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concept

Runx3 knockouts and stomach cancer

The challenge of identifying phenotypic defects directly attributable to loss of gene function

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Gene targeting often results in knockout mice that show several phenotypes, some of which may not directly relate to the intrinsic function of the disrupted gene. Hence, to study the biological function of genes using knockout mice, one must identify the defects that are directly due to the loss of the targeted gene. Runx3 is a transcription factor that regulates lineage-specific gene expression in developmental processes. Recently, two groups produced *Runx3* knockout mice. Two comparable defects were identified in both knockout strains, one involved neurogenesis and the other thymopoiesis. In addition, a stomach defect pertaining to gastric cancer was observed in one of the mutant strains, but not in the other. Here, we assess the differences between the two *Runx3* mutant strains and discuss further studies that could reconcile these discrepancies. This article highlights the difficulties of inferring gene function through the interpretation of knockout phenotypes.

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Introduction

Many knockout (KO) mice have multiple, and sometimes complex, phenotypic defects. Often, the null phenotype does not seem to recapitulate the known cellular function of the gene. This is frequently the case when the gene being studied has a distinct tissue- and/or temporal-specific function that is difficult to replicate in cell culture. Many homozygous KO mice die *in utero,* and those that are viable often have nutritional or immunological deficiencies that cause secondary phenotypes associated with aberrant growth and survival. Thus, the challenging aspect of analysis of gene function in KO mice is to identify the defects that are directly linked to the loss of function of the targeted gene. This study of Runx3 involvement in gastric cancer is a good example of such a challenge.

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The RUNX transcription factors

Mammalian RUNX3 belongs to the runt-domain family of transcription factors that act as regulators of gene expression in several important developmental pathways**.** The three *RUNX* genes, *RUNX1, RUNX2* and *RUNX3*, appeared early in evolution and have maintained extensive structural similarities (Fig. 1A; Eggers *et al.*, 2002; Kalev-Zylinska *et al.*, 2002; Levanon *et al.*, 2003). Members of the RUNX family show homology in a 128-amino-acid region known as the runt domain (RD), which directs the binding of RUNX proteins to DNA and mediates their interaction with the protein core-binding factor-β (CBF-β; Fig. 1B) (Ito & Bae, 1997; Speck, 2001). CBF-β enhances the binding of the RUNX proteins to their target DNA and is essential for their proper function (Adya *et al.*, 2000).

The *RUNX* genes are regulated at the transcriptional level by two promoters and at the translational level by an internal ribosome-entry site (IRES) and cap-dependent translation control (Fig. 1; Miyoshi *et al.*, 1995; Ghozi *et al.*, 1996; Geoffroy *et al.*, 1998; Pozner *et al.*, 2000; Bangsow *et al.*, 2001; Levanon *et al.*, 2001b; Rini & Calabi, 2001; Xiao *et al.*, 2001; Stewart *et al.*, 2002). All RUNX proteins bind to the same DNA motif and activate or repress the transcription of their target genes through the recruitment of common transcriptional modulators (Fig. 1B; Karsenty, 2000; Wheeler *et al.*, 2000). In spite of this, the *RUNX* genes have well-defined biological functions, which are reflected in their unique expression patterns (Simeone *et al.*, 1995; North *et al.*, 1999; Levanon *et al.*, 2001a; Chen *et al.*, 2002; Stricker *et al.*, 2002; Yamashiro *et al.*, 2002) and the distinct phenotypes that are shown by the corresponding KO mice (Otto *et al.*, 1997; Karsenty, 2000; Speck, 2001; Inoue *et al.*, 2002; Levanon *et al.*, 2002; Li *et al.*, 2002). *RUNX3* is located on human chromosome 1p36.1 (Levanon *et al.*, 1994) and seems to be the most ancient of the three *RUNX* genes (Bangsow *et al.*, 2001; Levanon *et al.*, 2003). This is consistent with its function in the neurogenesis of the monosynaptic reflex arc (Inoue *et al.*, 2002; Levanon *et al.*, 2002), the simplest neuronal response circuit found in the most primitive animals, such as hydra-like organisms.

