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The skeletal phenotypes of TRα and TRβ mutant mice

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Abstract

Analysis of mice harbouring deletions or mutations of thyroid hormone receptor α (TRα) and β (TRβ) have clarified the complex relationship between central and peripheral thyroid status and emphasised the essential but contrasting roles of thyroid hormone (T3) in skeletal development and adult bone. These studies indicate that TRα1 is the predominant TR expressed in bone and that T3 exerts anabolic actions during growth but catabolic actions in the adult skeleton. Examination of key skeletal regulatory pathways in TR mutant mice has identified growth hormone, insulin like growth factor 1 and fibroblast growth factor signalling and the Indian hedgehog/parathyroid hormone-related peptide feed back loop as major targets of T3 action in chondrocytes and osteoblasts. Nevertheless, although increased osteoclastic resorption is a major feature of thyrotoxic bone loss and altered osteoclast activity is central to the skeletal phenotype of TR mutant mice, it remains unclear whether T3 has direct actions in osteoclasts. Detailed future analysis of the molecular mechanisms of T3 action in bone will enhance our understanding of this emerging field and has the potential to identify novel strategies for prevention and treatment of osteoporosis.

Introduction

Thyroid hormone is essential for normal skeletal development and the maintenance of adult bone mass. Recent studies of thyroid hormone receptor (TR) mutant mice have advanced our understanding and demonstrated the relevance of this field to osteoporosis, a major public health burden that affects half of all women and one fifth of men over the age of 50, costing the European community €31 billion per annum (Kanis and Johnell 2005). Systemic thyroid hormone levels are maintained by the hypothalamus-pituitary-thyroid (HPT) feedback axis. The cellular actions of 3,5,3'-L-triiodothyronine (T3) are mediated by TRs, which act as hormone inducible transcription factors. Unliganded TRs bind thyroid hormone response elements (TREs) in T3 target genes and mediate transcriptional repression. T3 binding results in derepression and activation of gene transcription (Yen 2001). The *THRA* and *THRB* genes encode 3 functional receptors TRα1, TRβ1 and TRβ2 as well as a non-T3 binding isoform of unknown function TRα2 (Fig 1). TRα1 and TRβ1 are expressed widely and the ratio of TRα1 to TRβ1 is spatio-temporally regulated. Thus, T3-target tissues may predominantly display either TRα1 or TRβ1 responsiveness or show no TR-isoform specificity. TRβ2 has a more restricted pattern of expression and regulates sensory organ development (Jones, et al. 2007) as well as the HPT axis. In the skeleton chondrocyte and osteoblast lineages express TRα and TRβ mRNAs but in osteoclasts the position is less clear as studies have been restricted to precursor cells or *in situ* hybridisation analysis of osteoclastomas (Abu, et al. 2000; Kanatani, et al. 2004; Stevens, et al. 2000; Williams, et al. 1994). Several studies have demonstrated apparent expression of TR proteins in all bone cell lineages, but it is well recognised within the field that available TR antibodies are of low affinity, thus compromising the detection of endogenous TRs (Abu, et al. 1997; Robson, et al. 2000). For these reasons a comprehensive understanding of TR expression in bone is lacking and a detailed analysis of cell-specific and temporal expression of TR isoforms is required

Long bones are formed by endochondral ossification and the skull by intramembranous ossification. During endochondral ossification, mesenchyme-derived chondrocytes form a cartilage model, undergo hypertrophic differentiation and then apoptose. The surrounding collagen X-rich cartilage matrix calcifies and forms a scaffold for bone formation by osteoblasts. Organised columns of proliferating and differentiating chondrocytes persist in the growth plate until adolescence and mediate linear growth and the acquisition of peak bone mass. By contrast, in intramembranous ossification osteoblasts differentiate from mesenchyme to form bone directly. Adult bone structure and mechanical strength is preserved by a continuous process of skeletal remodelling during which precise coupling of osteoclastic bone resorption and subsequent osteoblastic bone formation is maintained.

The established view that skeletal responses to abnormal thyroid status result exclusively from altered T3-action in bone has been challenged recently by studies proposing a direct role for TSH in bone. We recently discussed this issue elsewhere (Bassett and Williams 2008) and the current review will therefore focus on analysis of T3 and TR action in bone.

Thyroid hormone receptor mutant mice

Several TRα knockout mice have been generated and this led to the identification of additional TRα isoforms expressed from a promoter within intron 7 of the *Thra* gene (Chassande, et al. 1997). As a result only $TR\alpha^{0/0}$ mice lack all TRα isoforms whereas other TRα mutants retain truncated isoforms with dominant negative activity (Fig. 1 and Table 1). This review will focus on mice lacking all TRα ($TR\alpha^{0/0}$) or all TRβ ($TR\beta^{-/-}$) isoforms and those harbouring dominant negative mutations of either TRα ($TR\alpha1^{PV/+}$, $TR\alpha1^{R384C/+}$) or TRβ ($TR\beta^{PV/PV}$).

Thyroid hormone actions during skeletal development are anabolic

The developing skeleton is exquisitely sensitive to thyroid status and childhood hypothyroidism is characterised by growth retardation, delayed bone age and short stature, whereas juvenile thyrotoxicosis accelerates growth and advances bone age but results in short stature due to premature fusion of the epiphyses (Boersma, et al. 1996; Rivkees, et al. 1988; Segni and Gorman 2001).

Although $TR\alpha^{0/0}$ mice are systemically euthyroid, juveniles display postnatal growth retardation with delayed endochondral ossifciation characterised by impaired chondrocyte differentiation and decreased mineral deposition (Bassett, et al. 2007b). $TR\alpha1^{R384C/+}$ mice, which harbour a dominant-negative mutation of TRα, are also euthyroid. They display a similar period of growth retardation and delayed endochondral ossification but additionally have reduced cortical bone thickness, abnormal cortical bone remodelling and impairment of intramembranous ossification (Fig. 2) (Bassett, et al. 2007a). $TR\alpha1^{PV/+}$ mice, which express a potent dominant-negative TRα mutant, display a more severe skeletal phenotype. $TR\alpha1^{PV/+}$ mice have persistent postnatal growth retardation, markedly delayed endochodral ossification, decreased mineralisation, reduced cortical bone thickness and impaired intramembranous ossification (O'Shea, et al. 2005). Similar findings have been reported in mice expressing the potent dominant negative receptor $TR\alpha1^{L400R}$ (Quignodon, et al. 2007). Consistent with the delayed bone development in juvenile $TR\alpha^{0/0}$, $TR\alpha1^{R384C/+}$ and $TR\alpha1^{PV/+}$ mice, skeletal expression of the T3-target genes fibroblast growth factor receptor 1 and 3 (FGFR1/3) (Barnard, et al. 2005; O'Shea P, et al. 2007;

Stevens, et al. 2003) were reduced (Barnard et al. 2005; Bassett et al. 2007a; Bassett et al. 2007b; O'Shea et al. 2005). Deletion or mutation of TRα does not affect systemic thyroid status but causes local skeletal hypothyroidism whilst the presence of a dominant-negative TRα leads to a more severe skeletal phenotype than receptor deficiency alone.

