Characterization of the Myc collaborating oncogenes Bmi1 and Gfi1

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Chapter 7

Enforced expression of Gfi1 alters craniofacial and tooth morphogenesis and induces osteoblastic neural crest cell tumors

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Enforced expression of Gfi1 alters craniofacial and tooth morphogenesis and induces osteoblastic neural crest cell tumors

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Gfi1 is a zinc-finger protein that acts as a transcriptional repressor due to a N-terminal SNAG-domain also present in Snail family of transcription factors, which mediate differentiation of epithelial cells to mesenchymal cells during embryonic development. Here we report that transgenic gfi1 expression interferes with proper intramembranous ossification, which results in hypoplastic calvarial bones and wide cranial sutures at birth, mimicking some defects seen in cleidocranial dysplasia. Defective ossification of frontal and squamosal bones correlates with the development of facial anomalies. Cephalic neural crest-derived alveolar bone and tooth morphogenesis is disturbed. From the age of 3 months, gfi1 transgenic mice start to develop osteoblastic neural crest cell tumors at the site of the mandible only. The incidence of these intermediate-grade fibroblastic osteosarcomas is increased in the absence of one functional Nf2 allele, mostly not associated with loss of Nf2 wild type allele. These data indicate that the zinc finger protein Gfi1 is able to regulate osteoblast and odontoblast differentiation.

[Key words: NF2, dentin, cleidocranial dysplasia, intermediate-grade osteosarcomas; craniofacial development]

The craniofacial skeleton follows a unique developmental pattern (Schilling 1997). Some cranial bones (supraoccipital, basioccipital, and exoccipital) are derived from cephalic mesoderm. However, the majority of skeletal elements in the head region originate from neural crest-derived ectomesenchyme, which participate in the formation of the maxillo-mandibular elements as well as the frontonasal mass, consisting of frontal, nasal, parietal and squamosal bones (Couly et al. 1993). Two mechanisms of bone formation have classically been distinguished, namely intramembranous and endochondral ossification (Hall and Miyake 2000; Olsen et al. 2000). The essential difference between them is the presence of an intermediate cartilaginous phase during endochondral ossification. Except for the supraoccipital bone, the majority of the calvarial bones form by intramembranous ossification. Intramembranous ossification occurs when mesenchymal precursor cells proliferate and subsequently differentiate directly into osteoblasts that mineralize immature bone tissue, which is progressively remodeled to mature lamellar bone.

The composite structure of the mammalian skull requires precise pre- and post-natal growth regulation of the individual calvarial elements. Disturbances of this process cause severe clinical manifestations in humans. Craniosynostosis, the premature fusion of one or more calvarial bones leading to skull deformity, is associated with gain-of-function mutations in the homeodomain of MSX2 (Jabs et al. 1993; Ma et al. 1996). Several results support the model in which Msx2 inhibits osteoblast differentiation and stimulates proliferation of cells at the extreme ends of the osteogenic fronts of the calvariae, facilitating closure of the sutures (Liu et al. 1995; Liu et al. 1999). Also mutations in the genes encoding
fibroblast growth factor receptors (FGFR)-1, -2 and -3, generating ligand-independent constitutive activation of the receptors, as well as the basic helix-loop-helix transcription factor TWIST, are known to cause craniostenosis (Muenke et al. 1994; Bellus et al. 1996; el Ghouzzi et al. 1997; Howard et al. 1997; Paznekas et al. 1998). *Twist* heterozygous mice exhibit limb and calvarial phenotypes reminiscent of the Saethre-Chotzen syndrome caused by *TWIST* mutations (Bourgeois et al. 1998).

On the other hand, there is an autosomal-dominant syndrome in humans and mice called cleidocranial dysplasia (CCD), characterized by hypoplastic clavicles and calvarial bones, delayed closure of the cranial fontanelles, dental anomalies and short stature (Mundlos et al. 1995; Mundlos 1999). Cbfal (AML3/PEBP2aA/RUNX2) is found heterozygously mutated in several CCD patients, and the γ-radiation-induced Ccd mouse mutant results from a deletion of one of the alleles of *Cbfal* (Sillence et al. 1987; Otto et al. 1997). The function of transcriptional activator CBFAl during skeletal development was further elucidated by the generation of Cbfal knockout mice, where loss of both alleles leads to a complete absence of bone owing to a lack of osteoblast differentiation (Komori et al. 1997; Otto et al. 1997).

