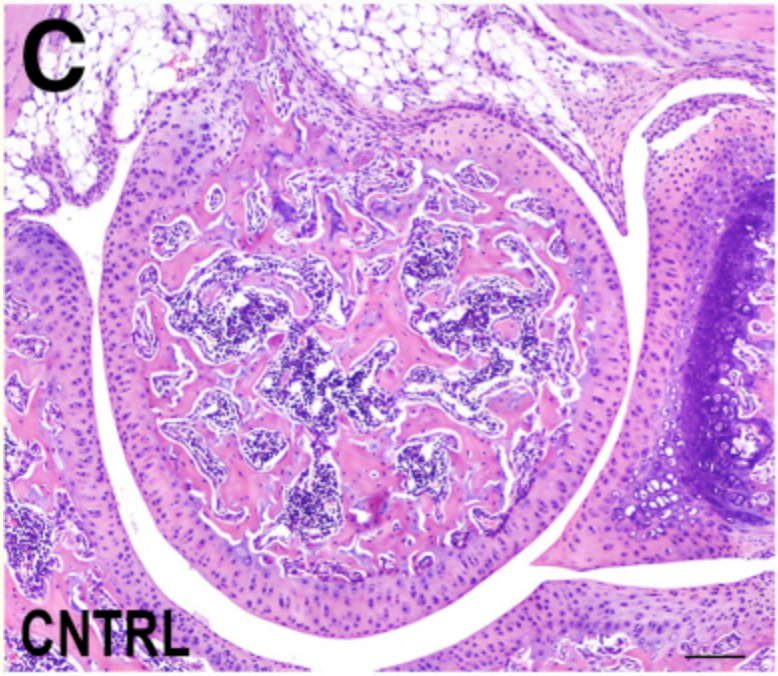
Cre is active in the soft tissues surrounding bone and cartilage of PRX1-Cre mice. **A–D:** Detection of fluorescence in frozen sections of tibias isolated from postnatal day 14 PRX1Cre-mTmG mice (**A**). Closeups of the synovium (**middle boxed area**; **B**), perimysium and epimysium (**top boxed area**; **C**), and one blood vessel (**bottom boxed area**; **D**) are shown. The enhanced green fluorescent protein signal indicates tissues and cells in which *cre* recombination occurred. Scale bars: 500 μm (**A**); 100 μm (**B–D**).

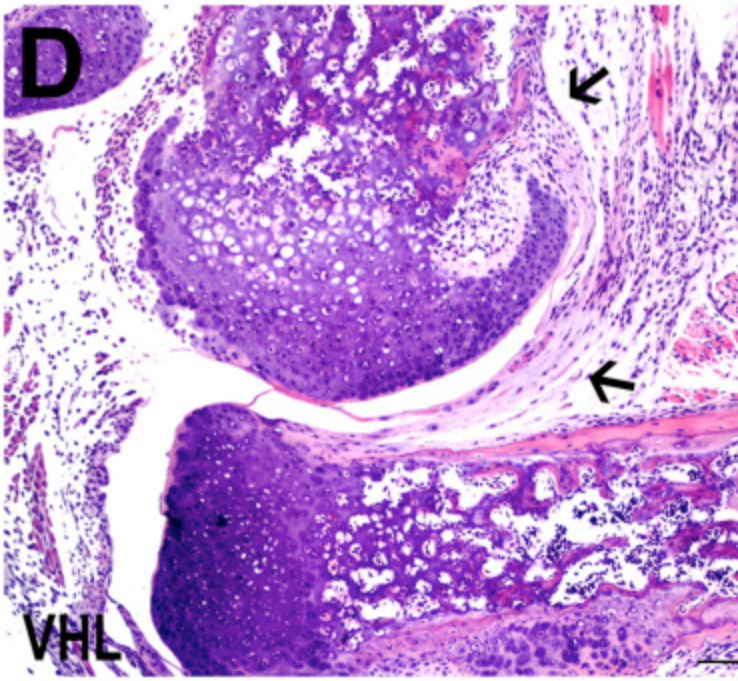
We next generated *Prx1-Cre;Vhl$^{f/f}$* mutants (VHL) as previously described,[16] and we analyzed the soft tissues that formed and surrounded their synovial joints. VHL mice were born with the expected mendelian frequency and reached adulthood. However, they were smaller than control littermates and died at approximately postnatal day 28 (p28), as already reported.[16]

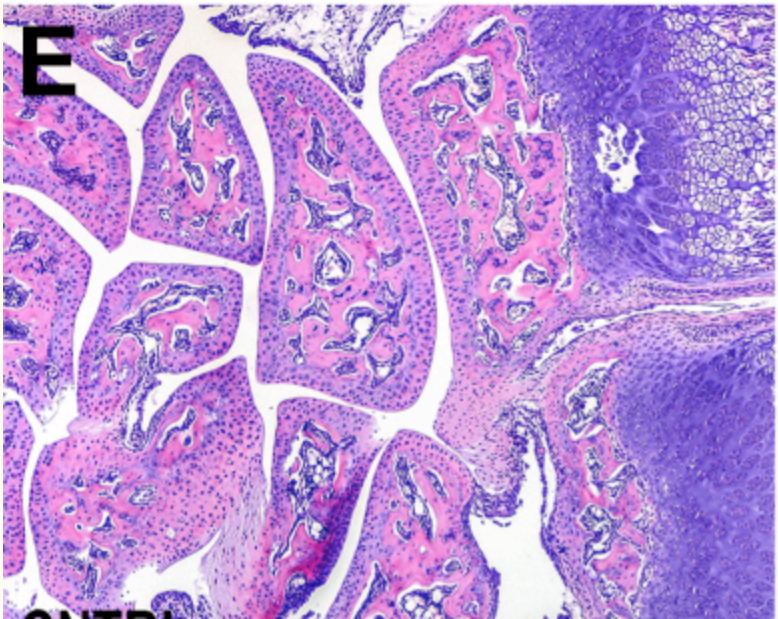
In agreement with our previous findings,[16] analysis of the synovial joints revealed that loss of Vhl did not alter either the joint specification (data not shown) or the joint cavitation process in mutant mice when compared with control littermates (Supplemental Figure S1). However, later in fetal development and perinatally, the synovial space in mutant mice appeared to be partially filled with fibrotic tissue (Supplemental Figure S1, B, D, and F). Curiously, the presence of knee menisci was never detected in VHL mutants either prenatally or postnatally (Supplemental Figure S1, C–F, and Figure 2, G and H). After birth, fibrosis of the synovial space was massive in VHL mice, and this was associated with partial destruction of the articular surface cartilage (Figure 2, A–F). The fibrotic lesions were particularly aggressive in the shoulder and in the elbow joints of most mutant mice (Figure 3). These aggressive lesions were formed by numerous spindyloid cells and a few multinucleated cellular elements; in addition, they had considerable accumulation of an amorphic and acellular material (Figure 3, B and C). Consistent with their fibroblastic appearance, spindyloid cells within the lesions expressed collagen Iα (Col1a1) mRNA (Figure 3, A–D, G, and H). The aggressive fibrosis eventually penetrated and replaced the growth plate and the adjacent bone (Figure 3, B and H). Fibrotic infiltrations of bone also occurred at the tendon insertions, where they caused erosions and destruction of bony structures, as seen on radiographs (Figure 3, E and F).


A
CNTRL


B
VHL


C
CNTRL


D
VHL


E

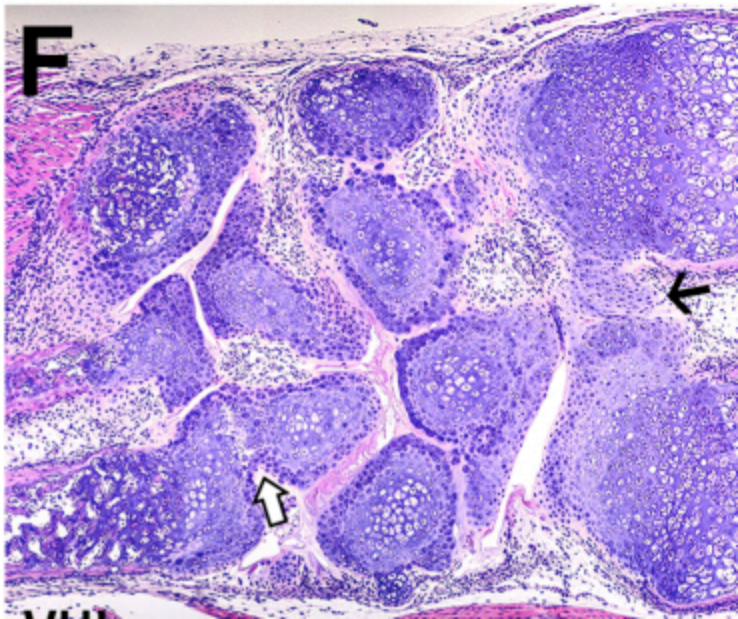

F

Figure 2

Deficiency of von Hippel Lindau (Vhl) in limb bud mesenchyme causes fibrosis of the synovial joints. Hematoxylin and eosin staining of postnatal day (p) 20 shoulders (**A** and **B**), p17 elbows (**C** and **D**), p17 wrists (**E** and **F**), and p18 knees (**G** and **H**) in control (CNTRL) (**A, C, E,** and **G**) and VHL (**B, D, F,** and **H**) mice. The **arrows** indicate the fibrosis (**B**) and point to the fibrotic infiltration (**D**). **F**: The **white arrow** indicates the fusion of small cartilaginous elements and the **black arrow** indicates the ectopic cartilage. The mutant knee joint is occupied by a massive fibrotic infiltration in **H**. Scale bar = 100 μm.