Two $TR\beta^{-/-}$ strains have been generated in different genetic backgrounds and both show similar skeletal phenotypes (Table 1) (Forrest, et al. 1996; Gauthier, et al. 1999). Deletion of TRβ results in elevated circulating TSH, T4 and T3 levels consistent with resistance to thyroid hormone (RTH). In contrast to TRα mutants, juvenile $TR\beta^{-/-}$ mice display advanced endochondral ossification, accelerated chondrocyte differentiation, increased mineral deposition and persistent short stature due to premature growth plate quiescence. Furthermore, cortical thickness is increased and intramembranous ossification advanced in $TR\beta^{-/-}$ mice (Figure 2) (Bassett et al. 2007b). $TR\beta^{PV/PV}$ mice express a potent dominant negative TRβ and display severe RTH with a 400-fold elevation of TSH and 15-fold elevation of T4. $TR\beta^{PV/PV}$ animals exhibit a more severe phenotype than $TR\beta^{-/-}$ mice with accelerated intrauterine growth characterised by advanced endochondral and intramenbranous ossification. Premature ossification results in persistent postnatal growth retardation, premature growth plate quiescence, increased mineral deposition and craniosynostosis (O'Shea, et al. 2003). Consistent with advanced skeletal development in $TR\beta^{-/-}$ and $TR\beta^{PV/PV}$ mice, expression of the T3 target genes *Fgfr1* and *Fgfr3* was increased (Barnard et al. 2005; Bassett et al. 2007a; Bassett et al. 2007b; O'Shea et al. 2003). Thus, deletion or mutation of TRβ disrupts the HPT axis resulting in increased circulating thyroid hormone levels and skeletal thyrotoxicosis. The presence of a dominant-negative TRβ leads to a greater elevation of systemic thyroid hormone concentration and a more severe skeletal phenotype than receptor deficiency alone.

In the developing skeleton reduced T3 action in TRα mutant mice results in delayed ossification and reduced mineralisation whereas increased T3action in TRβ mutant mice leads to advanced ossification and increased mineralisation. Thus, during growth T3 actions in bone are anabolic.

Thyroid hormone actions in adult bone are catabolic

Adult thyrotoxicosis results in both increased bone resorption and formation but uncoupling of these processes favours osteoclastic resorption and leads to a 10% net bone loss per remodelling cycle (Mosekilde, et al. 1990). Consequently thyrotoxicosis is an important cause of secondary osteoporotic fracture (Cummings, et al. 1995; Franklyn, et al. 1998; Mosekilde et al. 1990; Murphy and Williams 2004; Vestergaard and Mosekilde 2002; Vestergaard, et al. 2000) and even subclinical hyperthyroidism has been associated with decreased bone mineral

density and increased fracture risk in postmenopausal women (Bauer, et al. 2001; Heemstra, et al. 2006; Kim, et al. 2006; Morris 2007; Murphy and Williams 2004; Quan, et al. 2002).

Remarkably, delayed ossification and reduced mineralisation in juvenile TR$\alpha^{0/0}$ mice were accompanied by greatly increased trabecular bone mass in adults (Fig. 3) (Bassett et al. 2007b). Moreover, the robust and plate like trabeculae contained highly mineralized calcified cartilage indicating a trabecular bone remodelling defect. Consistent with such a defect TR$\alpha^{0/0}$ mice displayed reduced osteoclast numbers and activity (Bassett et al. 2007b). Trabecular bone mass increased progressively with age in TR$\alpha 1^{R384C/+}$ mice with adults showing osteosclerosis (Bassett et al. 2007a). Consistent with a remodelling defect, trabeculae were of increased thickness and connectivity, showed increased mineralisation with extensive retention of calcified cartilage and reduced osteoclast numbers and activity (Fig. 4). Remarkably, brief T3 supplementation during growth, sufficient to overcome transcriptional repression by TRα1R384C, ameliorated the adult skeletal phenotype (Bassett et al. 2007a) (Table 1). These data indicate that during development even transient relief from the transcriptional repression mediated by unliganded TRα1 (apo-TRα1) has long-term consequences for adult bone structure and mineralisation. Thus, in the adult skeleton, deletion or mutation of TRα results in persistently impaired bone remodelling. Similarly, the presence of a dominant-negative TRα leads to a more severe skeletal phenotype than receptor deficiency alone.

Despite a juvenile phenotype of accelerated growth and increased ossification, adult TR$\beta^{-/-}$ mice became progressively osteoporotic (Fig. 3)(Bassett et al. 2007a). Two TR$\beta^{-/-}$ strains in different genetic backgrounds were analysed (Bassett et al. 2007b; Gauthier, et al. 2001). In one strain cortical and trabecular bone mineralisation were both reduced and osteoclast numbers and activity increased (Bassett et al. 2007a; Bassett et al. 2007b). However, in the other strain only trabecular bone mineralisation was affected but this was also accompanied by an increase in osteoclast numbers and activity (Bassett et al. 2007a) (Fig. 4). Thus, in the adult skeleton deletion of TRβ results in accelerated remodelling and bone loss although the severity may depend on genetic background.

In the adult skeleton reduced T3-action in TRα mutant mice results in osteosclerosis whereas increased T3-action in TRβ mutant mice leads to osteoporosis. Thus, the actions of T3 are catabolic in adult bone.

Mechanism of T3 action

The opposing skeletal phenotypes in TRα and TRβ mutant mice provide compelling evidence for the complex interaction between central and peripheral thyroid status (Fig. 5). Thus, delayed ossification and impaired bone

remodelling in TRα mutant mice is secondary to disruption of T3 action in bone, whereas advanced skeletal development and osteoporosis in TRβ mutant mice is due to disruption of the HPT axis, elevated systemic thyroid hormone levels and local supraphysiological stimulation of TRα in bone (O'Shea P, et al. 2006). This model is supported by T3-target gene expression in skeletal cells of TR mutant mice and by the demonstration of higher levels of TRα mRNA expression in bone compared to TRβ (Barnard et al. 2005; Bassett et al. 2007a; Bassett et al. 2007b; Bookout, et al. 2006; O'Shea et al. 2005; O'Shea et al. 2003). Nevertheless, it is apparent that $TR\alpha^{0/0}TR\beta^{-/-}$ mice have a more severe skeletal phenotype than $TR\alpha^{0/0}$ mice, whilst $TR\alpha^{0/0}$ mice also remain sensitive to T4 treatment thus suggesting a residual role for TRβ in skeletal cells. In this context quantitative RT-PCR analysis has shown that TRα expression is 10 to 100 fold greater than TRβ expression in adult whole bone (Bookout, et al. 2006; O'Shea et al. 2003). However, since the temporo-spatial patterns of TRα and TRβ expression in skeletal cells are unknown a role for TRβ is possible. Furthermore, it is unclear whether individual skeletal cell co-express both TR isoforms or whether their patterns of expression are cell type specific. Current understanding is that TRα1 is functionally predominant in bone.

T3 action in the developing skeleton

In vitro T3 inhibits chondrocyte proliferation and promotes differentiation (Robson et al. 2000; Shao, et al. 2006) (Fig. 6). Since growth plate architecture and linear growth are frequently disrupted in TR mutant mice key regulators of endochondral ossification have been investigated as targets of thyroid hormone action.

Growth hormone (GH) and Insulin like growth factor 1 (IGF-1R) signalling pathways are thought to act as a point of convergence for many growth promoting factors since more than 80% of somatic growth can be attributable to actions of the GH/IGF-1 axis (Lupu, et al. 2001). IGF-1 is the major determinant of post natal growth, it mediates both GH dependent and independent effects and is implicated in chondrocyte recruitment, proliferation and hypertrophic differentiation (van der Eerden, et al. 2003). Examination of the GH/IGF-1 pathway revealed that GH receptor (GHR) and IGF-1 receptor (IGF-1R) mRNA expression was reduced in growth plate chondrocytes in $TR\alpha^{0/0}$ and $TR\alpha1^{PV/+}$ mice. Furthermore, phosphorylation of their secondary messengers signal tranducer and activator of transcription 5 (STAT5) and protein kinase B (AKT) was also impaired (Bassett et al. 2007b; O'Shea et al. 2005). By contrast, GHR and IFG-1R expression was increased in $TR\beta^{-/-}$ and $TR\beta^{PV/PV}$ mice (Bassett et al. 2007b; O'Shea et al. 2005). Thus, GH/IGF-1 signalling is also a local downstream mediator of T3 action in the growth plate (Fig. 6).