Besides giving rise to skeletogenic cells, ectomesenchyme cells from midbrain and rostral hindbrain will occupy the first branchial arch, and will produce the dental papilla structure. From this site odontogenic cells (odontoblasts, pulp cells) required for tooth development, as well as periodontal mesenchyme (cementoblasts, fibroblasts, osteoblasts) will arise (Ruch et al. 1995). The oral ectoderm produces the enamel organ from which ameloblasts will emerge. The dental basement membrane connects the developing enamel organ and dental papilla, and is involved in the critical and essential epithelial-mesenchymal interactions necessary for the functional differentiation of odontoblasts. Tooth development is regulated through reciprocal signaling by members of the fibroblast growth factor (FGF) family and transforming growth factor β (TGFβ) superfamily inducing expression of paired box- and homeodomain-containing transcription factors (Peters and Balling 1999).

The zinc-finger protein Gfi1 and its close homologue Gfi1B have been implicated in regulating myeloid and T cell development (Schmidt et al. 1998; Tong et al. 1998). The mouse gfi1 gene was identified as a common insertion site for Moloney murine leukemia virus (MoMLV)-induced lymphomas in myc and pim transgenic mice (van Lohuizen et al. 1991; Zörnig et al. 1996; Scheijen et al. 1997). Here we report that enforced expression of gfi1 induces multiple craniofacial abnormalities, which are linked to abnormal morphogenesis and result from defective cranial neural crest osteoblast and odontoblast differentiation. These data provide for the first time indications that the transcriptional repressor Gfi1 may regulate osteo- and odontogenesis, and can act as an oncogene outside the hematopoietic compartment.

### Results

**Gfi1 inhibits intramembranous ossification and induces craniofacial deformations**

To assess the role of gfi1 in lymphoid development and tumorigenesis, we previously generated Eu-pp-gfi1 mice and found that Gfi1 facilitates T cell selection and maturation, inhibits different modes of apoptosis, and predisposes mice to T lymphoblastic lymphomas and leukemia (Scheijen et al. submitted). Additionally, transgenic gfi1 expression expands the pool of granulocytic precursor cells in bone marrow and induces a CML-like disease (Scheijen and Berns, submitted). The presence of the *pim1* promoter (pp) in the transgenic construct not only allows expression in the hematopoietic compartment but also induces broad transgene expression during embryonic development, as has been confirmed by RNA in situ hybridization in Eu-pp-bmi1 transgenic mice (Alkema et al. 1995).

Two independent transgenic founder-lines (GFI37 and GFI39) had been obtained, which displayed comparable gfi1 transgenic expression levels and phenotypes in hematopoietic tissues (Scheijen et al., submitted). Interestingly, we observed that a fraction of the transgenic progeny displayed additional defects, which became apparent around the age of 3 weeks and were similar in both founder-lines. These consisted of short stature and craniofacial malformations, including ocular microsomia and asymmetric frontonasal dysostosis, which were evident on C57BL/6 as well as FVB background (Fig. 1A and 1B). The penetrance of this skeletal phenotype in transgenic progeny of both Eu-pp-gfi1 founder-lines was much
Craniofacial abnormalities in gfil transgenic mice

Figure 1. Craniofacial defects in gfil transgenic mice. (A) Fraction of adult C57BL/6 Ep-pp-gfil mice display specific craniofacial deformations, including ocular microsomia and asymmetric frontonasal dysostosis. (B) Similar phenotype of Ep-pp-gfil mice as described in (A) on FVB background. (C) - (F) Skeletal stainings of newborn wild type and Ep-pp-gfil mice, using Alcian Blue to stain cartilage and Alizarin Red for bone. (C) and (D) Dorsal view of the skull, showing diminished size and mineralization of calvarial bones, with open fontanelles in Ep-pp-gfil mice. (E) and (F) Lateral view on the skull demonstrates reduced size of the mandible, including alveolar ridge and condylar process, and hypoplastic squamosal bone in Ep-pp-gfil mice. Supraoccipital and exoccipital bones show comparable size and morphology in wild type and gfil transgenic animals. A, angular process; AR, alveolar ridge; CO, condylar process; E, exoccipital bone; F, frontal bone; I, interparietal bone; M, mandible; N, nasal bone; P, parietal bone; S, supraoccipital bone; SQ, squamosal bone.

stronger if the transgene had been inherited from the mother instead of the father. On average 70-80% of the transgenic progeny of a female Ep-pp-gfil parent and 5-10% of a male Ep-pp-gfil parent showed obvious skeletal defects.

The asymmetric anomalies observed in facial morphology prompted us to look in more detail at craniofacial development in Ep-pp-gfil mice. Therefore, skeletons were prepared from newborn wild type and Ep-pp-gfil transgenic mice of both founder-lines (GF137 and GF139), and stained with Alcian Blue and Alizarin Red dyes, which detect cartilage and bone tissue, respectively. Although there were individual variations, the most prominent defects observed at birth were in the skull, with hypoplasia and aplasia of the following calvarial membranous bones: frontal, parietal, interparietal, and squamosal bones (Fig. 1C-1F). The delayed ossification of the cranial bones resulted in an open anterior and posterior fontanelle, as well as wide cranial sutures. The frontal, parietal, and interparietal bones were significantly reduced in size, whereas the supraoccipital bone was almost normal.