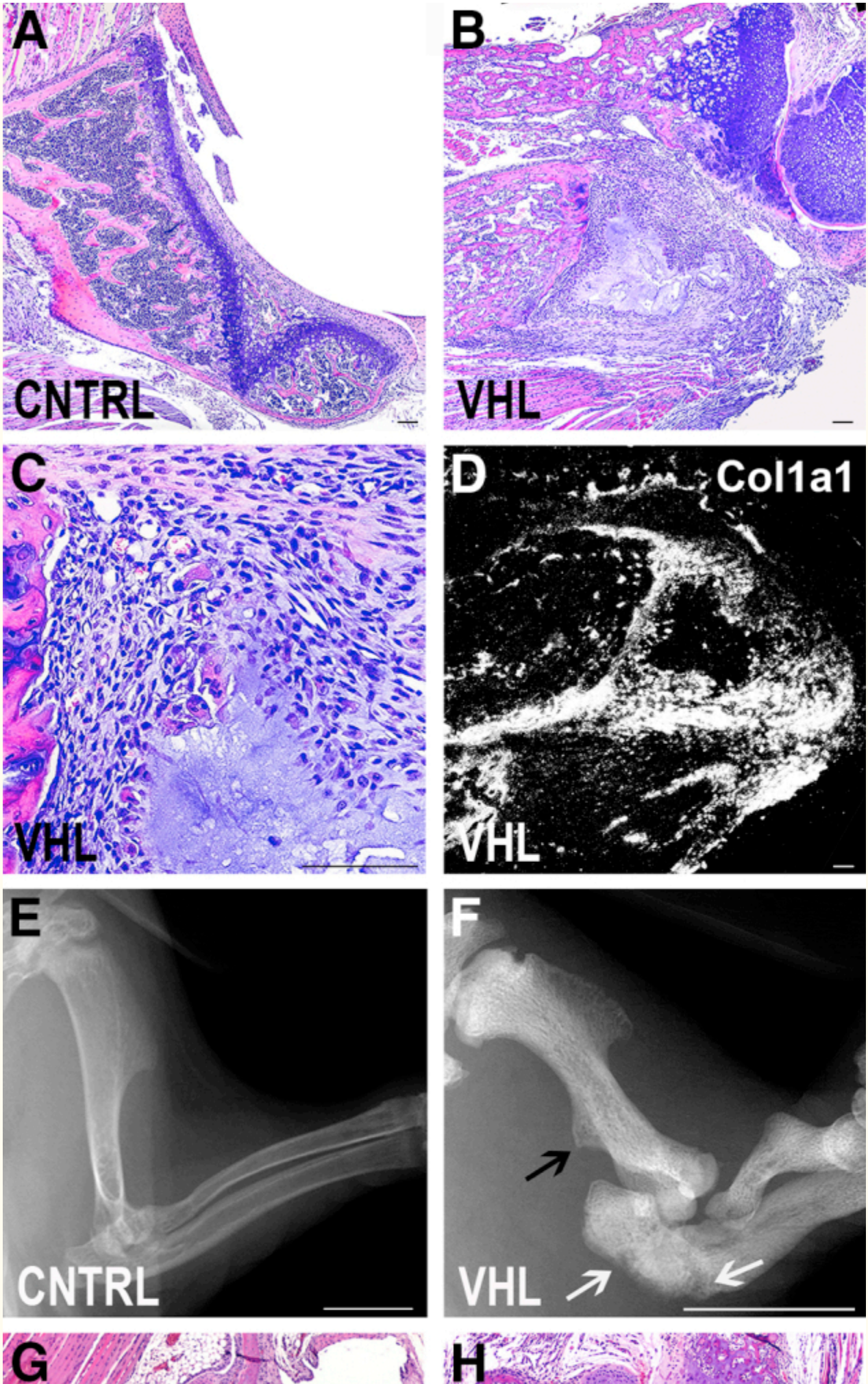

A
CNTRL
B
VHL
C
VHL
D
Col1a1
VHL
E
CNTRL
F
VHL
G
H

Figure 3

Aggressive synovial fibrosis in shoulder and elbow of von Hippel Lindau (VHL) mutants. **A–C:** Hematoxylin and eosin (H&E) staining of postnatal day (p) 18 scapulas in control (CNTRL) (**A**) and VHL (**B**) mice. **C:** Higher magnification of **B**. Aggressive fibrotic lesions are present in the shoulder of mutant mice. **D:***In situ* hybridization for detection of Col1a1 mRNA of p18 scapula in VHL mice. These fibrotic lesions express Col1a1 mRNA. **E** and **F:** Radiographs of p21 forelimbs in CNTRL (**E**) and VHL (**F**) mice. The **white arrows** (**F**) indicate the erosions of the ulna. The **black arrow** (**F**) points to the persistent deformity of the humerus observed in VHL mice. **G** and **H:** H&E staining of p20 elbows in CNTRL (**G**) and VHL (**H**) mice. The **black arrows** (**H**) highlight the infiltration of the aggressive fibrosis. Scale bars: 100 μm (**A–D**, **G,** and **H**); 0.5 cm (**E** and **F**).

Of note, we did not observe fibrosis of the synovial joints in mutant mice in which Vhl had been deleted exclusively in cells committed to either the chondrocyte[13] or the osteoblast lineage[14, 15] (Supplemental Figure S2, A–F) (some data not shown). In agreement with these findings, analysis of *Col2a1-Cre;mTmG$^{f/f}$* (COL2Cre-mTmG) mice revealed only a very low and spotted EGFP signal in the synovium, tendon, and ligaments of the synovial joints (Supplemental Figure S2G).

This finding is consistent with an early mesenchymal population being the target of the Prx1 enhancer, and it indicates that cells that give origin to the synovial fibrosis are not osteochondroprogenitors.

In addition to the massive fibrosis, loss of Vhl in limb bud mesenchyme led to the formation of foci of ectopic cartilage in the soft tissue surrounding the cartilaginous structures (Figure 2D). These data are in agreement with the notion that hypoxia signaling is not only necessary for chondrogenesis,[20] but it may also be sufficient.[28]

Lastly, fusion of small cartilaginous elements occurred in both forelimb and hindlimb VHL mutant autopods (Figure 2F) (some data not shown). Because no obvious impairment of joint specification could be detected in mutant samples at earlier time points,[16] we concluded that these fusions were not the result of a failure of synovial joints to be specified.

Loss of Vhl in Mesenchymal Progenitors of the Limb Bud Causes Soft Tissue Tumors Located in Close Proximity to Synovial Joints

The aggressive fibrosis described above postnatally was associated with the formation of discrete masses (Figures 4 and and5).5). The development of these masses mainly occurred in the scapula and elbow (Figure 4, A–J, and Figure 5) and, sporadically, also in the wrist (data not shown), knee (Figure 4, K and L), and sternum (Supplemental Figure S3, B–E). Of note, as with the long bones of the hindlimbs and forelimbs, the sternum originates from the lateral plate mesoderm, as confirmed by *cre* activity at this site[29] (Supplemental Figure S3A).

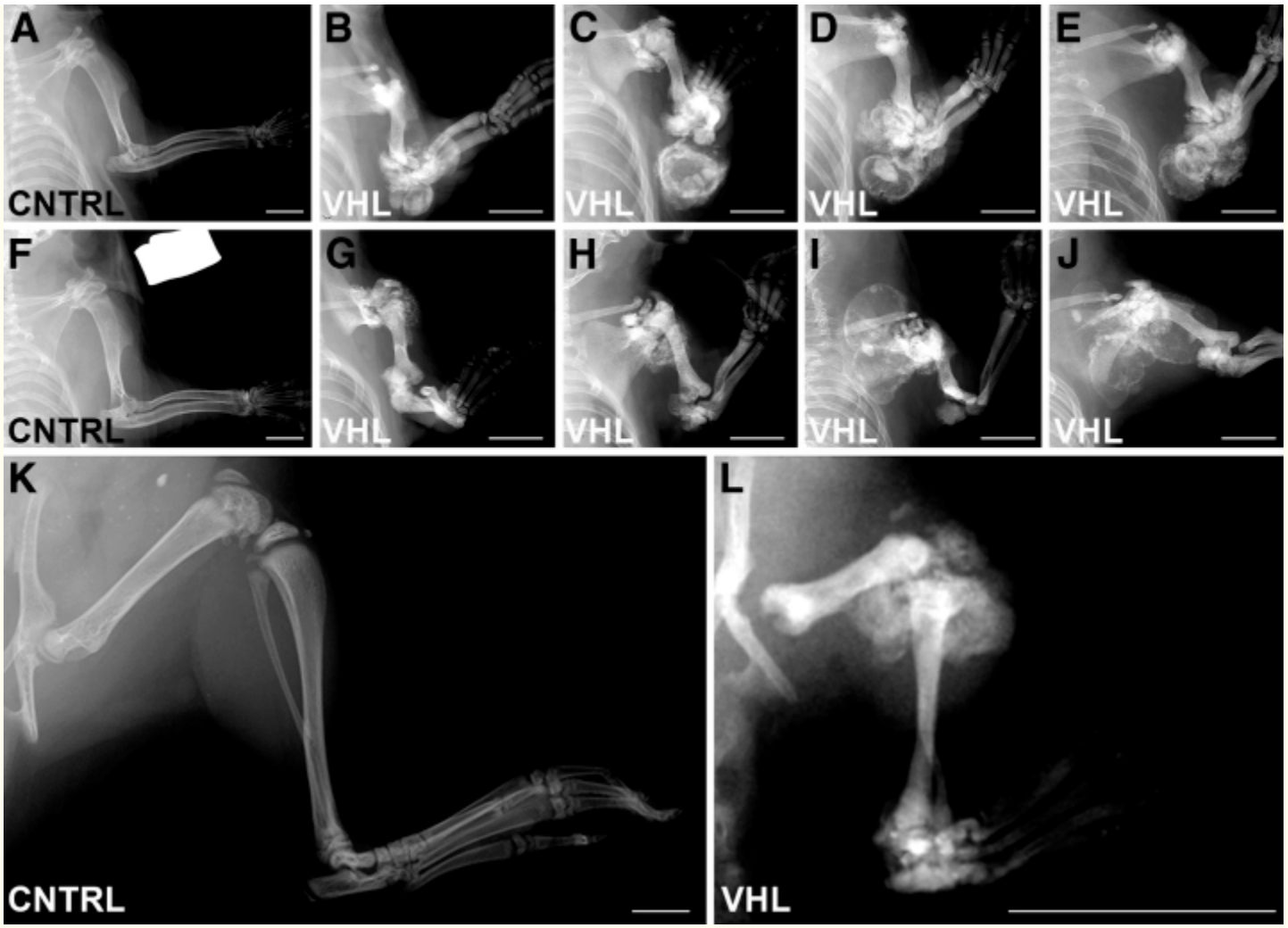


Figure 4

Presence of tumors in forelimbs and hindlimbs of von Hippel Lindau (VHL) mice. The aggressive fibrosis postnatally was associated with the formation of tumors. The development of these tumors mainly occurred in the scapula and elbow, and, sporadically, also in the knee. Radiographs revealed that the VHL masses were partially mineralized and were highly heterogeneous with irregular borders. Radiographs of postnatal day (p) 21 to 28 elbows (**A–E**), p21 to 28 shoulders (**F–J**), and p21 knees (**K** and **L**) in control (CNTRL) (**A, F,** and **K**) and VHL (**B–E**, **G–J**, and **L**) mice. Scale bar = 0.5 cm.