Fibroblast growth factors (FGFs) and their receptors have key roles in skeletal development with activating mutations of FGFR3 causing achondroplasia, the commonest form of genetic dwarfism. In the developing growth plate FGFR3 is expressed in reserve and proliferating chondrocytes and negatively regulates their proliferation and differentiation (Murakami, et al. 2004). By contrast, FGFR1 is expressed in prehypertrophic and hypertrophic chondrocytes and its location suggests a role in differentiation, matrix synthesis and apoptosis (Ornitz 2005). FGFR1 is also expressed in the osteoblast lineage with activating mutations result in Pfeiffer craniosynostosis. Investigation of the FGF/FGFR signalling pathway in TR mutant mice demonstrated *Fgfr3* and *Fgfr1* expression was reduced in growth plates of $TR\alpha^{0/0}$, $TR\alpha1^{R384C/+}$and $TR\alpha1^{PV/+}$ mice and *Fgfr1* expression was reduced in osteoblasts from $TR\alpha^{0/0}$ and $Pax8^{-/-}$ mice (Barnard et al. 2005; Bassett et al. 2007a; Bassett et al. 2007b; Bassett, et al. 2008; O'Shea et al. 2005; Stevens et al. 2003). By contrast, *Fgfr3* and *Fgfr1* expression was increased in growth plates of $TR\beta^{-/-}$ and $TR\beta^{PV/PV}$ mice and *Fgfr1* expression was increased in osteoblasts from $TR\beta^{PV/PV}$ mice (Barnard et al. 2005; Bassett et al. 2007a; Bassett et al. 2007b; O'Shea et al. 2003). Thus, FGF/FGFR signalling is a downstream mediator of T3 action in chondrocytes and osteoblasts (Fig. 6).

The pace of chondrocyte differentiation is precisely regulated by the Indian hedgehog/Parathyroid hormone-related peptide paracrine (Ihh/PTHrP) negative feedback loop. Prehypertrophoic chondrocytes secrete Ihh which diffuses to periarticular cells to induce synthesis of PTHrP. PTHrP, acting via its receptor PTHR1, then completes the loop by stimulating chondrocyte proliferation and inhibiting further hypertrophic differentiation (Dentice, et al. 2005; Vortkamp, et al. 1996). Although this pathway has not been studied in TR mutant mice previous experiments in thyroid-manipulated rats demonstrated increased PTHrP mRNA expression in hypothyroid animals and increased PTHrP receptor mRNA expression in growth plates of thyrotoxic animals (Stevens et al. 2000). Furthermore, recent studies in chicken tibia explants have shown that Ihh stimulates degradation of the type 2 deiodinase enzyme resulting in an induction of PTHrP expression (Dentice et al. 2005). Together these findings suggesting that thyroid hormone can inhibit chondrocyte proliferation and promote differentiation by local regulation of the Ihh/PTHrP negative feedback loop (Fig. 6).

T3 is essential for normal cartilage matrix synthesis. Heparan sulphate proteoglycans (HSPGs) are a key matrix component and are essential for functional FGF/FGFR signalling and extracellular diffusion of Ihh. Studies in thyroid manipulated rats and $TR\alpha^{0/0}\beta^{-/-}$ and $Pax8^{-/-}$ mice revealed reduced HSPG expression in thyrotoxic animals, increased expression in $TR\alpha^{0/0}\beta^{-/-}$mice and more markedly increased expression in hypothyroid rats and congenitally hypothyroid $Pax8^{-/-}$ mice (Bassett et al. 2008).

These studies suggest T3 co-ordinately regulates FGF/FGFR and Ihh/PTHrP signalling within the growth plate.

T3 actions in the bone remodelling cycle

In vitro studies suggest that thyroid hormone stimulates osteoblast proliferation, differentiation and apoptosis by direct and indirect actions. Thus, T3 increases synthesis of osteocalcin, type 1 collagen, alkaline phosphatase and matrix metalloproteinases 9 and 13 (Bassett and Williams 2003; Gouveia, et al. 2001; Huang, et al. 2000; Pereira, et al. 1999) and also regulates IGF-1, parathyroid hormone (PTH) and FGF signalling (Bassett and Williams 2003; Gu, et al. 2001; Huang et al. 2000; O'Shea P et al. 2007; Pepene, et al. 2001; Stevens et al. 2003) (Fig. 6). *In vivo*, activation of FGFR1 stimulates osteoblast proliferation and differentiation (Zhou, et al. 2000). Consistent with this, *Fgfr1* expression in osteoblasts is reduced in the hypothyroid skeleton and increased in thyrotoxic bone (Bassett et al. 2007a; O'Shea et al. 2003; Stevens et al. 2003). Although osteoclasts have been reported to express TRs (Abu et al. 1997; Abu et al. 2000; Allain, et al. 1992; Kanatani et al. 2004) it remains uncertain whether T3 regulates osteoclast differentiation directly or indirectly (Miura, et al. 2002; Siddiqi, et al. 1998; Varga, et al. 2004). Previous studies are contradictory; some demonstrating T3 acts direct in osteoclasts whilst others report indirect effects mediated via osteoblasts.

Future directions

Analyses of TR mutant and congenitally hypothyroid $Pax8^{-/-}$ mice has identified a key role for thyroid hormone and TRα1 in both the developing skeleton and adult bone. However, it remains unclear how the skeletal effects of apo-TRα1 differ from those of TRα1 deficiency and to what extent the skeletal effects of thyroid hormones result from central, systemic and local actions of T3. The more severe skeletal phenotype observed in congenitally hypothyroid $Pax8^{-/-}$ mice as compared with $TR\alpha^{0/0}\beta^{-/-}$ mice suggests that unliganded TRs may be more detrimental to skeletal development than TR deficiency (Bassett et al. 2008; Flamant, et al. 2002; Mansouri, et al. 1998) (Table 1). In support of this, amelioration of the $Pax8^{-/-}$ skeletal phenotype in $Pax8^{-/-}TR\alpha^{0/0}$ mice but not in $Pax8^{-/-}TR\beta^{-/-}$ mice suggests that some of the detrimental effects of hypothyroidism are mediated by unliganded TRα1 in bone (Flamant et al. 2002). Despite this it is important to note from an additional study (Mittag, et al. 2005) that deletion of TRα1 alone in $Pax8^{-/-}TR\alpha1^{-/-}$ mice did not prevent weight loss, early mortality and pituitary abnormalities although the skeletal consequences were not investigated. Thus, it remains possible that TRα2 has an additional and essential role in the manifestation of the $Pax8^{-/-}$ phenotype. This importance of apo-TRα1 is further supported by the more severe skeletal phenotype present in mice harbouring dominant negative mutations of TRα1 ($TR\alpha1^{R384C/+}$ and $TR\alpha1^{PV/+}$) as compared to mice lacking all

TRα isoforms ($TR\alpha^{0/0}$) (Bassett et al. 2007a; Bassett et al. 2007b; O'Shea et al. 2005) and by the amelioration of the adult skeletal phenotype in $TR\alpha1^{R384C/+}$ mice following a transient reversal of TRα1R384C apo-receptor activity during development (Bassett et al. 2007a). Nevertheless, a complete understanding of the molecular mechanism of thyroid hormone action in bone will require at least two experimental approaches. Firstly it is clear that phenotyping of the existing mouse models is incomplete and more detailed studies including quantitative histomorphometry, mechanical testing, and analysis of primary bone cell cultures will help to clarify the picture. However, such an approach cannot identify the cellular targets of T3-action in the skelton *in vivo* and this will require the use of cell specific gene targeting strategies.