Several defects were also observed in mandibles from Ep-pp-gfil mice. The coronoid, condylar, and angular processes of the mandible were hypoplastic, and the alveolar ridge was less pronounced compared to wild type control (Fig. 1E-1F). Structures of the middle ear like malleus, stapes, incus, as well as tympanic and exoccipital bones were not affected. Furthermore, at this age we found no evidence for axial skeletal transformation or major defects in bones that ossify through endochondral ossification in the appendicular skeleton. Thus the abnormalities evident in gfil transgenic mice concern diminished ossification of the craniofacial bones derived from posterior midbrain-derived neural crest cells.
Altered tooth and alveolar bone morphogenesis in gfi1 transgenic mice

Besides giving rise to craniofacial bone structures, the ectomesenchymal neural crest cells also form the odontogenic cells. Therefore, we analyzed tooth morphology and development in gfi1 transgenic mice. The hard portions of a tooth consist of three different tissues: dentin, enamel, and cementum. The bulk of the tooth is made up of dentin, which surrounds the pulp chamber. In the crown a layer of enamel covers dentin, whereas on the root there is a thin layer of cementum. The periodontal ligament binds the cementum-covered surface of the root to the alveolar bone. The dentin-producing odontoblasts are postmitotic polarized cells that form an epithelial layer around the periphery of the pulp cavity immediately beneath the inner surface of the dentin, extending one or more cytoplasmic processes into the pre-dentin and dentin.

Detailed analysis has indicated that cranial neural crest cells contribute to the formation of condensed dental mesenchyme, (pre-)odontoblasts, dentin matrix, cementum and periodontal ligaments, but not (pre-)ameloblasts and enamel (Chai et al. 2000).

Initial histological analysis indicated that enamel structure and cellular ameloblast morphology showed no differences between wild type and gfi1 transgenic mice (data not shown). However, the cellular structure of odontoblasts and dentin matrix of molars and incisors in mandible and maxilla was clearly different in mice transgenic for gfi1. Apical structures were most severely affected, but overall odontoblasts were not correctly arranged as a uniform epithelial layer of cells (Fig. 2). The production of predentin and dentin was diminished or absent, with frequent occlusions of odontoblasts into the pre-dentin (Fig. 2A and 2B) and dentin matrices (data not shown). These findings could either relate to a cell autonomous defect on the odontoblasts or...
im proper signaling during odontogenesis between ectodermal ameloblasts and neural crest-derived odontoblasts. Due to the hypoplastic dentin matrices, teeth of Eμ-pp-gfil mice were highly susceptible to mechanical damage as well as bacterial infections, with subsequent inflammatory response (Fig. 2C and 2D).

We also noted that the trabecular bone structure of alveolar bone in mandible and maxilla of Eμ-pp-gfil mice was clearly distinct from wild types. Whereas in wild type jaws alveolar bone was densely packed, showing more mineralized cancellous bone, this was clearly diminished in gfil transgenic animals. Instead more irregular hypercellularity of fibroblasts and some osteoblasts was observed, with only focal areas of alveolar bone (Fig. 2E and 2F). In addition, we found in one Eμ-pp-gfil mouse evidence of a preneoplastic lesion (PNL) within the alveolar bone at the age of 5 weeks (Fig. 2D). These results show that terminal differentiation of neural crest-derived odontoblasts as well as osteoblasts is disturbed in gfil transgenic mice, characterized by improper cellular organization and hypoplastic (pre-)dentin and alveolar bone structure.

Eμ-pp-gfil mice develop intermediate-grade mandibular osteosarcomas

Recently, we described that mice transgenic for the proto-oncogene gfil are predisposed to hematopoietic tumors, including thymic lymphomas, acute lymphoblastic T cell leukemia and CML-like disease (Scheijen and Berns, submitted). However it became apparent that from the age of 3 months, Eμ-pp-gfil mice of both founder-lines (GFI37 and GFI39) developed an additional tumor-type, which affected 30-40% of the gfil transgenic animals over a period of one year. The tumors were specifically localized at the site of the mandible and grew as a dominant tumor-mass protruding.
Mandibular osteosarcomas

All tumors were firm, larger than 1 cm in diameter and often grew out to a size of 3 to 4 cm. Histological analysis showed that the tumors enclosed the molar teeth of the lower jaw (Fig. 3B and 3C). The periodontal ligament was often still present (Fig. 3C), but normal alveolar bone was completely replaced by tumor cells that invaded and partially destroyed the cortical bone of the mandible (Fig. 3D). The tumors were diagnosed as either osteoblastoma-like osteosarcoma with patterns of osteoid deposition with prominent rimming polymorphic osteoblasts (Fig. 3E), or fibroblastic osteosarcomas with predominantly spindle-shaped neoplastic cells (Fig. 3D and 3F).