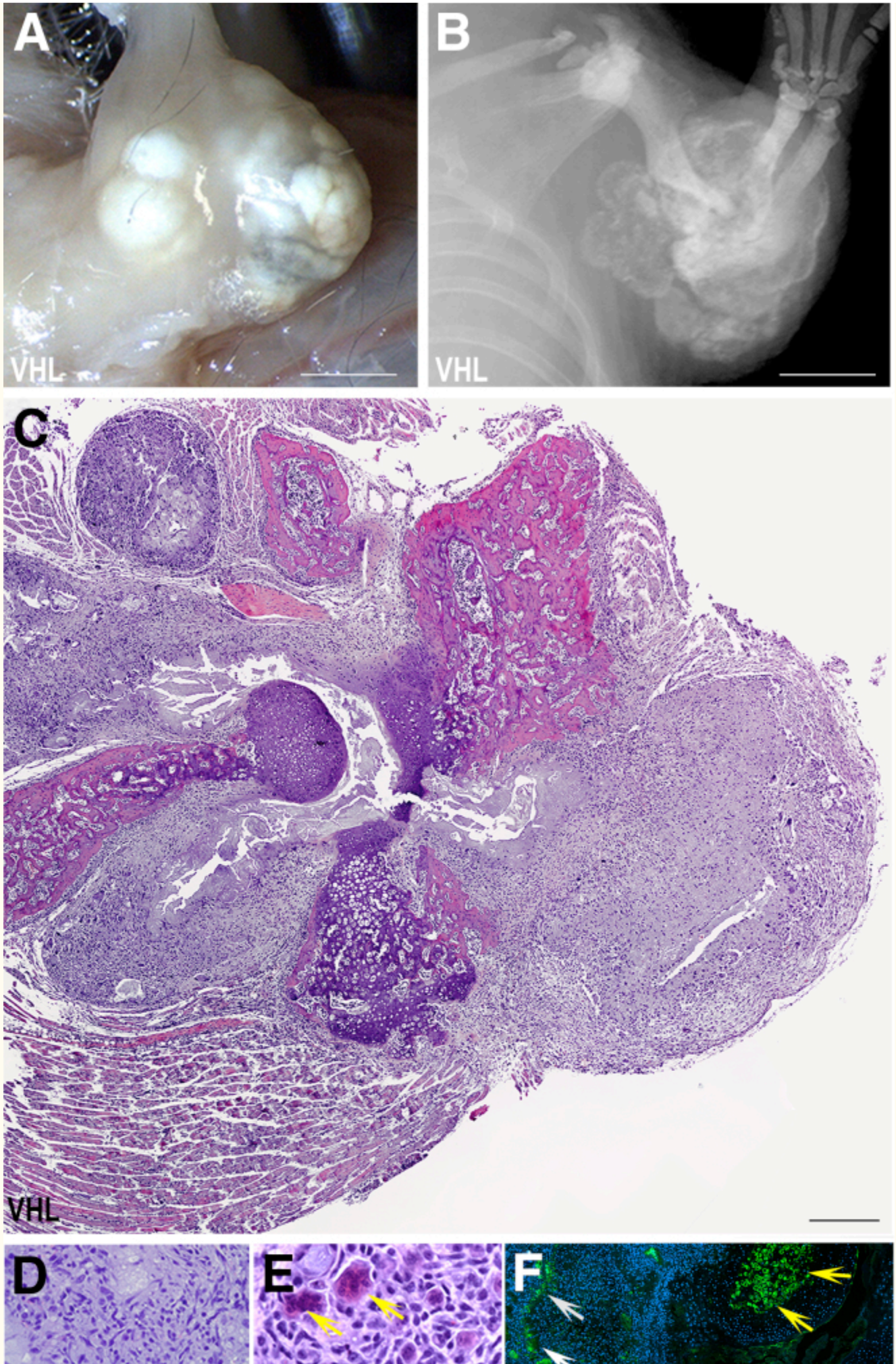

A
VHL
B
VHL
C
VHL
D
E
F

Figure 5

von Hippel Lindau (Vhl) deficiency leads to formation of soft tissue tumors in proximity to the synovial joints. **A:** Macroscopic appearance of a postnatal day (p) 28 elbow tumor in VHL mice. The tumor appears as a whitish and multilobulated mass. **B:** Radiograph of a p21 elbow tumor in VHL mice. **C:** Hematoxylin and eosin (H&E) staining of the VHL elbow tumor shown in **B**. The tumor aggressively invades adjacent tissues, such as cartilage, bone, and muscles. **D** and **E:** Higher magnifications of p21 elbow tumor in VHL mice. The tumor is formed by numerous spindyloid cells with irregular shape and by rare multinucleated cells with an intense eosinophilic cytoplasm indicated by the **yellow arrows** in **E**. **F:***In situ* cell death detection of a p21 VHL elbow tumor. The **yellow arrows** and the **white arrows** point at terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling–positive cells in the growth plate and in the tumor, respectively. **G:** Radiograph of a p21 shoulder tumor in VHL mice. Note multiple erosions of the mutant olecranon. **H:** H&E staining of the VHL shoulder tumor shown in **G**. An amorphic and acellular material abnormally accumulates in the tumor. Scale bars: 0.5 cm (**A**, **B** and **G**); 100 μm (**C–F** and **H**).

To systematically study these soft tissue lesions, VHL mice and control littermates were analyzed by radiography and histologic analysis at p7 to p28. These masses were overall more frequent in forelimbs than in hindlimbs (Figure 4), which is consistent with the finding that *cre* activity in forelimbs is detectable earlier than in hindlimbs.[19] Radiographs revealed that the VHL masses were partially mineralized and were highly heterogeneous with irregular borders (Figure 4, B–E, G–J, and L, and Figure 5, B and G). Macroscopically, they were whitish and multilobulated (Figure 5A). Histologically, they appeared to aggressively invade adjacent tissues, such as cartilage, bone, and muscles (Figure 5, C and H), and, like the aggressive fibrosis, were formed by numerous spindyloid cells with irregular shape (Figure 5D) and by rare multinucleated cells with an intense eosinophilic cytoplasm (Figure 5E). Interestingly, the spindyloid cells did not have any obvious nuclear atypia, and their overall proliferation rate was low (data not shown). Lastly, an amorphic and acellular material abnormally accumulated in the masses (Figure 5, C and H), most likely as a consequence of both cell death (Figure 5F) and synovial fluid being trapped within the lesions.

We next performed an IHC series to further phenotypically characterize the cells that formed these masses. No positive staining for desmin, CD31, von Willebrand factor, CD45, or cytokeratin was detected, which indicates that these cells did not belong to the muscular, endothelial, hematopoietic, or epithelial lineage (Figure 6, A and B, and Supplemental Figure S4, A–C). Conversely, they strongly stained positive for S100 and vimentin (Figure 6, C and D), which are both mesenchymal markers.[30, 31, 32, 33, 34] In addition, they also expressed high levels of Col1a1 mRNA (Figure 6E). In light of these data, we thus concluded that the spindyloid cells that formed the masses had a mesenchymal phenotype.

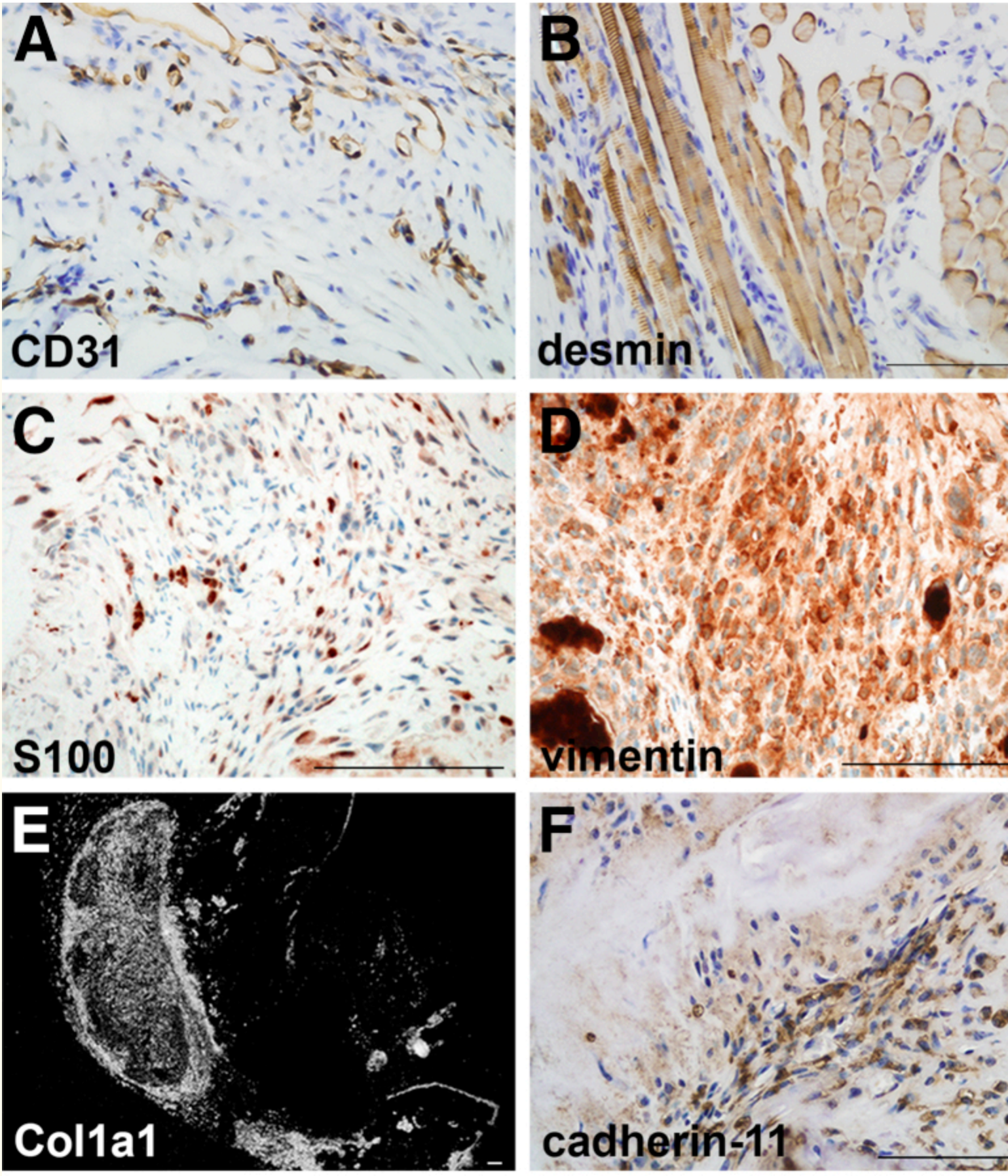

A
CD31
B
desmin
C
S100
D
vimentin
E
Col1a1
F
cadherin-11
G
2-loxP
H
HIF-1α

Figure 6

von Hippel Lindau (VHL) tumors are mainly formed by Vhl-deficient mesenchymal cells. **A** and **B:** Immunohistochemistry of CD31 (**A**) and desmin (**B**) in VHL tumors. Spindyloid cells within the tumor are negative for both CD31 and desmin, which indicates that these cells do not belong to either the endothelial or the muscular lineage. **C–F:** IHC of S100 (**C**), vimentin (**D**), and cadherin-11 (**F**) in VHL tumors. *In situ* hybridization for detection of Col1a1 mRNA (**E**). These cells have a mesenchymal phenotype because they strongly stain positive for S100, vimentin, and cadherin-11 and express high levels of Col1a1 mRNA. **G:** Efficient recombination of *Vhl* floxed allele in cells isolated from VHL tumors. Gels have been run under the same experimental conditions. **H:** Western blot analysis of hypoxia-inducible factor (Hif)-1α (Hif-1α) and HIF-2α (Epas1) in protein lysate from CNTRL periarticular soft tissue and from cells isolated from VHL tumors, respectively. Detection of α-tubulin was used as a loading control. Blots were run under the same experimental conditions. Tumor cells have increased accumulation of Hif-1α and Epas1. **I:***In situ* hybridization for detection of phosphoglycerate kinase-1 (PGK1) mRNA in a VHL tumor. The tumor expresses high levels of PGK1 mRNA, which is a classic downstream target of Hif-1α. Scale bar = 100 μm.

Notably, numerous spindyloid cells within the masses stained positively for cadherin-11 (Figure 6F). Cadherin-11 is expressed in fibroblast-like synoviocytes, and it is involved in the development of synovium and tendons.[35, 36] The positive staining results for cadherin-11 suggest that cells within the masses, or at least a subset of these cells, derived from the synovium and/or from tendon progenitors. Interestingly, lack of hemosiderin deposits (data not shown) ruled out the possibility that the masses observed in VHL mice could be the murine equivalent of a human condition known as pigmented villonodular synovitis,[37] which is characterized by overgrowth of the synovium, intense inflammation, and accumulation of hemosiderin deposit. In agreement with this conclusion, the virtual absence of CD3-positive staining also excluded the presence of a significant inflammatory component within the lesions (data not shown).

Intriguingly, cells surrounding the necrotic and amorphic areas expressed high levels of secreted phosphoprotein 1 (SPP1) mRNA (Supplemental Figure S4D). SPP1 is involved in numerous pathologic processes, and it is a marker of highly hypoxic tumors.[38, 39]

Taken together, our findings indicate that the masses observed in VHL mutant mice were mainly formed by mesenchymal cells and, despite the lack of nuclear atypia, exhibited features consistent with low-grade malignant tumors, such as local invasiveness, irregular margins, cell death, presence of multinucleated cells, high cell density, and cellular pleomorphism.

Xenotransplantation has become a widely used tool to determine the tumorigenicity of cells.[40] However, because it is classically quite challenging to succeed in generating xenografts with tumor fragments or cells isolated from low-grade malignant tumors of the soft tissue,[40] such an experimental approach was not pursued.

Of note, recombination of the *Vhl* conditional allele in mutant cells isolated from the masses was confirmed by genomic PCR analysis for the recombined (1-*loxP*) allele (Figure 6G). In addition, as predicted, increased accumulation of Hif-1α and Epas1 proteins occurred in these mutant cells when compared with controls, as indicated by Western blot analysis (Figure 6H). Moreover, most spindyloid cells within the tumors did not express Vhl as indicated by IHC (Supplemental Figure S5). Lastly, consistent with Hif-1α stabilization, high levels of expression of mRNA encoding for phosphoglycerate kinase-1, which is a classic downstream target of Hif-1α,[20, 41] were observed in the VHL masses (Figure 6I).