Analysis of TRα and TRβ mutant mice has demonstrated the complex relationship between central and peripheral thyroid status and established the predominant role of TRα1 in bone. These studies also highlight contrasting responses of the skeleton to T3 during developing and in adulthood. Although understanding of the molecular mechanism of T3-action in bone is still limited, coordinate regulation of key signalling pathways has now been identified in chondrocytes and osteoblasts. By contrast, the molecular mechanism of T3-action in the osteoclast lineage remains unclear. A more detailed understanding of the molecular basis of T3 action in bone will provide the rational for development of novel strategies for the prevention and treatment of osteoporosis.

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Figure Legends

Figure 1. Thyroid hormone receptor isoforms

Panel A. Schematic representation of the four functional domains of the thyroid hormone receptor.

Panel B. Products of the *Thra* and *Thrb* genes in the mouse. *Thra* has 2 promoters, TRα1 and TRα2 are derived from the 5' promoter whereas TRΔα1 and TRΔα2 are transcribed from an internal promoter in intron 7. TRα2 and TRΔα2 result from alternative splicing. TRα1 is a functional receptor whereas TRΔα1, TRα2 and TRΔα2 do not bind T3 and act as antagonists. *Thrb* also has 2 promoters and both N-terminal variants TRβ1 and TRβ2 bind T3 and act as functional receptors.

Figure 2. Skeletal development and growth in TR mutant mice.

Panel A. Skull vaults from P1 mutant mice and littermate controls stained with alizarin red (bone) and alcian blue (cartilage) showing sutures and anterior and posterior fontanelles. *Arrows* indicate delayed intramembranous ossification in $TR\alpha1^{PV/+}$ and $TR\alpha1^{R384C/+}$ mice and advanced ossification in $TR\beta^{-/-}$ and $TR\beta^{PV/PV}$ mice. Note that two $TR\beta^{-/-}$ strains in different genetic backgrounds were analysed $TR\beta^{-/-}$(a) (Bassett et al. 2007b; Gauthier et al. 2001) and $TR\beta^{-/-}$(b) (Bassett et al. 2007a; Forrest et al. 1996; Ng, et al. 2001). *Scale bar,* 1mm.

Panel B. Graphs of longitudinal growth in mutant mice and littermate controls; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ mean tibia/ulna length in mutant *vs*. control; two-tailed Students's *t* test.

Figure 3. Trabecular bone micro-architecture in adult TR mutant mice.

Panel A. Backscattered electron scanning electron microscopy (BSE-SEM) views showing trabecular bone in thyrotoxic wild type mice treated with daily sc injections of T3 (30ng T3/gram body weight) and congenitally hypothyroid $Pax8^{-/-}$ mice.

Panel B. , animals lacking either TRα or TRβ (panel B) and mice harbouring a dominant-negative mutation of TRα (R384C) (Panel C). *Scale bar*, 200μm.

Figure 4. Trabecular bone micro-mineralisation in adult TR mutant mice.

Quantitative backscattered electron scanning electron microscopy (qBSE-SEM) images showing mineralisation densities of trabecular bone from mice lacking TRβ and mice harbouring a dominant-negative mutation of TRα (R384C) (panel A). Mineralisation densities were derived from halogenated standards and images

pseudocoloured according to the palette shown in which high mineralisation density is grey. Panel B shows relative and cumulative frequency histograms of bone micro-mineralisation densities. *Black arrow* indicates the increased relative frequency of high mineralisation densities which correspond to retained calcified cartilage in TRα1$^{R384C/+}$ mice. Panel C shows higher power views of trabecular bone. *Dashed* w*hite arrows* indicate normal bone cement lines. W*hite solid arrows* indicate retained calcified cartilage within which the outlines of chondrocyte lacunae remain evident. *Scale bar* 200μm in all panels. ***, P<0.001 micromineralisation density in mutant *vs.* WT; Kolmogorov-Smirnov test.

Figure 5. Proposed molecular mechanism for TRα and TRβ mutant mice.

The pituitary expresses predominantly TRβ. T3 and TRβ act via a negative TRE to repress TSH transcription. The skeleton expresses predominantly TRα1 and T3 acts via TRα1 to activate target gene expression. Since the levels of TRα in the pituitary are low its absence in TRα$^{0/0}$ mice does not disrupt pituitary negative feedback and systemic TSH, T4 and T3 concentrations are normal. By contrast, because TRα predominates in bone its absence in TRα$^{0/0}$ mice results in impaired T3 responsiveness, skeletal hypothyroidism and reduced expression of T3-target genes. Similarly, in TRα1$^{PV/+}$ and TRα1$^{R384C/+}$ mice the levels of the dominant negative TRα receptor in the pituitary are insufficient to interfere with TRβ and disrupt TSH repression. However, in bone the higher concentrations of mutant receptor interfere with the actions of wild type TRα1. Thus, T3 responses are severely impaired and the skeleton is hypothyroid. In TRβ$^{-/-}$ mice, by contrast, the absence of TRβ disrupts pituitary T3 responses and impairs TSH repression leading to high circulating TSH, T4 and T3 concentrations. Since TRβ levels are low in bone T3 responses are unaffected in TRβ$^{-/-}$ mice and high circulating levels of thyroid hormone act via wild type TRα1 to induce skeletal thyrotoxicosis. Similarly in TRβ$^{PV/PV}$ mice the dominant negative TRβ disrupts TSH repression in the pituitary and results in grossly elevated TSH, T4 and T3 concentrations. The low levels of the mutant receptor in bone are insufficient to interfere with TRα1 and impair T3 responses and thus high circulating levels of thyroid hormone increase expression of T3-target genes resulting in severe skeletal thyrotoxicosis.

Figure 6. Role of thyroid hormone in endochondral ossification and bone remodelling.

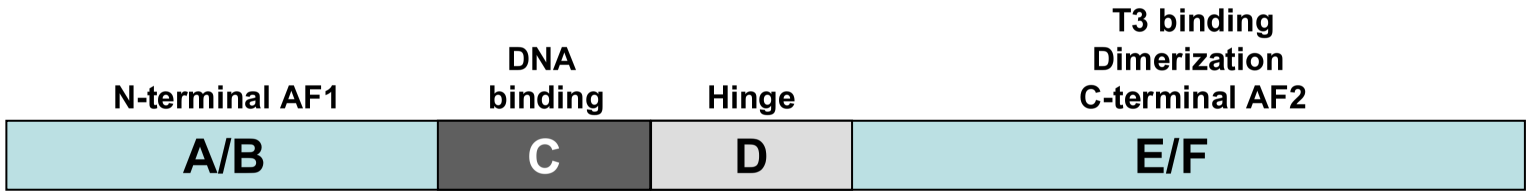
Panel A illustrates the role of T3 in the growth plate and shows the pattern of TR expression. Reserve cells undergo clonal expansion to form columns of proliferating chondrocytes which secrete cartilage matrix stained

blue in this section. Committed prehypertrophoic chondrocytes undergo hypertrophic differentiation, enlarge and subsequently apoptose, the residual mineralised cartilage matrix forming the scaffold for trabecular bone formation. In chondrocyte cultures T3 inhibits proliferation and increases expression of cyclin dependent kinase inhibitors $p21^{cip-1}$/$p27^{kip1}$. T3 stimulates matrix synthesis and expression of the differentiation markers collagen X, alkaline phosphatase (ALP), matrix metalloproteinase 13 (MMP13) and aggrecanase 2. Growth hormone/insulin like growth factor 1 (GH/IGF-1), Indian hedgehog/parathyroid hormone-related peptide (Ihh/ PTHrP) and fibroblast growth factor/ fibroblast growth factor receptor (FGF/FGFR) pathways are T3 targets. Expression of heparan sulphate proteoglycans (HSPGs), which are essential for FGF and Ihh signalling, is negatively regulated by T3.