To confirm that the observed mandibular tumors comprised of osteogenic tumor cells, \( \alpha(1)\text{collagen} \) and osteonectin mRNA expression levels were determined in 9 independent tumors. Indeed, all tumors turned out to be positive for both osteoblastic markers (Fig. 4A) (Ali et al. 1993). In addition, we performed Northern blotting to assess whether the osteosarcomas arose through a cell autonomous defect resulting from transgenic \( \text{gfi1} \) expression. Mandibular osteosarcomas were compared to \( \text{E}_{\mu}-\text{pp-gfi1} \)-induced acute T lymphoblastic lymphoma (T-ALL), with regard to \( \text{gfi1} \) and \( \beta\text{-actin} \) mRNA levels. Although the osteosarcomas expressed the expected 2.4kb \( \text{gfi1} \) transcript to a similar extent as the T cell lymphoma, we observed an additional smaller transcript of 1.7kb specifically in the osteosarcomas (Fig. 4B). Each transcript was also detected with a transgene specific \( U3\text{LTR} \) probe (data not shown), implying that both transcripts were derived from the \( \text{E}_{\mu}-\text{pp-gfi1} \) transgene. Western blot analysis, using a polyclonal antibody raised against the carboxy-terminus of Gfi1, showed only the normal 57-60kD post-translationally modified Gfi1 protein and revealed no additional smaller band(s) (Fig. 4C; data not shown). Also electrophoretic mobility shift assay (EMSA) on total cell extracts of Gfi1-induced osteosarcomas did not reveal any smaller sized fragment(s) binding the optimal consensus site for Gfi1. These data demonstrate that overexpression of Gfi1 induces frequently medium-grade craniofacial osteosarcomas only located in the lower jaw in \( \text{E}_{\mu}-\text{pp-gfi1} \) mice.

Haploinsufficiency for \( \text{Nf2} \) accelerates the onset and frequency of neural crest-derived osteosarcomas in \( \text{gfi1} \) transgenic mice

The specific location of the osteosarcomas in \( \text{E}_{\mu}-\text{pp-gfi1} \) transgenic mice, suggested that neural crest-derived osteoblastic cells in the mandible were targets for Gfi1-mediated oncogenic transformation. Comparable neural crest cell-derived osteosarcomas and benign osteomas have been described in \( \text{Nf2}^{-/-} \) mice, where predominantly osteogenic tumors arise in
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50-60% of animals older than one year (McClatchey et al. 1998), of which a small fraction is neural crest cell derived (Giovannini et al. 2000). Neurofibromatosis 2 (NF2) in humans is associated with a strong predisposition to the formation of schwannomas and meningiomas (Martuza and Eldridge 1988). This disease can only be recapitulated in mice where NF2 is bi-allelic site-specifically inactivated by P0 promoter-driven Cre-mediated recombination in myelinating Schwann cells (Giovannini et al. 2000). The NF2 tumor suppressor gene encodes an cytoskeletal-associated protein, termed merlin or schwannomin, which shows high structural similarity with the ERM proteins, ezrin, radixin, and moesin (Gusella et al. 1999).

To determine whether loss of NF2 function was a rate-limiting step in the onset of gfi1-induced mandibular osteosarcomas, NF2 heterozygous mutant (FVB x 129/OLA) mice were crossed with FVB Eu-pp-gfi1 transgenic animals of founder-line GFI39. F1, (129/OLA x FVB) Eu-pp-gfi1, Eu-pp-gfi1/NF2** and NF2** mice were followed in time for tumor development. After an observation period of 10 months it became apparent that the incidence of gfi1-induced mandibular osteosarcomas was increased in NF2** background (Fig. 5A). GFI39 mice on mixed (129/OLA x FVB) background had a similar tumor profile and latency as on an inbred FVB background. Whereas one third of the Eu-pp-gfi1 animals was still disease free after 10 months, 33% had developed hematopoietic tumors (lymphoblastic T cell lymphomas and CML-like disease), and another 33% mandibular osteosarcomas. In contrast, 63% of Eu-pp-
gfil/Nf2<sup>−/−</sup> mice succumbed to neural crest cell-derived osteosarcomas during the same observation period (Fig. 5B). In addition, there was a small but significant decrease in latency of Gfil-induced mandibular osteosarcomas in the absence of one functional Nf2 allele (Fig. 5B and 5C). Although all tumors still arose exclusively at the site of the mandible, we noticed that 4 of the 17 Eu-pp-gfil/Nf2<sup>−/−</sup> mice developed bi-lateral osteosarcomas, whereas this occurred only once out of 40 cases of osteosarcomas in GFI39 mice on FVB background.