Soft Tissue Tumors and Synovial Fibrosis Still Develop in Mutant Mice Lacking Both Vhl and Epas1 in Mesenchymal Progenitors of the Limb Bud

In light of our findings, we next asked whether the development of the VHL mesenchymal tumors was Epas1 dependent because it has been proposed that *Epas1* is an oncogene.[12, 42] For this purpose, we generated *Prx1-Cre;Vhl$^{f/f}$;Epas1$^{f/f}$* mice, in which both Vhl and Epas1 were conditionally deleted in

mesenchymal progenitors of the limb bud (VHL-HIF2 mice). Optimal efficiency of recombination of the *Epas1* conditional allele was confirmed as previously reported.[16, 43] Of note, VHL-HIF2 double mutant mice had an overall postnatal survival similar to VHL mice.

No obvious foci of ectopic cartilage could be detected in double mutants that lack both Vhl and Epas1 (data not shown) at least up to p28, but fusion of small cartilaginous elements still occurred in these double mutant mice (Figure 7, A and B). Synovial fibrosis was ameliorated but not fully corrected by loss of Epas1 (Figure 7B and Supplemental Figure S6, A, B, F, and G). Notably, loss of Epas1 rescued menisci formation; however, menisci appeared abnormally fibrotic in VHL-HIF2 mice (Supplemental Figure S6, K and L).

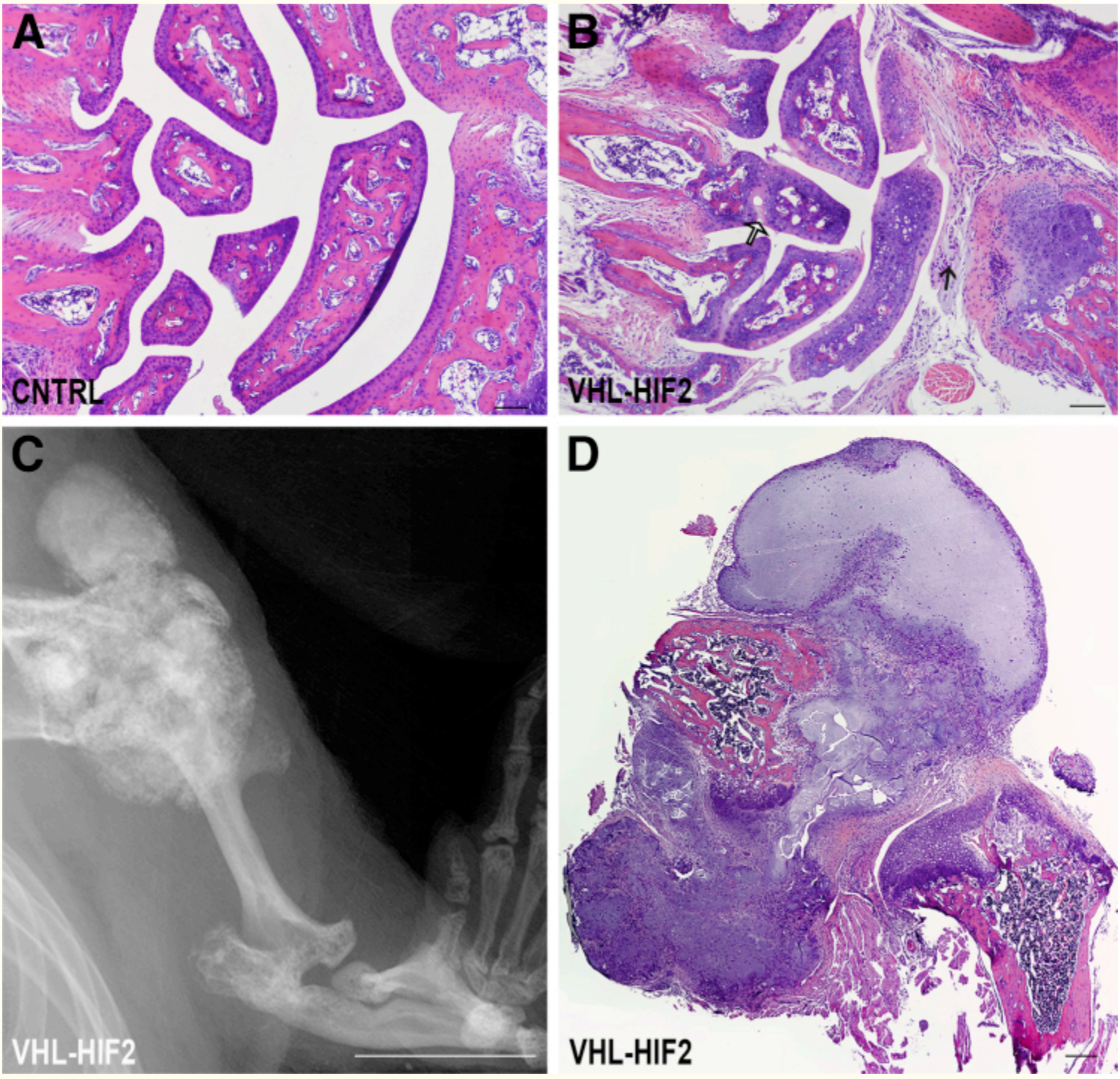


Figure 7

Development of von Hippel Lindau (VHL) tumors is mainly hypoxia-inducible factor (Hif)-2α (Epas1) independent. Tumors are still detectable in double mutant mice that lack both VHL and Epas1. **A** and **B:** Hematoxylin and eosin (H&E) staining of postnatal day (p) 21 wrists in control (CNTRL) (**A**) and VHL-HIF2 (**B**) mice. **B:** The **black arrow** and the **white arrow** indicate the fibrosis and the fusion of cartilaginous elements, respectively. **C:** Radiograph of a p21 shoulder tumor in VHL-HIF2 mice. **D:** H&E staining of the VHL-HIF2 shoulder tumor shown in **C**. Scale bars: 100 μm (**A**, **B**, and **D**); 0.5 cm (**C**).

More importantly, VHL-HIF2 mutant mice still developed tumors in proximity to synovial joints (Figure 7, C and D). These masses were phenotypically similar to the ones observed in VHL single mutants as shown by macroscopic inspection, radiographs (Figure 7C), and routine histologic analysis (Figure 7D). However, the overall penetrance of these lesions was 70% in the single VHL mutants but only 47% in the double mutants, as indicated by radiographs at p21 to p28 (Figure 8D).

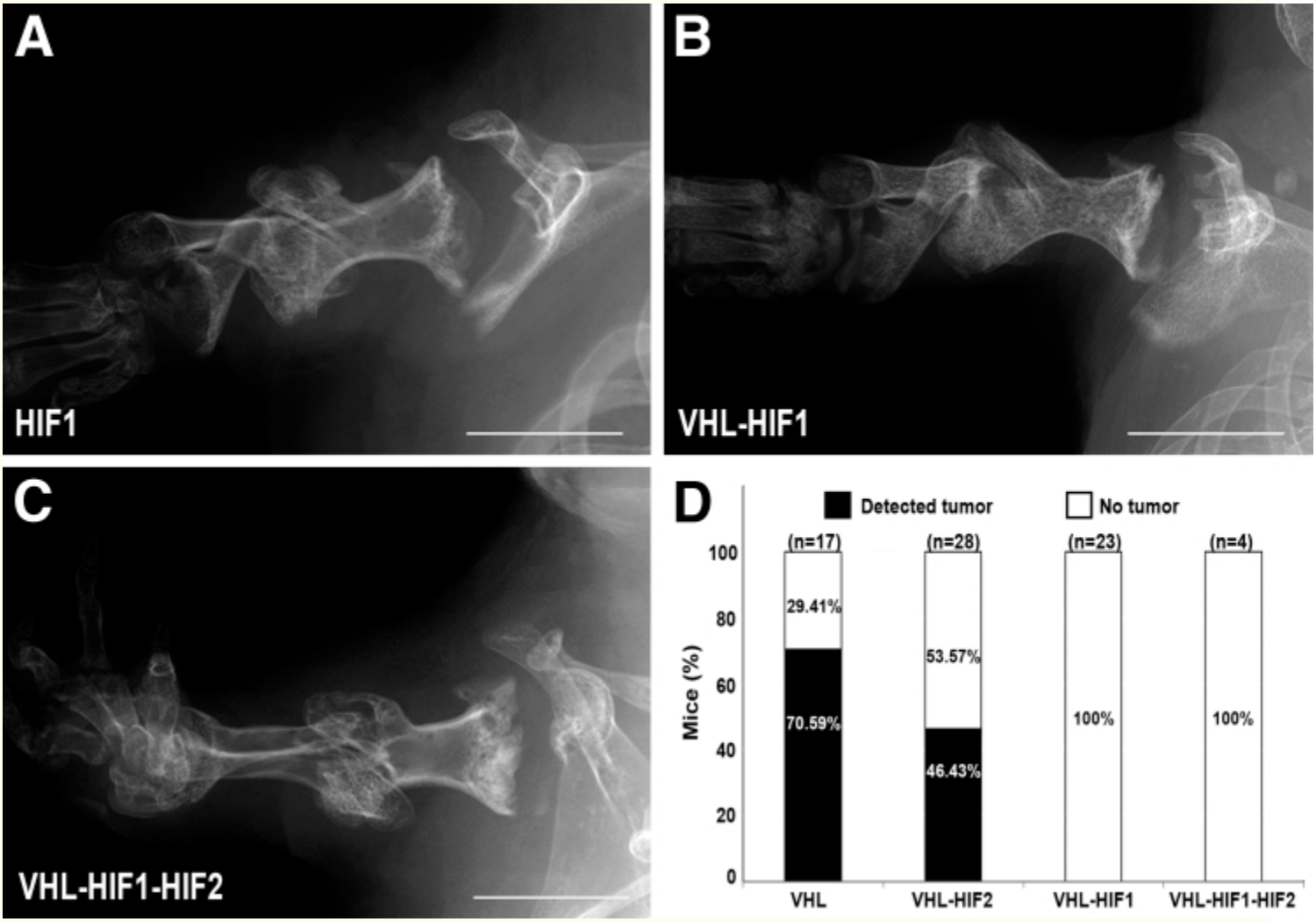


Figure 8

Hypoxia-inducible factor (Hif)-1α (Hif-1α) is necessary for the onset of von Hippel Lindau (VHL) tumors. Tumors do not develop in double mutant mice that lack both VHL and Hif-1α. **A–C:** Radiographs of postnatal day (p) 21 forelimbs in HIF1 (**A**), VHL-HIF1 (**B**), and VHL-HIF1-HIF2 (**C**) mice. **D:** Percentage of VHL, VHL-HIF2, VHL-HIF1, and VHL-HIF1-HIF2 mice presenting tumors detectable by radiography. Scale bar = 0.5 cm (**A–C**).

Notably, whereas tumors in VHL mice had been observed both in proximity to the scapula and at the elbow site with an overall distribution of 55% and 45%, respectively, the totality of the VHL-HIF2 masses was detected exclusively in the scapular region. The biological relevance of this finding is elusive at this stage,

but it may be associated with a specific gene expression profile along the proximal/distal axis of the developing limb.[44]

All in all, these data indicate that Epas1 is not absolutely necessary for the development of the tumors observed in VHL mutant mice. However, loss of Epas1 considerably reduced the onset of the tumors in VHL mice. Moreover, Epas1 is also partially responsible for the fibrosis of their synovial joints as its absence ameliorated the fibrotic infiltration of synovial joints observed in VHL mice.

Development of Soft Tissue Tumors in VHL Mutant Mice Is Hif-1α–Dependent

Since Epas1 is not absolutely necessary for the onset of the mesenchymal tumors present in VHL mutant mice, we next studied the involvement of Hif-1α in this process. For this purpose, we generated *Prx1-Cre;Vhl$^{f/f}$;Hif1a$^{f/f}$* mice (VHL-HIF1), in which both Vhl and Hif-1α had been deleted in mesenchymal progenitor of the limb bud. These mice were born with the expected mendelian frequency, but, as with VHL mice, premature death occurred at approximately p28, and thus, we were not able to analyze and compare VHL and VHL-HIF1 mutant mice at time points later than p28.