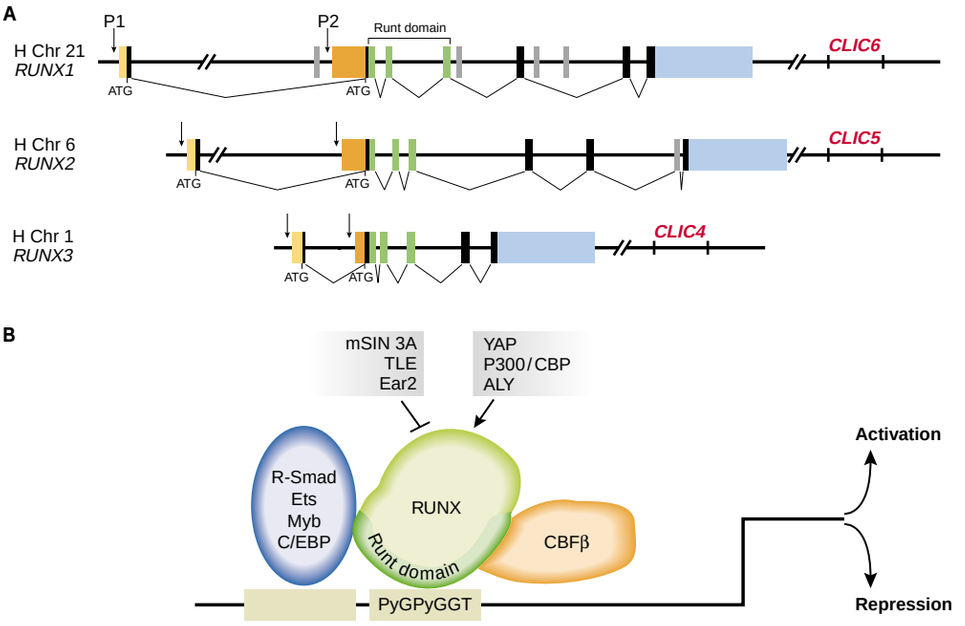

Fig. 1 | The mammalian *RUNX* genes: structure and mode of function. (**A**) The three mammalian *RUNX* genes have similar genomic organization with two promoters (P1 and P2) and a very large first intron. The two promoters give rise to two biologically distinct 5′ untranslated regions (UTRs) (yellow and orange). In humans and mice, each gene resides on different chromosomes (human 21, 6 and 1, and mouse 16, 17 and 4, respectively). The highly conserved runt domain is encoded by the three exons marked in green. Exons comprising the transactivation domain are shown in black and grey and the 3′ UTR in blue. *Runx3* is the smallest and simplest of the three genes. (**B**) The runt domain directs binding to the RUNX DNA-motif PyGPyGGT at the promoter of target genes, and protein–protein interactions withcore-binding factor-β (CBF-β). The RUNX proteins bind to the same DNA motif and either activate or repress transcription through interactions with other transcription factors (blue ellipse) and co-activators (arrows), or co-repressors (blocked line). Of note, due to lack of space, only a few examples of RUNX transcriptional co-modulators are indicated.

Runx3, gastric mucosa hypertrophy and stomach cancer

Stomach cancer is the second most common form of malignancy and is a major contributor to cancer mortality throughout the world (Fuchs & Mayer, 1995; Parkin *et al.*, 1999). Gastric carcinomas have been linked with the loss of homozygosity at various chromosomal loci, but no single gene that accounts for the majority of cases has been identified. Recently, Li and colleagues (Li *et al.*, 2002) reported that the gastric mucosa of *Runx3* null mice showed hyperplasia and concluded that lack of RUNX3 is causally related to human gastric cancer. Consistent with this, an analysis of *RUNX3* in human stomach cancer cell lines and primary human tumours revealed hemizygosity in 40% of the tumours analysed, and silencing by promoter hypermethylation in 60% of the tumours, the latter figure rising to 90% in the advanced stage tumours (Li *et al.*, 2002). Furthermore, silencing by hypermethylation of the *Runx3* promoter was also observed in cell lines derived from N-methyl-M-nitrosourea-induced mouse stomach carcinomas (Guo *et al.*, 2002).