Mutational analysis of both germline and somatic alterations in the human NF2 gene has supported the tumor suppressor model, revealing a wide variety of inactivating mutations, the vast majority of which are predicted to produce a truncated protein, due to insertions, deletions and premature terminations (Gutmann et al. 1998). Less common, missense mutations are found that produce dysfunctional proteins, which can not form Merlin intramolecular complexes, necessary to fulfil a schwannoma growth-suppressive function (Sherman et al. 1997; Gutmann et al. 1999).

Tumors in mice carrying one germline mutation of Nf2, display in all cases examined loss of the remaining wild type Nf2 allele (McClatchey et al. 1998; Giovannini et al. 2000). However, it is not clear whether the frequently occurring smaller osteoma lesions in Nf2<sup>−/−</sup> mice display loss of heterozygosity (LOH). Therefore, we decided to check the Nf2 status in 16 osteosarcomas of Eu-pp-gfil/Nf2<sup>−/−</sup> mice, using Southern blot analysis on genomic DNA isolated from the tumors. Except for one tumor (GNF10), all mandibular osteosarcomas showed still the presence of the wild type allele (Fig. 5D). Interestingly, the animal with LOH for Nf2 was one of two mice that presented with an osteosarcoma very early in time, namely 74 days.

To confirm that Nf2 expression was absent in tumor GNF10 and to assess whether mutations had not inactivated Nf2 expression in the other tumor samples, Western blot analysis was performed on total cell extracts of Eu-pp-gfil/Nf2<sup>−/−</sup> osteosarcomas. All osteosarcomas showed clear Nf2 expression with the exception of tumor GNF10 (Fig. 5E). High Gfil levels confirmed that also in the context of Nf2 heterozygosity mandibular osteosarcomas were induced by transgenic gfil expression (Fig. 5E). We noted that Gfil protein had a different mobility in tumor GNF10. Whether there is a functional implication for this observation remains to be established. All together these data indicate that haploinsufficiency for Nf2 is sufficient to accelerate the onset and increase the frequency of mandibular osteosarcomas in Eu-pp-gfil mice. However, we can not rule out the presence of low amounts of dysfunctional or truncated Nf2 proteins that act as dominant-negative molecules. Loss of Nf2 function is associated with a significant decrease in tumor latency.

**Discussion**

This study illustrates that overexpression of the transcriptional repressor Gfil affects terminal differentiation of mainly calvarial osteoblasts and odontoblasts, which both are derived from neural crest-derived cells and mineralize via non-endochondral ossification. Post-mitotic osteoblasts secrete an extracellular matrix (ECM) that is at first unmineralized, osteoid, and this tissue is converted to bone when carbonate apatite crystals are deposited on type I collagen. Events similar to those occurring in osteogenesis also take place during the formation of dentin: after exiting the cell cycle, odontoblasts terminally differentiate and secrete an ECM that is at first unmineralized, the predentin. The transformation of predentin to dentin involves changes in the ECM and deposition of carbonate apatite crystals within and around collagen fibrils.

Eu-pp-gfil mice display at birth hypoplastic frontal, parietal, interparietal and squamosal bones, showing defective intramembranous ossification. However, the cranial supraoccipital bone in addition to the appendicular skeleton show no significantly reduced ossification at this age. These findings indicate that transgenic gfil expression only disturbs intramembranous ossification and not endochondral ossification. Furthermore, we found no evidence of axial skeletal transformations due to deregulation of Hox gene expression, as has been noticed in mice transgenic for the Polycomb group (Pc-G) gene bmi1 (Alkema et al. 1995).

Only at the age of 3 weeks, a small fraction of the Eu-pp-gfil mice appears significantly smaller in size than their wild type littermates. Longitudinal growth of the appendicular skeleton is mainly dependent on endochondral ossification at the epiphyseal growth plate, where chondrocytes produce a cartilage anlage that is replaced by bone (Hall and Miyake 2000; Olsen et al. 2000). In a mouse model of inducible osteoblast ablation, it
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was firmly established that also osteoblasts along with chondrocytes are required for longitudinal growth of the skeleton (Corral et al. 1998). Therefore it seems that even osteoblast function outside the skull might be affected by Gf1 expression. The small size of some of the gfil transgenic mice correlates with the presence of malformations in the periocular and frontonasal area. Mild and severe forms of ocular microsomia with completely closed eyelids, and asymmetric facial dysostosis are observed. We postulate that these anomalies most likely are secondary to defective ossification of the frontonasal bones.

At present we have no definitive explanation for the observed difference in skeletal defects that correlates with inheriting the transgene from a male or female parent. A subset of mammalian genes is monoallelically expressed in a parent-of-origin manner. These genes are subject to an imprinting process that epigenetically marks alleles according to their parental origin during gametogenesis. Since in Eμ-pp-gfil mice this phenomena is present in two independent founder-lines, imprinting of the transgene itself is most like not the right explanation. Rather the presence of an independent imprinted modifier may exert an effect, resulting in a stronger penetration of the skeletal phenotype in progeny of a female Eμ-pp-gfil parent.