VHL-HIF1 double mutant mice were phenotypically similar to mutant mice lacking only Hif-1α in limb bud mesenchyme[45] (HIF1) (Figure 8, A and B). Of note, VHL-HIF1 synovial joints were completely altered and characterized by severe disruption of the articular surfaces and partial fusion of bony epiphysis (Supplemental Figure S6, A, D, F, I, K, and N). Therefore, synovial fibrosis, fusion of small cartilaginous elements, and presence of ectopic cartilage and menisci could not be evaluated in these double mutant mice.

However, we were able to establish that VHL-HIF1 mice did not develop soft tissue tumors at least up to p21 to p28, as shown by X-rays (Figure 8, B and D).

To address any potential issue of redundancy and complementarity between Hif-1α and Epas1, VHL-HIF1-HIF2 triple mutant mice were generated. These mutant mice were born with the expected mendelian frequency, but they also died prematurely around p28. Notably, none of the VHL-HIF1-HIF2 triple mutant mice we studied showed any detectable sign of soft tissue tumors by X-rays (Figure 8, C and D). Similarly to HIF1 and VHL-HIF1 mice (Supplemental Figure S6, C, D, H, I, M and N), triple mutant mice also displayed extreme deformities of the limbs and fusion of bony elements; presence of synovial fibrosis and menisci could thus not be completely assessed (Supplemental Figure S6, A, E, F, J, K, and O).

Taken together, these findings indicate that Hif-1α has an essential and nonredundant role in the onset and development of soft tissue tumors in mice that lack Vhl in mesenchymal progenitors of the limb bud.

Development of Soft Tissue Tumors in VHL Mutant Mice Is Ctgf Dependent

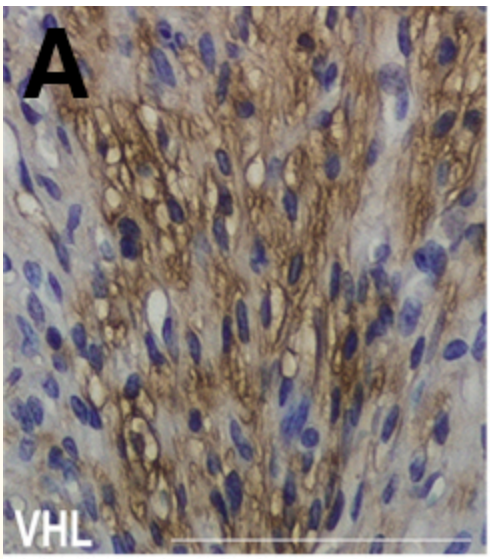
It has been reported that both loss of Vhl and stabilization of HIF cause fibrosis through a variety of molecular mechanisms, including up-regulation of profibrotic cytokines such as transforming growth factor-β1 and Ctgf.[46]

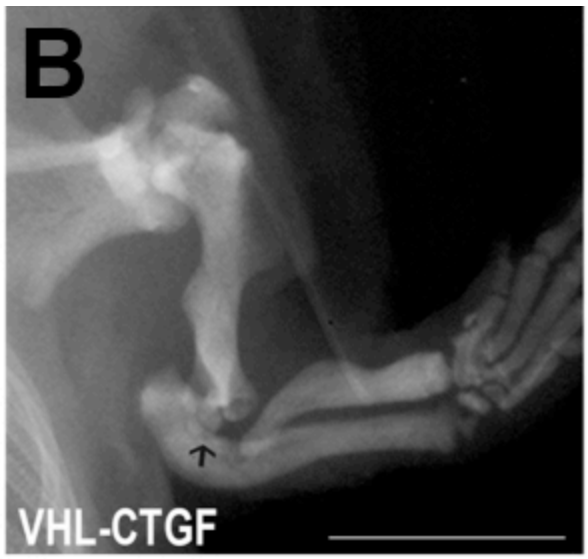
Ctgf (or Ccn2) is a matricellular protein involved in numerous biological processes, including cell proliferation, adhesion, chemotaxis, and extracellular matrix production.[46] Ctgf is also necessary for proper endochondral bone development.[47]

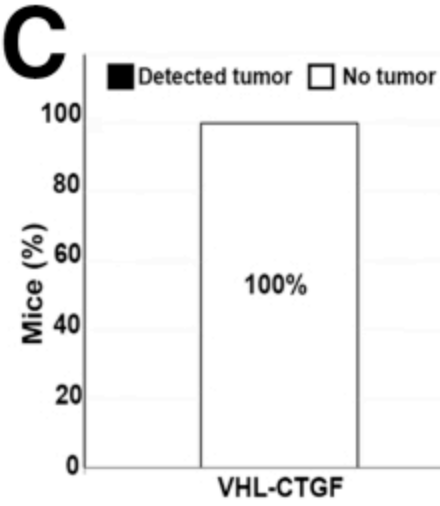
More importantly, Ctgf is hypoxia inducible in a Hif-1α–dependent manner,[46, 48] and the expression of Ctgf is up-regulated in a variety of fibrotic disorders.[49, 50]

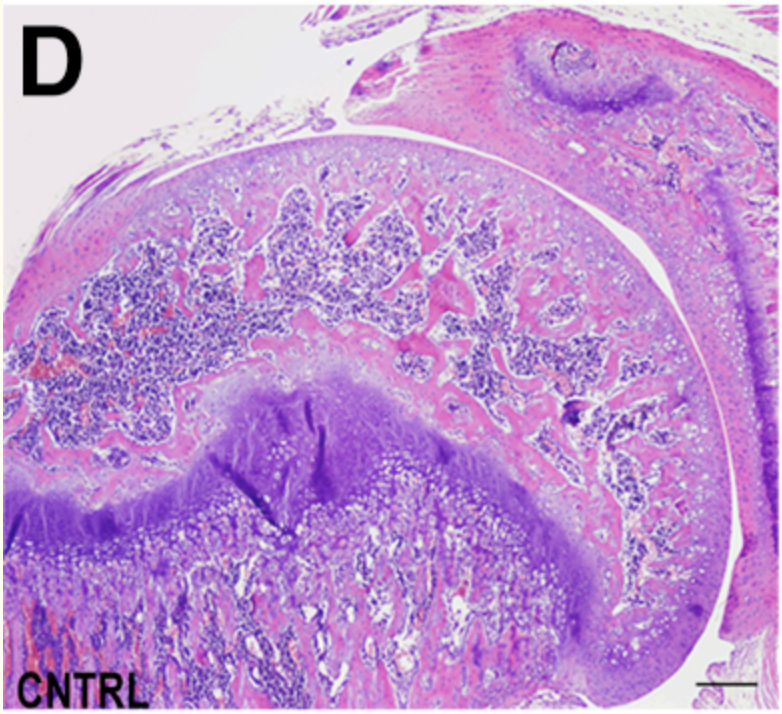
In light of these findings and in the attempt to understand why Hif-1α is necessary for the formation of mesenchymal tumors, we analyzed the role of Ctgf in the context of Vhl-deficient limb bud mesenchyme.

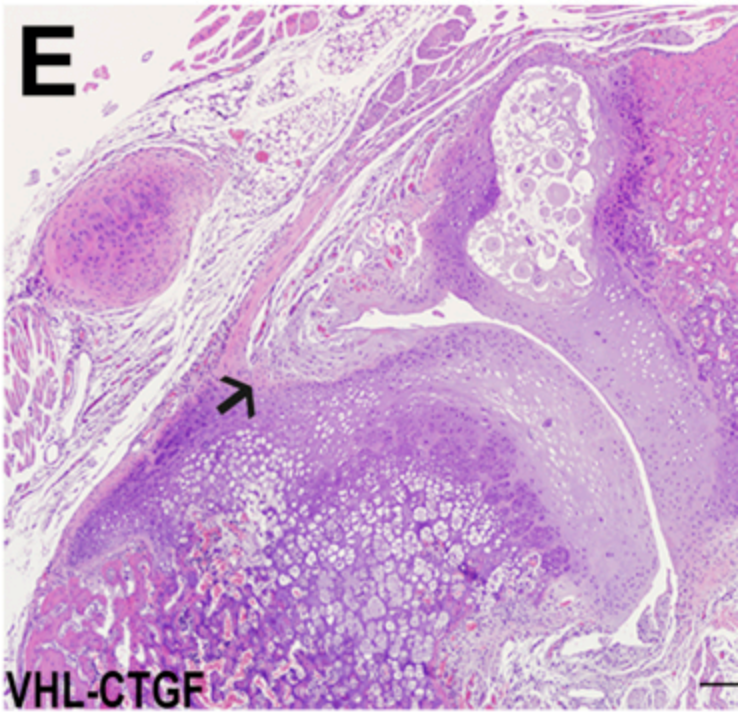
IHC confirmed high levels of expression of Ctgf in the VHL tumors (Figure 9A). We thus generated *Prx1-Cre;Vhl*$^{f/f}$*;Ctgf*$^{f/f}$ (VHL-CTGF) mice lacking both Vhl and Ctgf in limb bud mesenchyme.