Panel B shows the role of T3 in the bone remodelling cycle, which is characterised by osteoclastic bone resorption followed by osteoblast migration, osteoid matrix deposition and mineralisation. Osteoclast differentiation requires direct osteoblast-osteoclast interaction mediated by receptor activator of nuclear factor-κB (RANK) and its ligand RANKL. Osteoprotegerin (OPG) is a decoy receptor secreted by osteoblasts that antagonises this interaction. Osteoclast differentiation also requires co-stimulation by osteoblast derived cytokines. Thyroid hormone stimulates osteoblast proliferation, differentiation and apoptosis directly and also indirectly by regulating IGF-1 and FGF signalling. It remains uncertain whether osteoclast differentiation is regulated directly by T3 or indirectly via T3 actions in osteoblasts.

Figure 1

A


N-terminal AF1
DNA binding
Hinge
T3 binding
Dimerization
C-terminal AF2
A/B
C
D
E/F

B

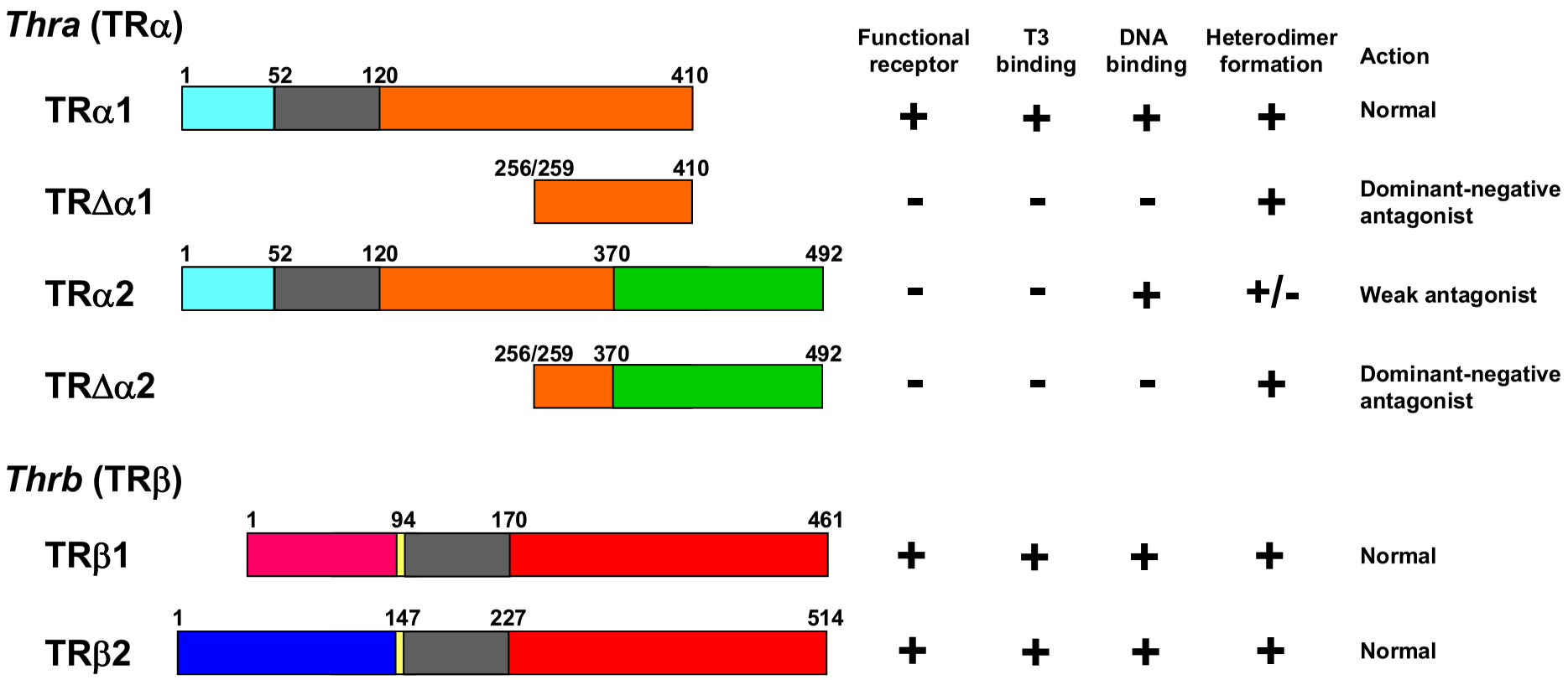

Thra (TRα)
Functional receptor
T3 binding
DNA binding
Heterodimer formation
Action
TRα1
1 52 120 410
+ + + +
Normal
TRΔα1
256/259 410
- - - +
Dominant-negative antagonist
TRα2
1 52 120 370 492
- - + +/-
Weak antagonist
TRΔα2
256/259 370 492
- - - +
Dominant-negative antagonist
Thrb (TRβ)
TRβ1
1 94 170 461
+ + + +
Normal
TRβ2
1 147 227 514
+ + + +
Normal