In newborn gfil transgenic mice also mandibles display clear diminished ossification, which results in smaller alveolar ridge, and less pronounced condylar and angular process. Histological analysis at later age indicates that alveolar bone in both mandible and maxilla has distinctive morphology in Eμ-pp-gfil mice. Instead of homogeneous cancellous bone there are many hypercellular regions consisting of fibroblasts and undifferentiated osteoblasts, interspersed with only focal areas of ossification. Similar to the majority of cranial bones and compact mandibular bone is intramembranous ossification responsible for mineralization of neural crest-derived alveolar cancellous bone.

The defects in craniofacial intramembranous ossification found in Eμ-pp-gfil mice bears resemblance to cleidocranial dysplasia (CCD). CCD is a dominantly inherited skeletal defect associated with heterozygous inactivating mutations of CBFAl, and characterized by hypoplasia of the clavicles and cranial bones, open fontanelles and dental anomalies (Mundlos 1999). The runt related transcription factor CBFAl (AML3/PEBP2α/RUNX2) represents one of three different mammalian DNA-binding CBFα subunits, which form a heterodimer with the unrelated common CBFβ subunit (Westendorf and Hiebert 1999). Heterozygous Cbfal+ mice have specific bone defects that recapitulate the phenotype of CCD in humans (Otto et al. 1997). In addition, the cbfal gene is found deleted in the radiation-induced mouse model of CCD (Sillence et al. 1987; Otto et al. 1997). Homozygous Cbfal− mice show a block in global osteoblast development from mesenchyme and thus no ossification (Komori et al. 1997; Otto et al. 1997). These findings implicate CBFAl as an important regulator of osteogenesis by controlling the expression of bone-specific genes like osteocalcin, osteoponitin and α(I) collagen (Ducy et al. 1997).

Tooth development is also severely disturbed in cbfal+ mice, illustrated by the misshapen and severely hypoplastic tooth organs that lack overt odontoblast and ameloblast differentiation and normal dentin and enamel matrices (D'Souza et al. 1999). It has been argued that CBFAl is not involved in tooth initiation and early morphogenesis but regulates key epithelial-mesenchymal interactions that control advancing morphogenesis. Enforced gfil expression has also a distinct effect on odontoblast differentiation, where at later stages of tooth development (pre-)dentin matrices are severely disorganized. During normal tooth development, the dentin-producing odontoblasts, which are also derived from cranial neural crest cells, form a layer of columnar cells around the periphery of the pulp cavity, immediately beneath the inner surface of the dentin. Junctional complexes, which form a terminal web at the base of the cells, join neighbouring odontoblasts (Ruch et al. 1995). Key signaling events at the junction of mesenchymal odontoblasts and epithelial ameloblasts, contribute to proper tooth morphogenesis (Peters and Balling 1999). Histological analyses demonstrate evident defects in cellular organization of the columnar odontoblasts and diminished production of predentin and dentin in Eμ-pp-gfil mice. In contrast, ameloblast differentiation seems not to be affected, but this could be related to spatial differences in transgene expression. Primary tooth germ initiation and odontogenic patterning are clearly not influenced by transgenic gfil expression.

One possible explanation for the reduction in mineralization of osteoblasts and odontoblasts as observed in Eμ-pp-gfil mice is...
that Gfi1 may directly inhibit cbfa1 expression, by negatively regulating the cbfa1 promoter, or prohibit indirectly CBFA1 transcriptional activity. It has been shown that Gfi1 acts as a transcriptional repressor, due to the N-terminal SNAG transcription repression domain (Grimes et al. 1996; Zweidler-Mckay et al. 1996), also present in the zinc finger proteins of the Snail family of transcription factors, which are implicated in mediating differentiation of epithelial cells to mesenchymal cells during embryonic development (Sefton et al. 1998; Cano et al. 2000). Sequence analysis of the cbfa1 promoter tells us that there are potential Gfi1-binding sites, but additional experiments need to address this issue in more detail. Alternatively, Gfi1 expression could interfere with advanced tooth morphogenesis by acting upstream of diffusible signaling molecules, like BMPs, produced by the neural crest derived odontoblasts or epithelium that in turn regulate gene expression within the enamel organ or dental papilla (Thesleff and Sharpe 1997).