These observations prompt the question: does the loss of Runx3 in the KO mice cause gastric tumorigenesis? In the case of the *Runx3*$^{-/-}$ mice described by Li *et al.* (2002) (hereafter referred to as *Runx3* type I KO), which were inbred on a C57BL/6 background, most progeny died of starvation soon after birth and none survived for more than 10 days. Therefore, studies of tumorigenesis in these mice were not feasible. However, another strain of *Runx3* KO mice (*Runx3* type II KO), which were bred on a heterogeneous genetic background (ICR and MF1), produced progeny of which a significant number survived for several months (Levanon *et al.*, 2002). Intriguingly, newborn *Runx3* type II KO mice did not show hyperplasia of the gastric epithelium and did not develop gastric tumours (Levanon *et al.*, 2002). This observation strongly suggests that loss of Runx3 is not necessarily associated with gastric neoplasia, and that the mucosal hypertrophy observed in the newborn *Runx3* type I KO mice might be related to the strain on which the study was performed. It is interesting to note that the C57BL/6 strain is more susceptible to *Helicobacter felis* infection, which often results in a severe gastric phenotype that is characterized by increased proliferation of the mucosal epithelium (Wang *et al.*, 1998), than other strains such as BALB/c or C3H/HeJ. Therefore, as suggested by Lund van Lohuizen (Lund van Lohuizen, 2002), the possible involvement of *Helicobacter* in the aetiology of stomach lesions in *Runx3* KO mice should not be overlooked.

As mentioned earlier, CBF-β is essential for the activity of all the Runx proteins (Adya *et al.*, 2000); consequently, CBF-β -deficient mice recapitulate the Runx1 null phenotype, as they lack definitive haematopoiesis and die due to haemorrhaging at embryonic day 12.5 (Adya *et al.*, 2000; Speck, 2001). Recently, three groups produced new strains of CBF-β-mutant mice in which the early haematopoietic defect was reversed (Kundu *et al.*, 2002; Miller *et al.*, 2002; Yoshida *et al.*, 2002). The progeny of these mice were born, but died within one day of birth. As the stomach defect in

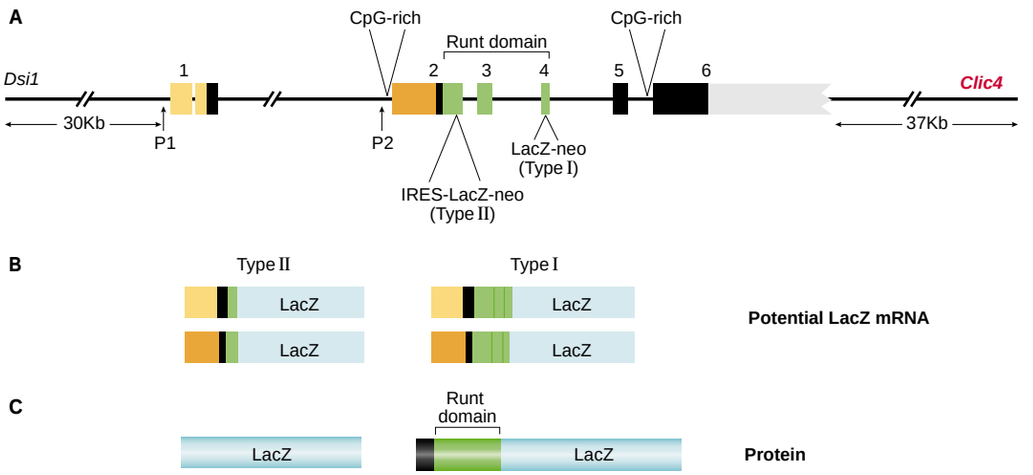

Fig. 2 | The *Runx3* genomic locus including the neighbouring genes and the potential lacZ messenger RNA and protein products. (**A**) Genomic organization showing the two promoters (P1 and P2) and six exons. The different targeting sites in exons 2 and 4 used by Levanon *et al.* (2002) and Li *et al.* (2002), respectively and the corresponding targeting cassettes are indicated, as are the distances between *Runx3* and the *Dsi-1* and *Clic4* loci. The positions of the CpG-rich islands at the 5′ and 3′ regions of *Runx3* are marked. The various lacZ mRNAs that are transcribed from the P1 and P2 promoters in both type I and type II knockout mice are shown in (**B**) as are the corresponding protein products (LacZ and runt domain–LacZ) in (**C**). The yellow and orange segments represent the 5′ untranslated regions of P1 and P2 respectively. The green segments denote the runt domain (RD) and the blue segments represent the LacZ protein. Of note, the genomic organization of Runx3 (Bangsow *et al.* 2001) which is shown here including the number of *Runx3* exons (1–6) differs from that in Li *et al.*, (2002) due to the omission of exon 1 in the latter.