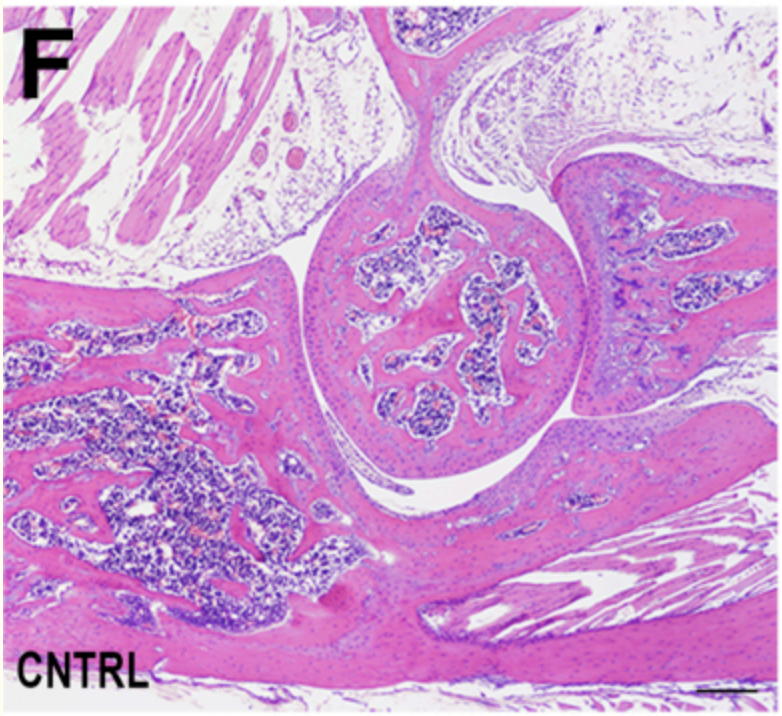

A
VHL


B
VHL-CTGF


C
Detected tumor
No tumor
100
80
60
40
20
0
Mice (%)
100%
VHL-CTGF


D
CNTRL


E
VHL-CTGF


F
CNTRL

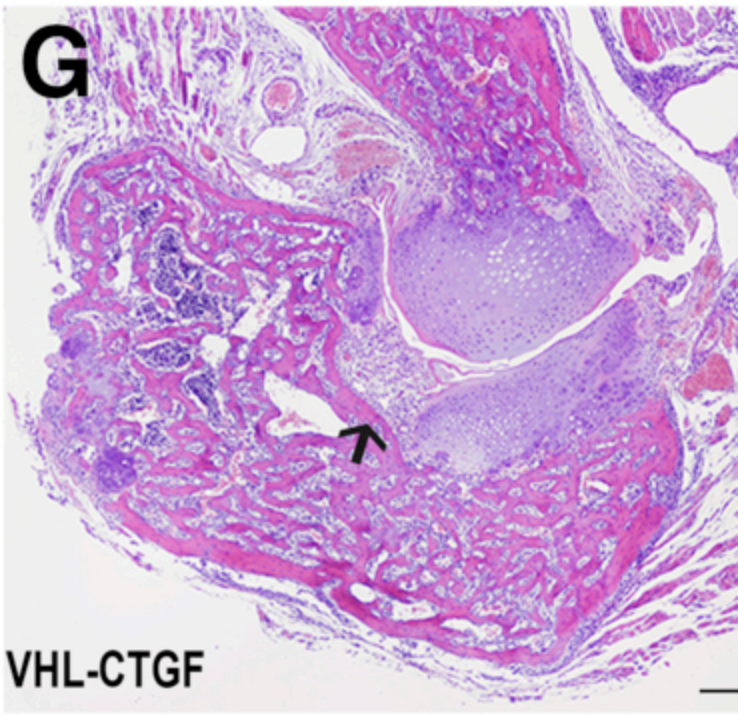

G
VHL-CTGF

Figure 9

Connective tissue growth factor (Ctgf) is necessary for the onset of von Hippel Lindau (VHL) tumors. Severity of the fibrosis markedly decreases and tumor lesions are not detectable anymore in double mutant mice that lack both VHL and Ctgf. **A:** Immunohistochemistry of Ctgf in a VHL tumor. **B:** Radiographs of a postnatal day (p) 28 VHL-CTGF forelimb. The **black arrow** indicates the initial erosion of the ulna. **C:** Percentage of VHL-CTGF mice presenting tumors detectable by radiography (n = 18). Hematoxylin and eosin staining of p28 shoulders (**D** and **E**), p28 elbows (**F** and **G**), and p28 knees (**H** and **I**) in control (CNTRL) (**D, F,** and **H**) and VHL-CTGF (**E, G,** and **I**) mice. **Black arrows** point to the fibrosis in the synovial space (**E**), infiltration of the fibrosis in the collapsing growth plate (**G**), and menisci (**I**). Scale bars: 75 μm (**A**); 0.5 cm (**B**); 100 μm (**D–I**).

VHL-CTGF double mutant mice had an overall postnatal survival similar to VHL single mutants. We studied these double mutants at p28 by radiography and routine histologic analysis. Radiography revealed that none of the VHL-CTGF mice had any evidence of the tumors found in the VHL single mutants (Figure 9, B and C). Fibrosis of the synovial joint was still present in both their forelimbs and hindlimbs, but it was considerably milder than in VHL single mutants. In particular, because it occurred in VHL single mutants, histologic analysis revealed that the fibrotic lesions in VHL-CTGF double mutant mice started around tendon/capsule insertions or in the proximity of the synovium (Figure 9, D and E), and they invaded the growth plate and the surrounding bony structures, causing erosions detectable by radiography (Figure 9, B, F, and G). This fibrotic process acquired a somehow more aggressive phenotype only in a small cohort of VHL-CTGF double mutant mice (n = 4 of 18), but it was never associated with the formation of discrete masses.

Of note, as opposed to the VHL mice, the presence of knee menisci was easily identifiable in VHL-CTGF double mutant mice, although their structure was altered by the fibrotic infiltration (Figure 9, H and I).

Taken together, these data indicate that loss of Ctgf ameliorated the fibrotic phenotype observed in VHL mutant mice and prevented the formation of mesenchymal tumors at least up to p28. These findings suggest that the aggressive fibrosis and the formation of discrete soft tissue tumors observed in VHL mutant mice is likely mediated by Hif-1α through up-regulation of Ctgf.

Discussion

This is the first study reporting the development of severe fibrosis of the synovial joints and Hif-1α− and Ctgf-dependent soft tissue tumors on loss of Vhl in mesenchymal progenitor cells.

VHL deficiency has been correlated with the development of proliferative lesions in various tissues and organs.[10, 11, 51, 52, 53, 54] In particular, heterozygous missense mutations of the *VHL* gene have been identified in the VHL syndrome.[9] This syndrome is characterized by a dominantly inherited tendency to develop pheochromocytoma, clear cell renal carcinoma, and hemangioblastomas in the central nervous system and retina.[9] Interestingly, to this end, an augmented predisposition to develop mesenchymal tumors of the soft tissue has not been reported in patients with the VHL syndrome. Our findings, however, suggest that activation of the hypoxia signaling pathway could indeed be an important pathogenetic event in the development of mesenchymal tumors of the soft tissue in humans. To test this possibility, further investigations are warranted.

Notably, loss of Vhl in chondrocytes or in cells of the osteoblast lineage did not cause any fibrosis or tumor of the soft tissue.[13, 14, 15] This finding indicates that the development of fibrosis of the synovial joints and the formation of soft tissue masses in mutant mice that lack Vhl in limb bud mesenchyme are secondary to loss of this protein in an early and unique mesenchymal population, rather than in chondrocytes, osteoblasts, or osteochondroprogenitors (Figure 10). Moreover, our findings of IHC, such as the positive staining results for cadherin-11, suggest that this unique subset of mesenchymal cells could be mainly contributed by the

progenitors of tendon, ligament, or synovium cells, although a role for the periendothelial cells cannot be excluded because they also express the *cre* transgene in Prx1-Cre mice.

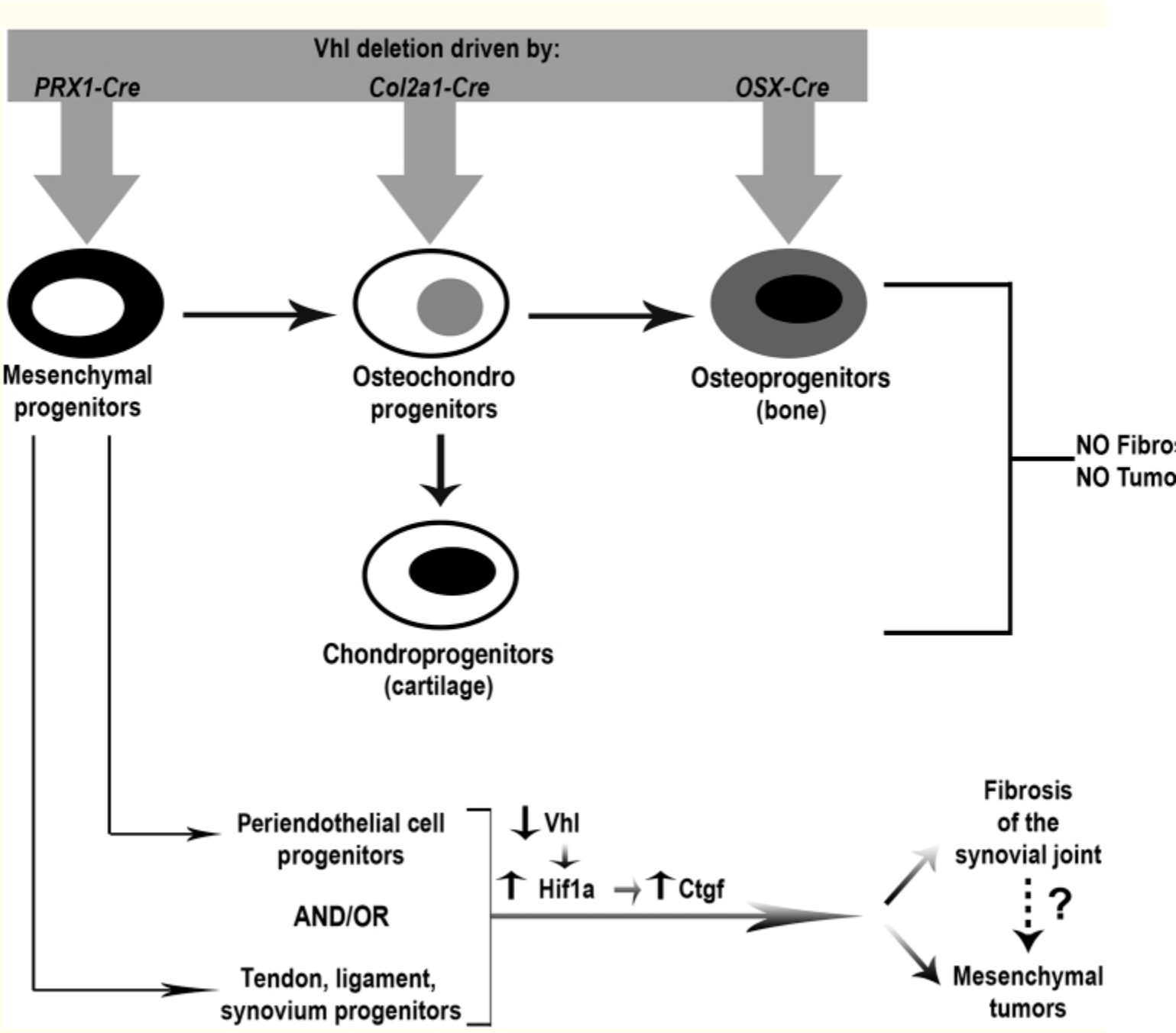


Figure 10

Up-regulation of Hypoxia-inducible factor (Hif)-1α (Hif-1α) and connective tissue growth factor (Ctgf) on loss of von Hippel Lindau (Vhl) in a subset of mesenchymal progenitors leads to fibrosis of the synovial joints and tumors of the soft tissue.

The tumors characterized in this study are often hypercellular, as indicated by histologic findings. Paradoxically, a significant impairment of proliferation of Vhl-deficient murine embryonic fibroblasts and of growth plate chondrocytes has been previously reported.[13, 55, 56, 57] It is thus possible that crosstalk between Vhl-deficient mesenchymal cells and the microenvironment may be critical for the onset and development of the masses observed in VHL mutant mice. Cells in which Hif-1α and Epas1 are stabilized through the loss of Vhl could represent a specific niche for additional cell types that may contribute, with yet unknown mechanisms, to the development of the soft tissue masses observed in VHL mutant mice. Along these lines,

it is intriguing that multinucleated cells with intense eosinophilic cytoplasm are a consistent histologic feature of the masses we report in this study. The biological relevance of this finding requires further evaluation. In any event, multinucleated cells are often present in malignant mesenchymal tumors of the soft tissue.[58]

A specific role of Epas1 in the development of renal fibrosis on loss of Vhl has been previously suggested.[59] In our genetic model, loss of Epas1 partially ameliorates the massive fibrosis of the synovial space in VHL mutant mice. Our data suggest that Epas1 overexpression in mesenchymal progenitors, in association with Hif-1α stabilization, may lead to fibrotic degeneration.

Moreover, our study indicates that Epas1 is not absolutely necessary for the development of soft tissue tumors on deletion of VHL in limb bud mesenchyme. However, loss of Epas1 considerably reduced the penetrance of the VHL tumors; thus, Epas1, in association with Hif-1α, may still play a role in the onset of these mesenchymal tumors.

It has been reported that both loss of Vhl and stabilization of HIF cause fibrosis through distinct mechanisms.[60, 61, 62, 63, 64] Along these lines, up-regulation of profibrotic cytokines, such as Tgfb1 and Ctgf,[46] of inhibitors of metalloproteineases,[46, 65] and of enzymes involved in postranslational modifications of collagens, such as lysyl oxidase and collagen prolyl-4-hydroxylases,[46, 66, 67] have been reported to have an important function in mediating Vhl- or Hif-dependent tissue fibrosis. Notably, Ctgf plays profound roles in malignant tumors in major organs and tissues by modulating the actions of key molecules involved in tumorigenesis.[68] Our study suggests that Hif-1α–dependent up-regulation of Ctgf is the critical event that leads to the formation of fibrosis and soft tissue tumors in the specific developmental setting of synovial joints on loss of Vhl.[9]

Acknowledgments

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Footnotes

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L.M. and C.M. contributed equally to this work.

Disclosures: None declared.