In addition, Gfi1 might alter expression levels of homeodomain-containing transcription factors, such as Mxi1/Msx2 (Foerst-Potts and Sadler 1997; Winograd et al. 1997), Dlx1/Dlx2 (Qiu et al. 1997), Cart1 (Zhao et al. 1996), Pitx1/Pitx2 (Lin et al. 1999; Lu et al. 1999) and Mhox (Martin et al. 1995), known to control craniofacial mineralization in a more restricted and highly specific manner. These homeodomain-containing genes control patterning of the first branchial arch, since at this anterior location Hex genes are not expressed. However, since we do not find any defects in tooth initiation or absence of specific craniofacial bone structures in Eu-pp-gfi1 mice, this explanation might be less likely. Issues for the immediate future are to determine the exact expression pattern of the Eu-pp-gfi1 transgene and potential target genes during skeleto- and odontogenesis, and whether endogenous gfi1 is normally expressed in osteoblasts and during tooth development.

Enforced gfi1 expression has not only an effect on terminal differentiation of osteoblasts, but is also able to trigger unscheduled proliferation in these cells, leading to the onset of intermediate-grade osteosarcomas. A remarkable observation is the specificity of the mandible for these tumors to occur. Studies in young Eu-pp-gfi1 mice indicated the presence of early pre-neoplastic lesions in alveolar bone of the mandible. Clearly, there is a strong correlation between the altered morphology of neural crest cell-derived alveolar bone and the susceptibility to the onset of osteosarcomas at this location. A similar tumor phenotype is observed in mice heterozygous mutant for the tumor-suppressor gene Nf2. Nf2 allele mice do not develop schwannomas like the human counterpart, but instead develop osteogenic tumors (McClatchey et al. 1998) of which some are derived from cranial neural crest and show LOH for Nf2 (Giovannini et al. 2000). In addition, Nf2 allele mice develop many osteomas, i.e. small osteoblastoma like lesions in the skull (Giovannini et al. 2000). Absence of one functional Nf2 allele enhances the frequency and slightly accelerates the onset of Gfi1-induced mandibular osteosarcomas, underscoring the fact that these osteoblastic tumors are derived from neural crest cell origin. Except for one tumor, this is not associated with loss of the wild type Nf2 allele as determined by Southern blot analysis and confirmed by immunoblotting for Nf2 expression. However, we can not firmly exclude the possibility that small deletions, insertions or mutations may produce dysfunctional Nf2 proteins that would act as dominant negative molecules, by preventing the formation of Merlin intramolecular complexes, necessary to fulfil growth suppressive function (Sherman et al. 1997). Alternatively, reduction in Nf2 expression is already sufficient to elicit increased initiation of Gfi1-induced osteoblast transformation, and relates to the presence of osteoma lesions in the skulls of Nf2'' mice, which may not require loss of Nf2 expression.

The fact that we only observe osteosarcomas arising from the mandible and never form the maxilla may argue that there are differences between the two populations of cranial neural crest-derived cells in the gfi1 transgenic mice. This could either relate to an intrinsic difference between mandible and maxilla mesenchymal cells. Indeed homeobox expression studies have indicated that induction of Dlx5 expression is a unique property of the mandible as a response to regulatory signals derived from both mandibular and maxillary arch epithelium, whereas Dlx2 expression was induced in both mandibular and maxillary primordium (Ferguson et al. 2000). Alternatively, the spatial and temporal expression pattern of the gfi1 transgene may be different between the two sides of the jaw, and more detailed RNA expression analysis needs to address this aspect.

Conventional osteosarcomas in humans are often associated with inactivation of p53 or pRb function (Miller et al. 1996; Pompetti et al. 1996). Overexpression or alterations of p53...
have been documented in approximately 30% of the high-grade osteosarcomas, and altered p53 is associated with highly aggressive variants of osteosarcomas. In a small percentage of cases, alterations of p53 are associated with the amplification of the MDM2 gene, and correlates with metastatic osteosarcoma (Lorando et al. 1997). Human patients with germline RB mutations have 2000 times the normal risk for osteosarcoma, which is associated with allelic loss of the wild type gene (Toguchida et al. 1988; Toguchida et al. 1989). The RB gene is also frequently altered in sporadic osteosarcomas, causing the absence of a functional pRb protein (Wada et al. 1994). Furthermore, mice transgenic for SV40 large T, which binds and functional inactivates pRb and p53, display facial neural crest-derived osteosarcomas (Lensen et al. 1993).

Inter crossing the Ep-pp-gfil transgene onto a Rb- or p53- background did however not reveal any significant acceleration in the onset of gfil-induced neural crest-derived osteosarcomas (data not shown). Therefore, inactivation of p53 or pRb seems not to be a rate-limiting step in generating the mandibular osteosarcomas. More detailed molecular characterization of the mandibular osteosarcomas on RNA and protein expression levels of the individual genes or gene products involved in the Rb and p53 pathway could provide additional information on this aspect. Alternatively, Gfi1 itself might act downstream on the ARF-Mdm2-p53 and/or pl03'-pRb pathway and affect the function of critical targets of these signaling cascades. Interestingly, there are some indications that CBFA1 could function as a target of pRb. Cbfal-null mutant embryos have a significant increased amount of primitive nucleated erythrocytes, reminiscent of a block in erythropoiesis (Otto et al. 1997), which is also observed in Rb- embryos (Clarke et al. 1992; Jacks et al. 1992). Furthermore, recent experiments have indicated that pRb enhances CBFA1-mediated transactivation by binding to the C-terminus of CBFA1, and CBFA1 inhibits cell growth and colony formation in wild type but not Rb- 3T3 cells. (D. Thomas, pers. commun.). Whether there is a genuine connection between regulation of CBFA1 activity by Gfi1 and pRb signaling remains to be established.