Runx3 type I KO mice was detected immediately after birth and before suckling commenced, it would be interesting to look for mucosal hyperplasia in these new CBF-$\beta^{-/-}$ rescue mutants. Moreover, the early death of the Runx3 KO C57BL/6 pups precluded the analysis of tumour development in these mice. However, as breeding them into an ICR background significantly extended their lifespan (Inoue *et al.*, 2002), it is now possibile to examine stomach tumorigenesis in these mice. It would be equally interesting to breed the *Runx3* type II KO mice onto a C57BL/6 background and to examine the newborns for gastric mucosa hyperplasia.

Is Runx3 intrinsically required in the gastric epithelium?

The phenotypic differences between the two *Runx3* KO strains also extend to the expression pattern of Runx3 during development. Analysis of heterozygous *Runx3*$^{LacZ/+}$ embryos of both type I and type II KO mice revealed X-gal staining in sensory ganglia, epidermal appendages and developing skeletal elements (Levanon *et al.*, 2001a, 2002; Li *et al.,* 2002). In addition, *Runx3* RNA was detected by *in situ* hybridization in the stomach epithelium of type I KO mice and strong X-gal staining was observed in the stomach and intestine of E14.5 embryos (Li *et al.,* 2002). These data do not correspond with data for the type II KO mice, in which no Runx3 expression was detected in the stomach, either by LacZ or immunostaining with specific Runx3 antibodies (Levanon *et al.,* 2001a). The latter studies also showed that Runx1 is highly expressed in epithelia, including the gastric epithelium, whereas Runx3 expression is restricted to the mesenchymal tissues (Levanon *et al.*, 2001a; Yamashiro *et al.*, 2002). The genetic backgrounds of the two types of KO mice could account for the phenotypic differences observed, but what is the underlying cause of these gene expression discrepancies?

Differences in the targeting constructs

One important difference between the two mutant strains is the structure of the targeting vectors used for creating the KO mice. In type II KO mice, *Runx3* was disrupted by inserting a LacZ–neomycin (neo) cassette into exon 2, the first exon of the RD (Fig. 2). Here, expression of LacZ is mediated by the distal P1 and proximal P2 promoters of *Runx3*, and by the IRES of the vector, thus generating a free LacZ protein (Fig. 2). In *Runx3* type I KO mice, however, the LacZ–neo cassette was inserted in frame at the carboxyl terminus of the RD, creating a RD–LacZ fusion protein (Fig. 2). As the C terminus of the RD is important for DNA binding (Nagata *et al.*, 1999; Rudolph & Gergen, 2001), the fused RD–LacZ protein would be expected to bind very poorly to DNA. However, as this fused product retains most of the RD, it may bind to the Runx partner protein CBF-β (Bravo *et al.*, 2001) and therefore exert a dominant-negative effect (Michaud *et al.*, 2002), particularly in the stomach of *Runx3* type I KO mice where the RD–LacZ protein is highly expressed. A potential target for this negative effect is Runx1, which is expressed in the gastric mucosa of developing mouse embryos (Levanon *et al.*, 2001a). So far, there has been no functional analysis of Runx1 activity in the stomach and intestine of either *Runx3* type I or *Runx3* type II KO mice, and this may prove to be informative.

Structural differences within the targeted genomic locus

In addition to the structural difference in the LacZ product of the two constructs, the genomic targeting site also differs in the KO types. In type II KO mice the LacZ–neo cassette was inserted into exon 2, whereas in *Runx3* type I KO mice it was placed ~10 kb downstream, near the 3′ end of exon 4 (Fig. 2).