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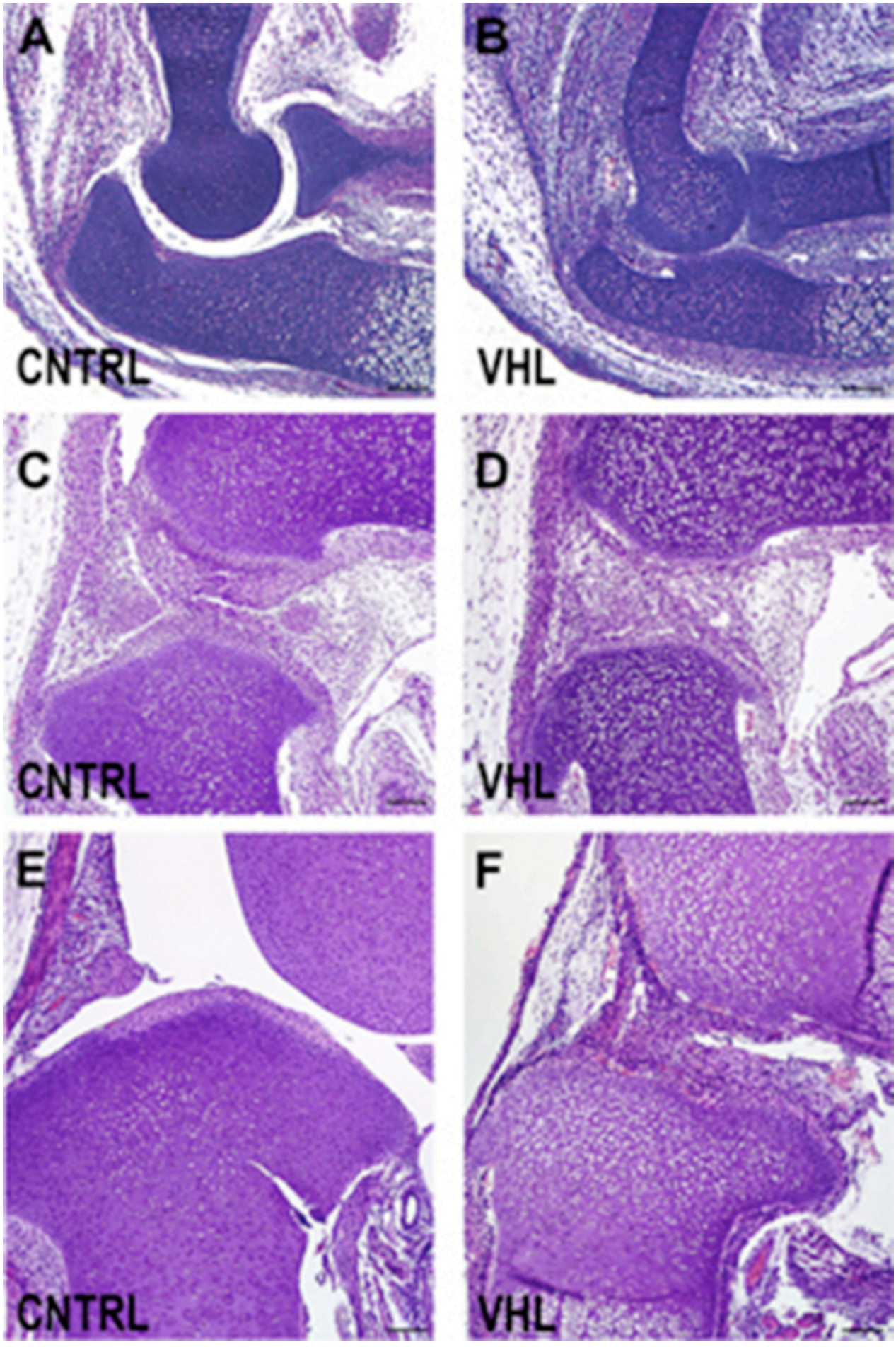
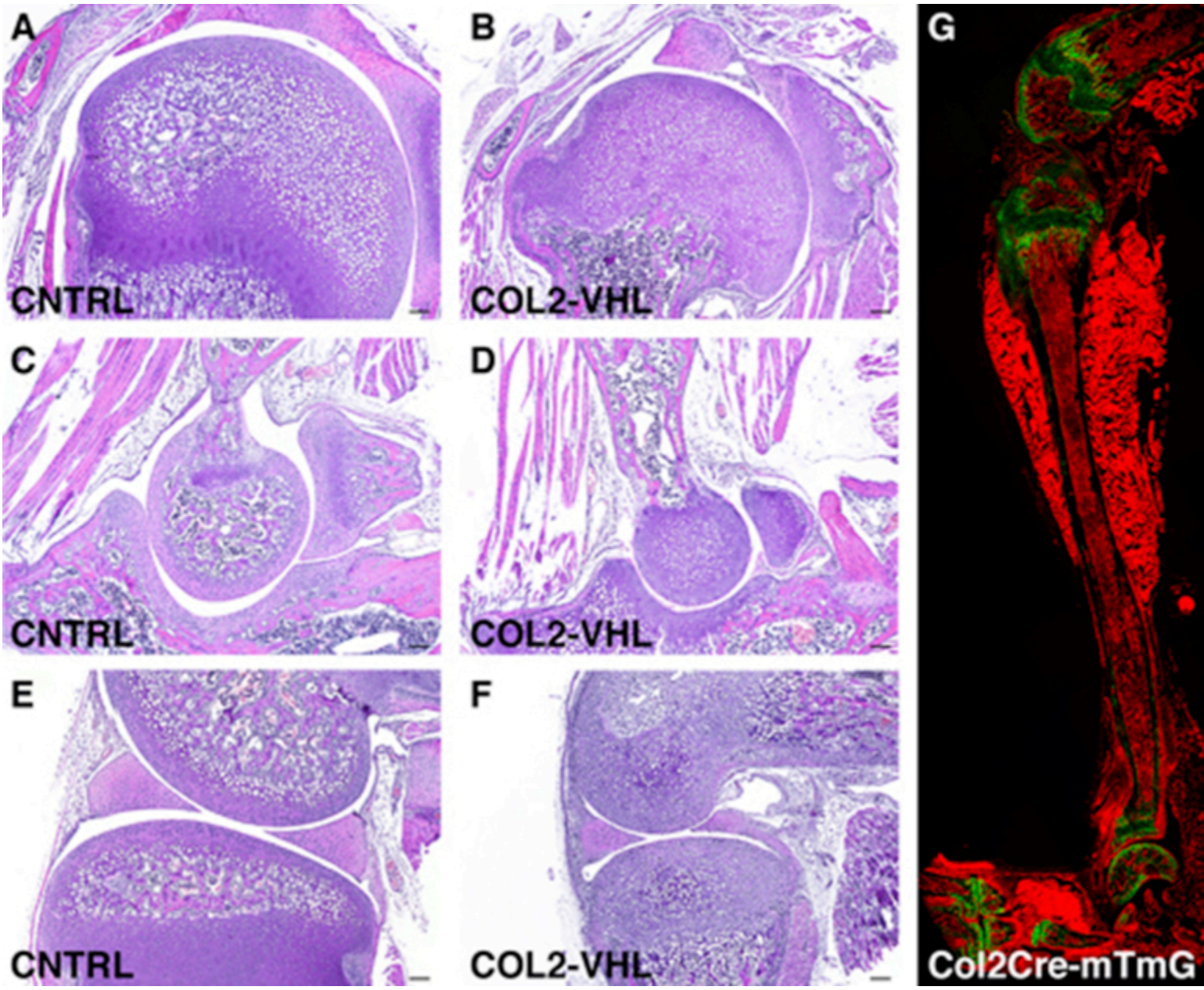
Supplemental Data

Supplemental Figure S1


A
CNTRL
B
VHL
C
CNTRL
D
VHL
E
CNTRL
F
VHL

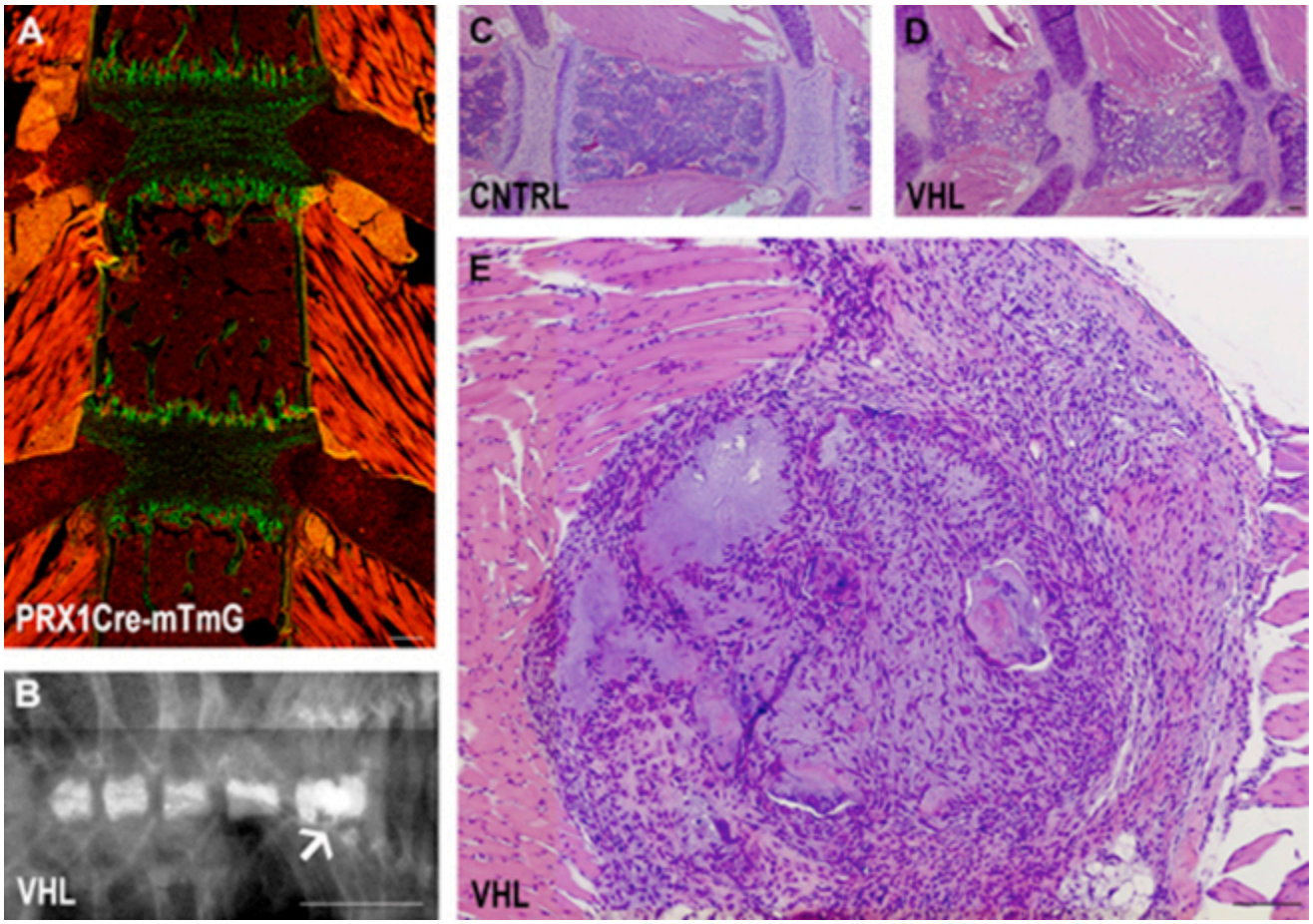
Synovial joint development in absence of von Hippel Lindau (Vhl) in limb bud mesenchyme. Synovial space in mutant mice that lack Vhl appears to be partially filled with fibrotic tissue as early as embryonic stage E15.5. Hematoxylin and eosin staining of E15.5 elbows (**A** and **B**), embryonic stage E17.5 knees (**C** and **D**), and newborn knees (**E** and **F**) in control (CNTRL) (**A, C,** and **E**) and VHL (**B, D,** and **F**) mice. Scale bar = 100 μm.