Figure 2

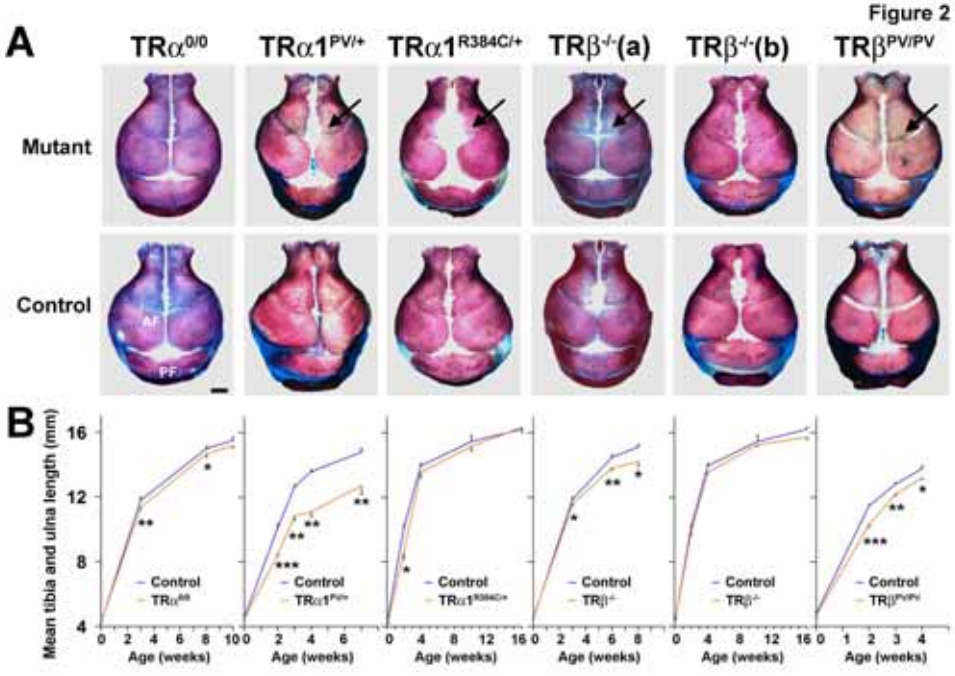


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Figure 3

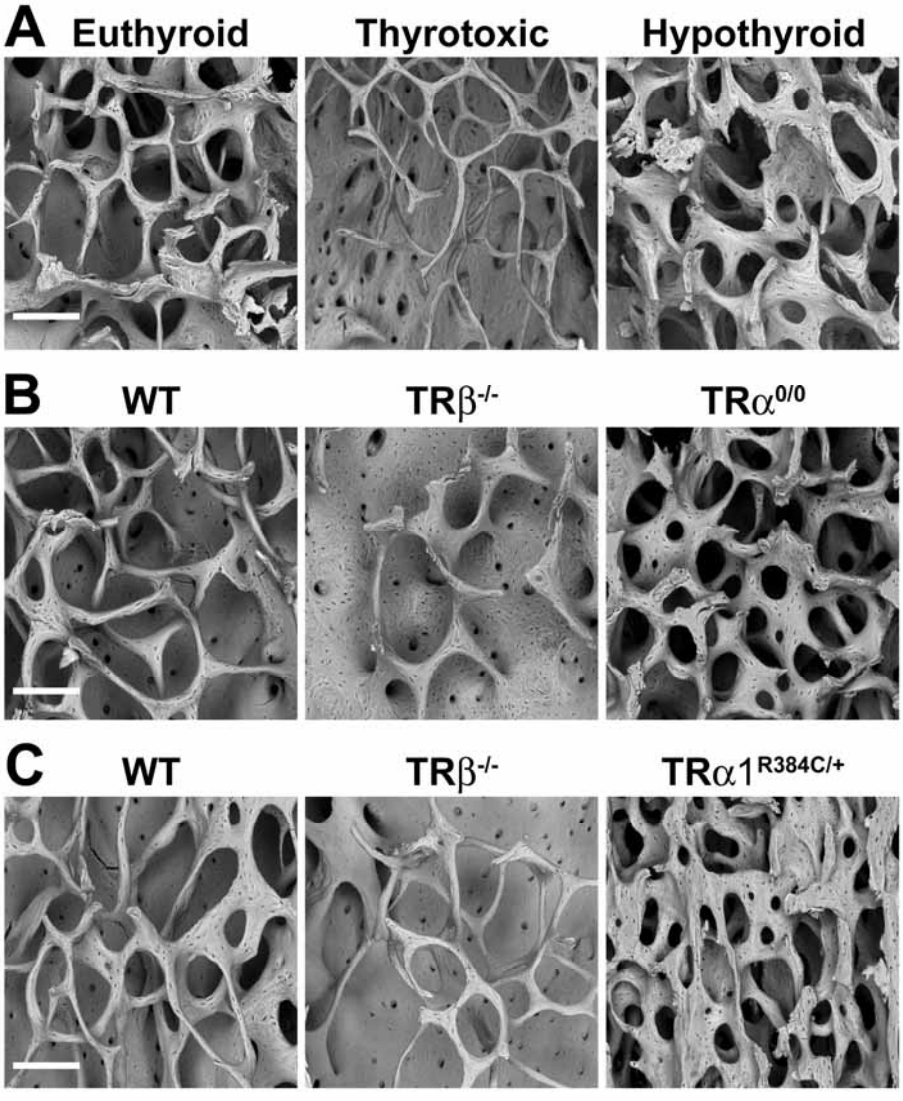

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Figure 4

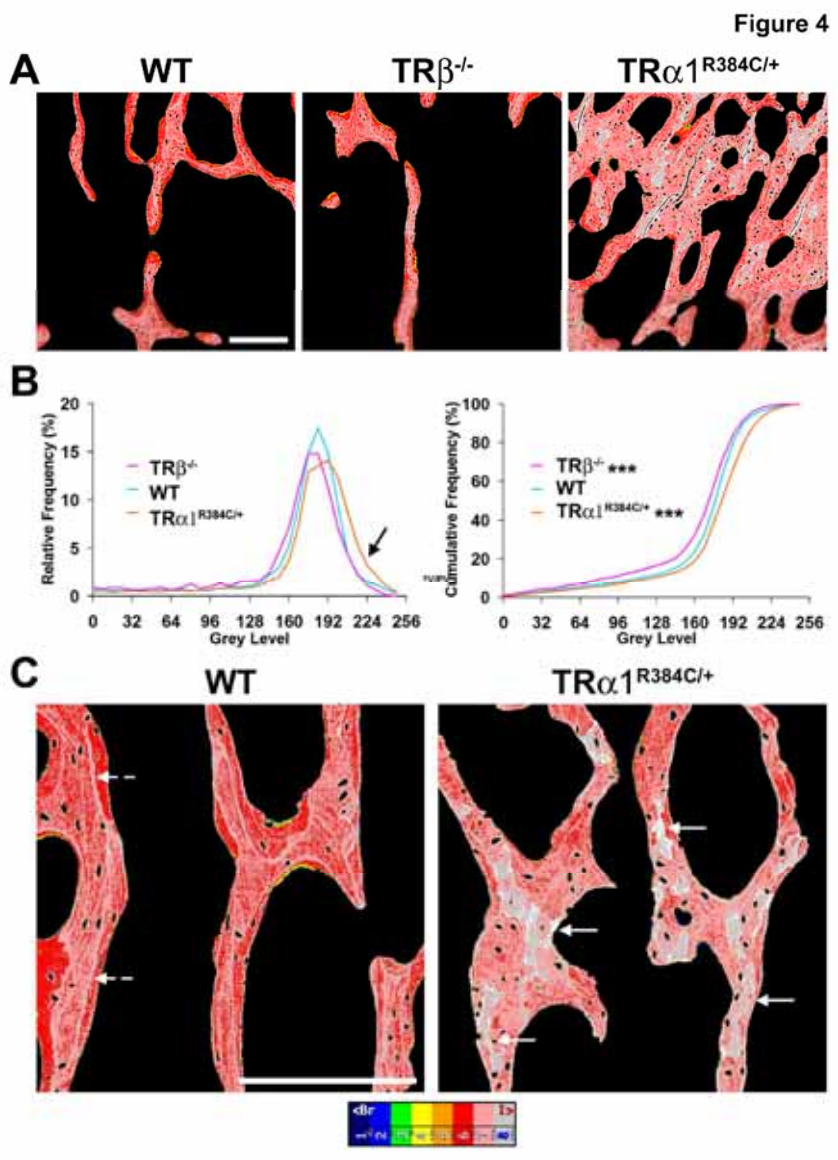

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Figure 5

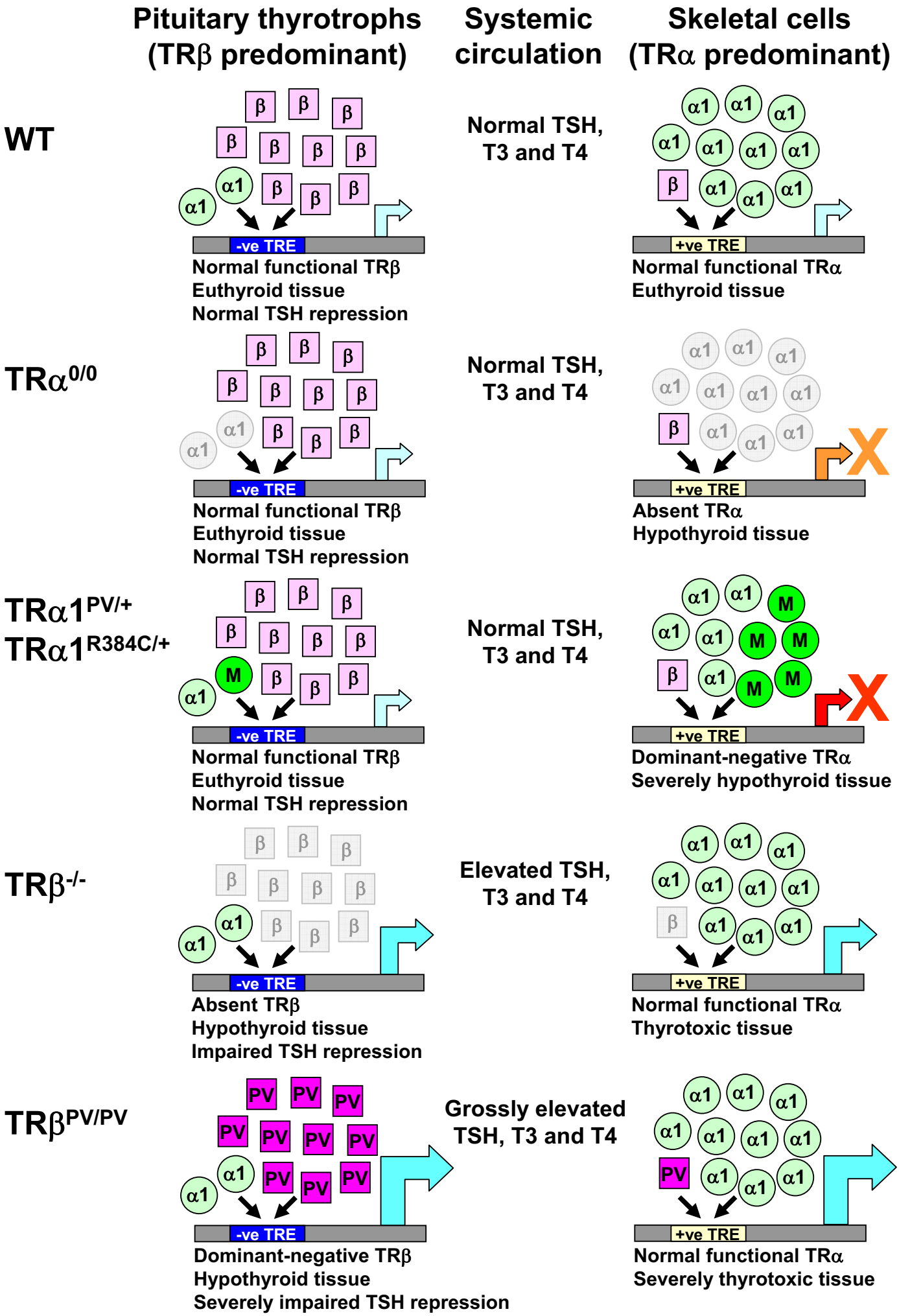
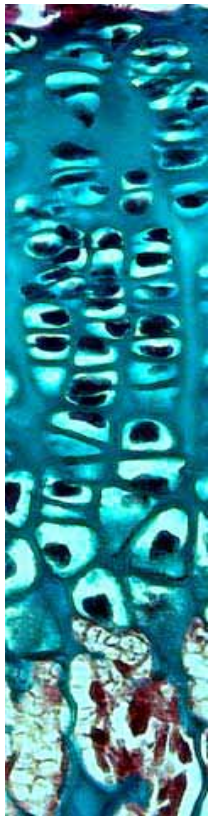

Pituitary thyrotrophs (TRβ predominant)
Systemic circulation
Skeletal cells (TRα predominant)
WT
Normal TSH, T3 and T4
-ve TRE
+ve TRE
Normal functional TRβ
Euthyroid tissue
Normal TSH repression
Normal functional TRα
Euthyroid tissue
TRα0/0
Normal TSH, T3 and T4
-ve TRE
+ve TRE
Normal functional TRβ
Euthyroid tissue
Normal TSH repression
Absent TRα
Hypothyroid tissue
TRα1PV/+
TRα1R384C/+
Normal TSH, T3 and T4
-ve TRE
+ve TRE
Normal functional TRβ
Euthyroid tissue
Normal TSH repression
Dominant-negative TRα
Severely hypothyroid tissue
TRβ-/-
Elevated TSH, T3 and T4
-ve TRE
+ve TRE
Absent TRβ
Hypothyroid tissue
Impaired TSH repression
Normal functional TRα
Thyrotoxic tissue
TRβPV/PV
Grossly elevated TSH, T3 and T4
-ve TRE
+ve TRE
Dominant-negative TRβ
Hypothyroid tissue