How might this affect the gastric phenotype? Long-distance effects on neighbouring genes are well-known phenomena. As

pointed out by Balmain (2002), *Runx3* is subject to upregulation in T-cell lymphomas by the insertion of murine leukemia virus (MuLV) at the *Dsi1* locus, located 30 kb upstream of its P1 promoter (Stewart *et al.*, 2002) (Fig. 2). If this, or another transposable element is present in the strain used to generate the type I KO mice, it might also affect *Runx3* expression in the stomach. Interestingly, a gene that encodes the chloride intracellular channel 4 (*Clic4*) is located downstream of *Runx3,* close to the 3′-end of the gene (Figs 1,2), and downregulation of *Clic4* is known to abrogate p53-dependent apoptosis (Fernandez-Salas *et al.*, 2002). Therefore, an intriguing possibility, is that the targeting of *Runx3* exon 4 might have affected the expression of *Clic4* in *cis,* leading to attenuation of apoptosis in the gastric mucosa of *Runx3* type I KO mice. In particular, the introduction of the *neo* gene, which is driven in the *Runx3* type I KO mice by the phosphoglycerate kinase (PGK) promoter that is known to influence neighbouring genes (Scacheri *et al.,* 2001), may have affected *Clic4* expression. In this regard, it is worth noting that several *Clic4* expressed sequence tags (ESTs) have previously been isolated from stomach complementary DNA libraries (for example, NCBI, UniGene Cluster Hs.25035). Analysis of *Runx3* mutant mice for MuLV integration, as well as analysis after removal of the LacZ-neo cassette by Cre-recombinase–mediated excision, should help to clarify these issues.

Is the *RUNX3* P1 promoter methylated in gastric cancer?

What is the connection between the downregulation of *RUNX3* and human gastric cancer? Furthermore, how might the hypermethylation of the *RUNX3* promoter that has been observed in primary human gastric tumours fit into the puzzle?

Gastric carcinomas, as with many other human tumours, are associated with multiple genetic alterations that affect numerous genes. These changes include genetic instability, reactivation of telomerase, inactivation of tumour-suppressor genes and activation of oncogenes (Yasui *et al.*, 2000). Thus, it could be considered possible that epigenetic silencing of *RUNX3* in human gastric tumours reflects a secondary event induced by the malignant transformation.

The regulation of RUNX3 by two promoters may also be a factor in its potential silencing, as only the P2 promoter was evaluated for hypermethylation in gastric tumours (Li *et al.,* 2002). In contrast to the P2 promoter, which is located within a conserved CpG island (Fig. 2), the environment of the P1 promoter is CpG poor (Bangsow *et al.*, 2001), so it is unlikely to be a target for methylation. Silencing of the P2 promoter by hypermethylation could therefore be accompanied by an upregulation of the P1 promoter (Stewart *et al.*, 2002). Such a P2-to-P1-promoter switch has been previously observed for *RUNX1* (Pozner *et al.*, 2000). This promoter switch should not be overlooked as it could result, as with RUNX1, in an enhanced production of alternatively spliced isoforms of RUNX3 (Levanon *et al.*, 1996), some of which could lack various parts of the carboxy-terminal region of the protein. As this missing domain is considered to be the transactivation domain of RUNX3, such isoforms may act as dominant-negative regulators. Moreover, RUNX3 isoforms that lack the trans-activation domain, but have transcription start sites at the P1 promoter, may not be detected by the RT–PCR (PCR after reverse transcription) primers used by Li *et al.*, (2002). Examination of the methylation status of the *RUNX3* P1 promoter in primary gastric tumours should shed light on this.

Perspectives

The discrepancies between the two *Runx3* mutant strains will have to be reconciled through further investigation. However, it is worth noting that Runx3 KO mice have two phenotypic defects that are comparable in both type I and type II, despite variations in strain and targeting strategy. These are a prominent sensory-motor defect (Inoue *et al.*, 2002; Levanon *et al.*, 2002) and a well-defined defect in thymopoiesis (Taniuchi *et al.*, 2002; Woolf *et al.*, 2003)**.** In both cases, Runx3 is readily detected in the affected tissues and a cell-intrinsic requirement for Runx3 has been shown. However, *Runx3* has several alternatively spliced isoforms (Bangsow *et al.,* 2001), the expression of which has not been carefully evaluated in either of the two Runx3 KO strains. Thus, the 'down but not out' phenomenon seen in other KO models (see Kos *et al*., 2002) should also be considered here. Furthermore, the association of Runx3 deficiency with defects in cytotoxic T-cell development (Taniuchi *et al.*, 2002; Woolf *et al.* 2003) may cause secondary phenotypes that are not directly linked to Runx3 activity. Thus, the phenotypic differences described here pose the challenge of distinguishing between defects that are directly due to loss of Runx3 and those that are unrelated, non-cell-autonomous or secondary.

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