Supplemental Figure S2



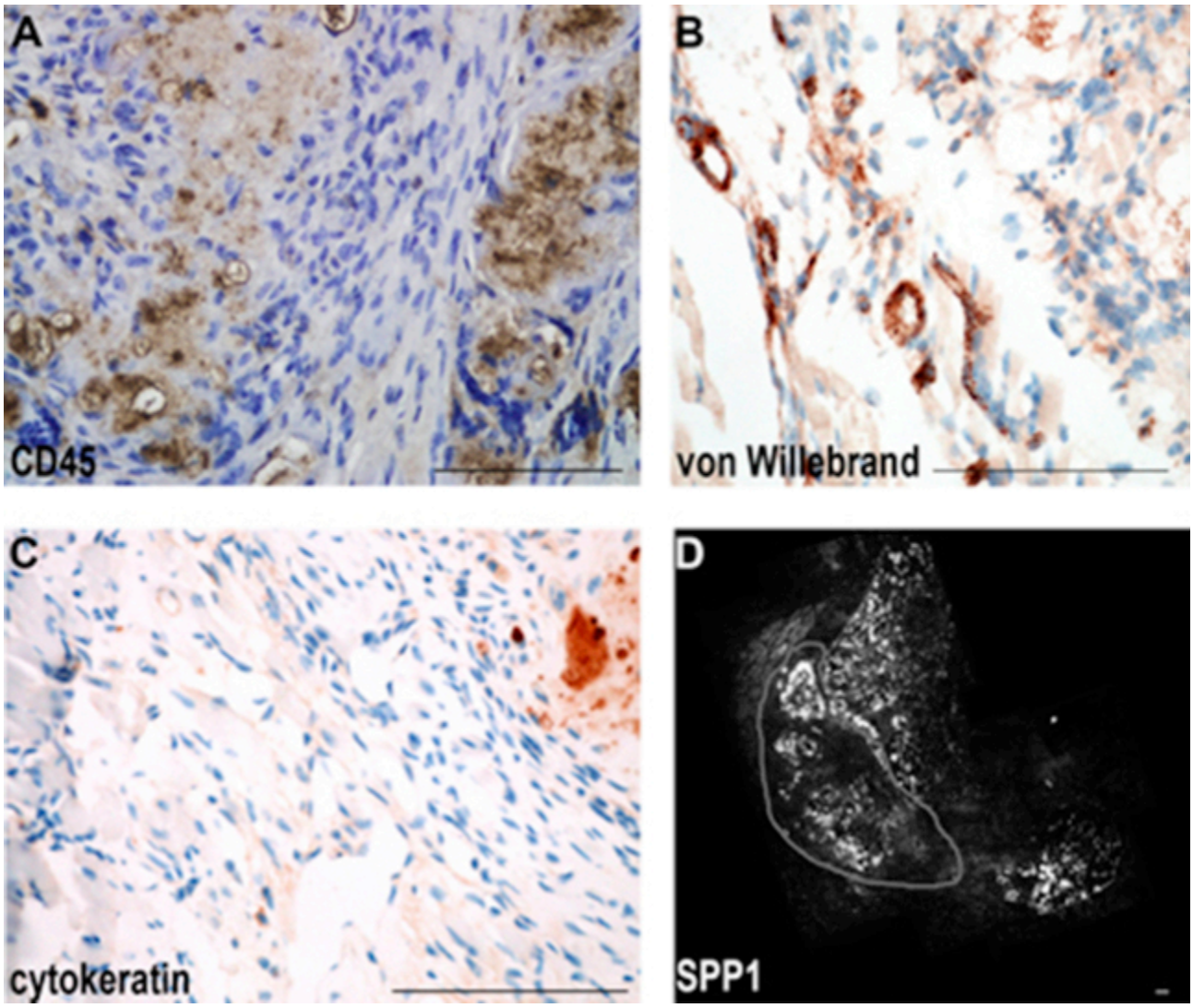
Cre activity in postnatal hindlimbs of COL2-Cre mice. Fibrosis of the synovial joints does not occur in mutant mice in which von Hippel Lindau (Vhl) has been deleted exclusively in cells committed to the chondrocyte lineage. Hematoxylin and eosin staining of postnatal day (p) 14 shoulders (**A** and **B**), elbows (**C** and **D**), and knees (**E** and **F**) in control (CNTRL) (**A**, **C**, and **E**) and COL2-VHL (**B**, **D**, and **F**) mice. **G:** Detection of fluorescence in fresh frozen sections of tibias isolated from p21 COL2Cre-mTmG mice. COL2Cre-mTmG reporter mice have a strong enhanced green fluorescent protein signal in the growth plate and trabecular bone, whereas very low levels of green fluorescence can be detected in synovium, tendon, and ligaments. Scale bar = 100 μm.

Supplemental Figure S3



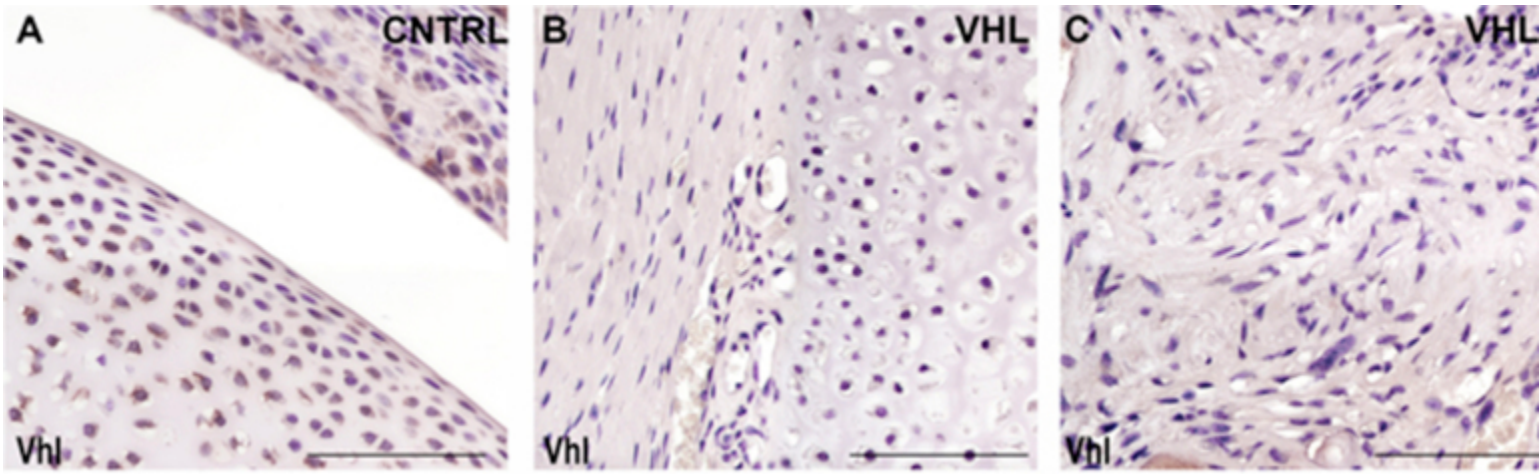
von Hippel Lindau (VHL) tumor development in the manubrium sterni. Tumors sporadically form in the sternum of mutant mice that lack Vhl. Of note, as with long bones of hindlimbs and forelimbs, the sternum originates from the lateral plate mesoderm, as confirmed by *cre* activity at this site. **A:** Detection of fluorescence in a frozen section of sternum isolated from postnatal day (p) 14 PRX1Cre-mTmG mice. **B:** Radiographs of p21 sternum in VHL mice. The **arrow** highlights the lesion close to the manubrium sterni. **C** and **D:** Hematoxylin and eosin (H&E) staining of p24 sternebra in control (CNTRL) (**C**) and VHL (**D**) mice. **E:** H&E staining of a p24 VHL tumor in proximity to the sternum. Scale bars: 100 μm (**A, C–E**); 0.25 cm (**B**).

Supplemental Figure S4



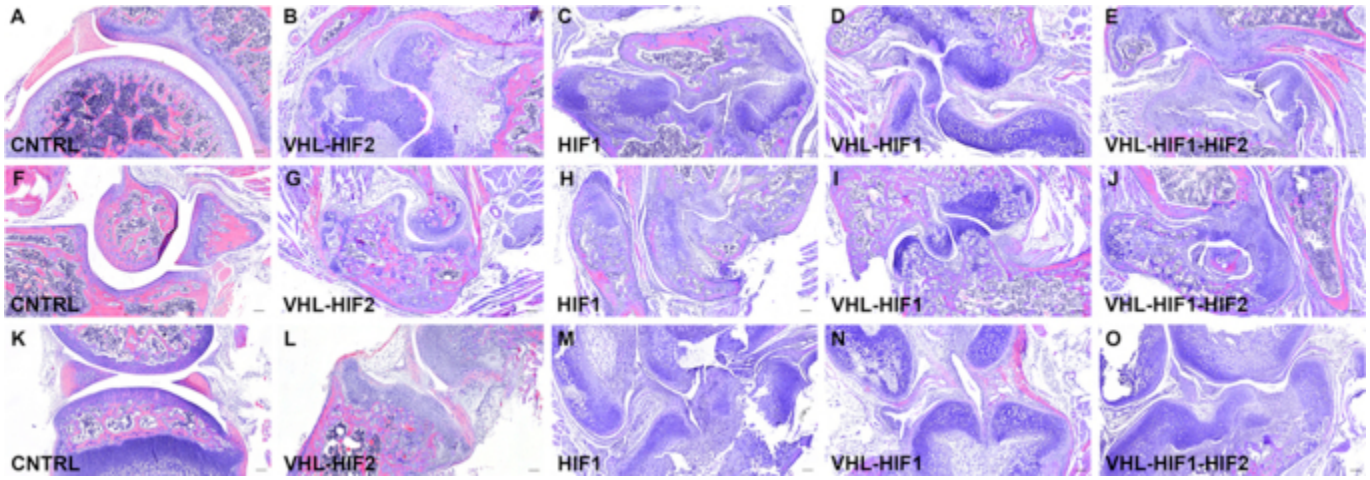
Phenotypical characterization of von Hippel Lindau (VHL) tumor cells. **A–C:** Immunohistochemistry of CD45 (**A**), von Willebrand factor (**B**), and cytokeratin (**C**) in VHL tumors. Spindyloid cells within the tumor are negative for CD45, von Willebrand factor, and cytokeratin, which indicates that these cells do not belong to the hematopoietic, endothelial, or epithelial lineage. **D:***In situ* hybridization for detection of phosphoprotein 1 (SPP1) mRNA in a VHL tumor. The gray contours define the tumor. Note the positive signal also in VHL bone. SPP1 is involved in numerous pathologic processes, and it is a marker of highly hypoxic tumors. Scale bar = 100 μm.

Supplemental Figure S5



von Hippel Lindau (Vhl) expression is absent in VHL tumors. **A–C:** Immunohistochemistry of Vhl in control (CNTRL) (**A**) and VHL (**B**) growth plates. IHC of Vhl in a VHL tumor (**C**). Spindyloid cells within the tumor lesion lack Vhl expression. Scale bar = 100 μm.

Supplemental Figure S6



Joint phenotype in the absence of von Hippel Lindau (Vhl), hypoxia-inducible factor (Hif)-1α (Hif-1α), or HIF-2α (Epas1) in limb bud mesenchyme. Fibrosis is ameliorated in the joints of mutant mice that lack both Vhl and Epas1. Synovial fibrosis, fusion of small cartilaginous elements, presence of ectopic cartilage, and menisci could not be evaluated in double and triple mutant mice that lack both Vhl and Hif-1α or Vhl and Hif-1α and Epas1, respectively, because of the severe deformities of the mutant bones and the partial fusion of the epiphyses. Hematoxylin and eosin staining of postnatal day 21 shoulders (**A–E**), elbows (**F–J**), and knees (**K–O**) in CNTRL (**A**, **F**, and **K**), VHL-HIF2 (**B**, **G**, and **L**), HIF1 (**C**, **H**, and **M**), VHL-HIF1 (**D**, **I**, and **N**), and VHL-HIF1-HIF2 (**E**, **J**, and **O**) mice. Scale bar = 100 μm.