Severely impaired TSH repression
Normal functional TRα
Severely thyrotoxic tissue

Figure 6

A

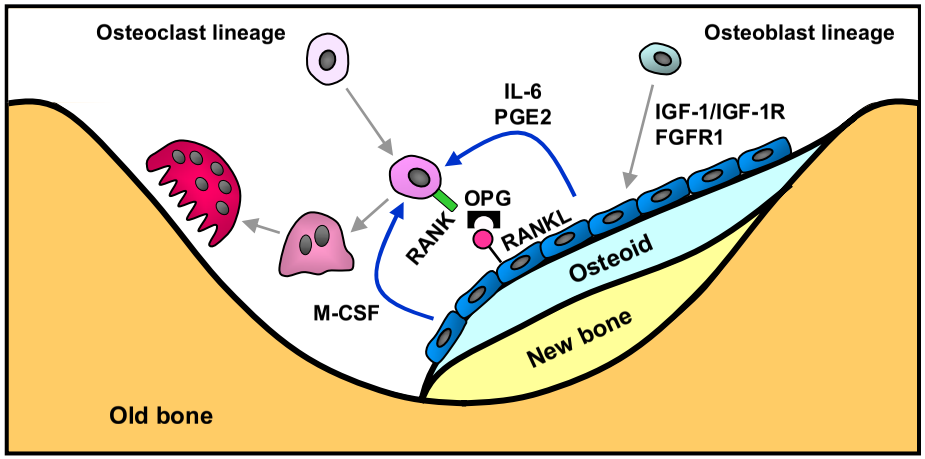
Endochondral ossification



T3 action on critical signalling pathways	Growth plate	TR expression	Direct T3 action
↑GHR, ↑IGF-1R → Increased recruitment		Reserve zone (TRα1/α2/β1)	Stimulates recruitment
↓PTHrP, ↓PTHR1 → Reduced proliferation		Proliferative zone (TRα1/α2/β1)	Inhibits Proliferation Expression of *HSPGs*
↑FGFRs		Prehyper-trophic zone (TRα1/α2/β1)	Simulates Differentiation Matrix synthesis Expression of $p21^{cip-1}$/$p27^{kip1}$ *Collagen X* *ALP* *MMP13* *Aggrecanase-2*
↑GHR, ↑IGF-1R → Increased differentiation		Hypertrophic zone (No TRs)	
		Primary spongiosum (TRα1/α2/β1)	

B

Bone remodelling cycle



Bone resorption | **Bone formation**

Osteoclasts (may express TRs)

T3 may have direct actions in osteoclasts

T3 may simulates osteoclastogenesis by its action in osteoblasts

T3 stimulates expression of *IL-6 and PGE2*

Osteoblasts (express TRα1/α2/β1)

T3 stimulates Osteoblast proliferation, differentiation and apoptosis Expression of *FGFR1* *IGF-1/IGF-1R* *Osteocalcin* *Collagen I* *ALP* *MMP9, MMP13*