In summary, our data suggest that deregulation of Gfi1 expression levels interferes with proper osteoblast and odontoblast differentiation and consequent intramembranous ossification, craniofacial and tooth development. Alterations in neural crest-derived alveolar bone structure correlates with a high predisposition for mandibular osteosarcomas in Ep-pp-gfil transgenic mice, which arise more frequent in a Nf2- background. This report, together with recent data on thymocyte development, lymphoblastic T cell tumors and chronic myeloid leukemia in Ep-pp-gfil mice (Scheijen et al., submitted; Scheijen and Berns, submitted), clearly establishes Gfi1 as an important transcription factor that is able to control cell differentiation, proliferation and apoptosis in many different cell lineages. Moreover, this study provides indications that deregulation of GFI1, located on human chromosome lp22, could be implicated in human craniofacial malformations and osteosarcomas.

Materials and methods

Mice and genomic DNA analysis

The generation of Ep-pp-gfi1 mice has been described elsewhere (Scheijen et al., submitted). Intercrossing the Nf2- allele was done by mating male FVB Ep-pp-gfi1 (GFI39 line) with female (FVB x 129/OLA) Nf2- mice. For this purpose we used the Nf2-KO mice as described in Giovannini et al. (2000). Genotyping of gfi1 transgenic and Nf2- mice was done by Southern blot analysis on EcoRV-digested, or BamHI-EcoRV-digested genomic DNA isolated from tail-biopsies, using gfi1 cDNA or probe A (0.5-kb KpnI-BamHI fragment) as [32P]dATP radio-labeled probes. Similar BamHI-EcoRV digest was used to analyze the Nf2 status in Ep-pp-gfi1 mandibular osteosarcomas and score for LOH. Conditions for hybridization have been described elsewhere (Scheijen et al. 1997).

Histological analysis and skeletal staining

Mice were sacrificed when presenting with an overt mandibular tumor. At necropsy the tumors were excised and frozen down at -80°C or fixed in formalin, when necessary 0.5M EDTA treated to decalcify mineralized tissues and paraffin-embedded. Alternatively, young gfi1 mice were sacrificed at the age of 4 weeks and maxilla and mandible were fixed in 4% paraformaldehyde/PBS. Tissues were sectioned at 5 µm and stained with hematoxylin and eosin (H&E).

For skeletal staining, newborn mice were sacrificed, deskinned, eviscerated, and fixed in 95%
ethanol for 24 h. Genomic DNA was isolated from skin and used for genotyping. The skeletons were stained for 24 h with 0.015% Alcian Blue, dissolved in 75% ethanol/20% glacial acetic acid for. Thereafter, they were rinsed with ethanol and fixed for another 24 h. Samples were cleared in 1%-KOH for 6 h, and stained with 0.005% Alizarin Red in 2%-KOH for 3 h. Samples were subsequently placed with 1-day intervals in decreasing concentrations 2%-KOH mixed with increasing concentrations of glycerol.

**RNA analysis**

Total RNA was isolated with TRizol (Gibco BRL) according to the instructions of the supplier. Samples of 15-µg RNA were separated on a 1% agarose/parafomaldehyde-containing gel, blotted onto nitrocellulose and hybridized under standard conditions with [α-32P]dATP radio-labeled gfi1, β-actin, osteonectin (a gift from K. Bechler), or α(I)collagen (a gift from K. Kratochwil) cDNA probes.

**Western blotting**

Total cell extracts were generated by 2 x 4 sec sonicication of frozen tissue samples in ice-cold lysis buffer (250 mM NaCl, 0.1% NP40, 50 mM HEPES pH 7.0 and 5 mM EDTA, supplemented with protease inhibitors (Complete, Boehringer Mannheim). Undissolved material was sedimented by centrifugation for 10 min at 14,000 rpm. Samples corresponding to 40 µg of protein (Biorad Bradford protein assay) were separated on a SDS-polyacrylamide gel and transferred to Immobilon-P membranes (Millipore). Polyclonal antibodies against gfi1 (M-19), NF2 (N-19), and Actin (C-11) were used, followed by horseradish peroxidase conjugated secondary antibodies (Biosource). The specific protein products were visualized with ECL (Amersham).

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