Table 1. Skeletal phenotypes of thyroid hormone receptor mutant mice

Model	Genotype	Endocrine status	Juvenile skeleton	Adult skeleton	Skeletal thyroid status	Refs
TRα mutants						
TRα$^{-/-}$	No TRα1/α2 TRΔα1 and Δα2 preserved	Hypothyroid (T_4 0.1x T_3 0.4x, TSH 2x) GH normal	Severe growth delay Delayed endochondral ossification Impaired chondrocyte differentiation Reduced mineralisation	Die by weaning unless T_3 treated	NR	Chassande et al. 1997 Fraichard et al. 1997 Gauthier et al. 1999
TRα1$^{-/-}$	No TRα1/Δα1 TRα2/Δα2 preserved	Mild Hypothyroidism (T_4 0.7x T_3 1x, TSH 0.8x)	No growth retardation of skeletal phenotype	NR	NR	Wikstrom et al. 1998
TRα2$^{-/-}$	No TRα2/Δα2 TRα1/Δα1 preserved TRα1 over-expression	Mild Hypothyroidism (T_4 0.8x T_3 0.7x, TSH 1x) GH normal IGF1 low	No growth retardation Normal endochondral ossification	Reduced bone mineral density Reduced cortical bone	NR	Salto et al. 2001
TRα$^{0/0}$	No TRα transcripts TRβ unaffected	Euthyroid Normal GH and IGF1	Transient growth delay Delayed endochondral ossification Impaired chondrocyte differentiation Reduced mineralisation	Osteosclerosis Increased trabecular bone volume Reduced osteoclastic bone resorption	Hypothyroid	Bassett et al. 2007b Gauthier et al. 2001
TRα1$^{PV/+}$	Heterozygous dominant-negative TRα receptor	Mild thyroid failure (T_4 1x T_3 1.2x, TSH 1.7x) GH normal	Severe persistent growth retardation Delayed intramembranous and endochondral ossification Impaired chondrocyte differentiation Reduced mineralisation	NR	Hypothyroid	Kaneshige et al. 2001 O'Shea et al. 2005
TRα1$^{R384C/+}$	Heterozygous dominant-negative TRα receptor (10-fold reduced affinity for T3)	Euthyroid adults Mild hypothyroidism P10-35 (T_4 0.8x T_3 0.7x, TSH 0.7x) (GH reduced in juveniles)	Transient growth delay Delayed intramembranous and endochondral ossification Impaired chondrocyte differentiation	Osteosclerosis Increased trabecular bone volume Increased mineralisation Reduced osteoclastic bone resorption	Hypothyroid	Bassett et al. 2007a Tinnikov et al. 2002
TRα1$^{R398H/+}$	Heterozygous dominant-negative TRα receptor	Euthyroid (T_4 1.1x T_3 1.1x, TSH 3.4x)	NR	NR	NR	Liu et al. 2003
TRα1$^{AMI/+}$ SYCP1-Cre	Cre inducible dominant-negative receptor TRα1^{L400R} (Early global expression)	Euthyroid (T_4 1x T_3 0.9x, TSH 0.3x) Reduced GH (0.4x)	Severe persistent growth retardation Delayed endochondral ossification	NR	NR	Quignodon et al. 2007
TRβ mutants						
TRβ$^{-/-}$	No TRβ transcripts TRα unaffected	RTH and goitre (T_4 4x T_3 4x, TSH 8x)[1] (T_4 3x T_3 3x, TSH 2.6x)[2]	Persistent short stature Advanced endochondral and intramembranous ossification Increased mineralisation	Osteoporosis Reduced mineralisation Increased osteoclastic bone resorption	Thyrotoxic	Bassett et al. 2007a Bassett et al. 2007b Forrest et al. 1996 [1]Gauthier et al. 2001 [2]Ng et al. 2001
TRβ2$^{-/-}$	No TRβ2 TRβ1 preserved	Mild RTH (T_4 3x T_3 1.3x, TSH 2.5x)[1] Small decrease in GH (T_4 1x T_3 1.5x, TSH 1.2x)[2]	No reported growth abnormality	NR	NR	[1]Abel, et al 1999 [2]Ng et al. 2001
TRβ$^{PV/PV}$	Homozygous dominant-negative	Severe RTH and goitre (T_4 15x T_3 9x, TSH >400x) Reduced GH	Accelerated prenatal growth Persistent postnatal growth retardation Advanced intramembranous and	NR	Thyrotoxic	Bassett et al. 2007a Kaneshige, et al. 2000 O'Shea et al. 2005

	TRβ receptor		endochondral ossification Increased mineralisation			O'Shea et al. 2003
TRβ$^{\Delta 377I/\Delta 377I}$	Homozygous dominant-negative TRβ receptor	Severe RTH and goitre (T_4 15x T_3 10x, TSH >50x)	No growth phenotype	NR	NR	Hashimoto et al. 2001
TRα and TRβ compound mutants						
TRα$^{-/-}$β$^{-/-}$	No TRα1/α2 or TRβ TRΔα1/Δα2 preserved	RTH and small goitre (T_4 10x T_3 10x, TSH >100x)	Growth delay similar to TRα$^{-/-}$ Delayed endochondral ossification Impaired chondrocyte differentiation Reduced mineralisation	Die at or near weaning	NR	Bassett et al. 2007b Gauthier et al. 1999
TRα1$^{-/-}$β$^{-/-}$	No TRα1/Δα1 or TRβ TRα2/Δα2 preserved	RTH and large goitre (T_4 60x T_3 60x, TSH >160x) Reduced GH/IGF1	Persistent growth retardation Delayed endochondral ossification Reduced mineralization	Reduced trabecular and cortical bone mineral density. GH treatment corrects growth but not ossification defect	NR	Gothe, et al. 1999 Kindblom et al. 2005 Kindblom et al. 2001 O'Shea et al. 2005 O'Shea et al. 2003
TRα2$^{-/-}$β$^{-/-}$	No TRα2/Δα2 or TRβ TRα1/Δα1 preserved TRα1 over-expression	Mild Hypothyroidism (T_4 0.7x T_3 0.8x, TSH 1x)	Transient growth delay	NR	NR	Ng et al. 2001
TRα$^{0/0}$β$^{-/-}$	No TRα/TRβ transcripts	RTH and goitre (T_4 13x T_3 14x, TSH >200x) Reduced GH/IGF1	More severe phenotype than TRα$^{0/0}$ Growth delay Delayed endochondral ossification Impaired chondrocyte differentiation Reduced mineralisation	NR	Hypothyroid	Flamant et al. 2002 Gauthier et al. 2001
TRα, TRβ and Pax8 compound mutants						
Pax8$^{-/-}$	Apo TRα and TRβ receptors	No Thyroid (No T_4 or T_3, TSH 1900x)	More severe growth defect than TRα$^{0/0}$β$^{-/-}$ Severe persistent growth retardation Severely delayed endochondral ossification Impaired chondrocyte differentiation	Majority die by weaning Coarse plate like trabeculae with impaired trabecular remodeling Reduced cortical thickness Reduced mineralisation	Hypothyroid	Bassett et al. 2008 Flamant et al. 2002 Mansouri et al. 1998
Pax8$^{-/-}$TRα1$^{-/-}$	No T_3 or TRα1/Δα1 TRα2/Δα2 preserved Apo TRβ receptors	No Thyroid (No T_4 or T_3, TSH NR)	Severe growth retardation similar to Pax8$^{-/-}$ Skeletal phenotype not reported	Die by weaning	NR	Mittag et al. 2005
Pax8$^{-/-}$TRα$^{0/0}$	No T_3 or TRα Apo TRβ receptors	No Thyroid (No T_4 or T_3, TSH >400x)	Growth retardation less than Pax8$^{-/-}$ and similar to TRα$^{0/0}$β$^{-/-}$ Delayed endochondral ossification Mice survive to adulthood	NR	NR	Flamant et al. 2002
Pax8$^{-/-}$TRβ$^{-/-}$	No T_3 or TRβ Apo TRα receptors	No Thyroid (No T_4 or T_3, TSH >400x)	Severe growth retardation similar to Pax8$^{-/-}$ Severely delayed endochondral ossification	Die by weaning	NR	Flamant et al. 2002

Abbreviations: TR, thyroid hormone receptor T4, thyroxine T3, triiodothyronine, TSH, thyroid-stimulating hormone NR, not reported GH, growth hormone IGF-1, insulin like growth factor 1 RTH, resistance to thyroid hormone P10-35, postnatal day 10 to 35.