TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling

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Bone resorption and remodeling is an intricately controlled, physiological process that requires the function of osteoclasts. The processes governing both the differentiation and activation of osteoclasts involve signals induced by osteoprotegerin ligand (OPGL), a member of tumor necrosis factor (TNF) superfamily, and its cognate receptor RANK. The molecular mechanisms of the intracellular signal transduction remain to be elucidated. Here we report that mice deficient in TNF receptor-associated factor 6 (TRAF6) are osteopetrotic with defects in bone remodeling and tooth eruption due to impaired osteoclast function. Using in vitro assays, we demonstrate that TRAF6 is crucial not only in IL-1 and CD40 signaling but also, surprisingly, in LPS signaling. Furthermore, like TRAF2 and TRAF3, TRAF6 is essential for perinatal and postnatal survival. These findings establish unexpectedly diverse and critical roles for TRAF6 in perinatal and postnatal survival, bone metabolism, LPS, and cytokine signaling.

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Members of the tumor necrosis factor receptor (TNFR) superfamily are known to mediate important and diverse physiological functions, including apoptosis, osteoclastogenesis, and immune system regulation (Liu et al. 1996; Anderson et al. 1997; Lacey et al. 1998). The specific biological outcomes governed by these cell surface receptors are dependent not only on stimulation by their cognate ligands but also on various cytoplasmic signal transducing proteins (Liu et al. 1996; Arch et al. 1998). TNFR-associated factors (TRAFs) have been implicated in mediating signals induced by a subset of TNFR family members, including TNFR1, TNFR2, LTβR, CD30, and CD40 (Arch et al. 1998). Members of the TRAF family of proteins are characterized by a conserved carboxy-terminal TRAF-C domain, a coiled-coil TRAF-N domain and, excluding TRAF1, an amino-terminal ring finger domain (Rothe et al. 1994; Cao et al. 1996b). The TRAF-C domain mediates interactions among TRAF proteins as well as their association with members of the TNFR superfamily. The ring finger domain is thought to be essential for downstream signaling (Rothe et al. 1995; Cao et al. 1996a; Takeuchi et al. 1996).

Several members of the TRAF family, including TRAF2, TRAF5, and TRAF6 have been implicated in regulating signals from various TNFR family members, leading to the activation of nuclear factor-κB (NF-κB) (Cheng et al. 1995; Rothe et al. 1995; Ishida et al. 1996a,b; Nakano et al. 1996; Hsu et al. 1997; Marsters et al. 1997; Song et al. 1997; Muzio et al. 1998). NF-κB is a ubiquitously expressed transcription factor that controls a plethora of genes, including those involved in regulating apoptosis, immune responses, and embryonic development. For example, signaling of CD40, a receptor expressed on B lymphocytes and certain accessory cells such as dendritic cells (Banchereau et al. 1994), involves the recruitment of TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 (Pullen et al. 1998). Although the physiological roles of individual TRAF proteins in CD40 signaling remain to be clarified, ex vivo data have suggested a potentially distinguished role for TRAF6. Of the six TRAFs
described to date, TRAF6 shows the least homology to the prototypical TRAF domain sequence (Cao et al. 1996b). TRAF6 binds to a cytoplasmic domain of CD40, which is distinct from that containing the binding sites for TRAF1, TRAF2, TRAF3, and TRAF5 (Ishida et al. 1996a). Furthermore, the TRAF6-binding region is thought to be required for CD40-mediated NF-κB activation (Cao et al. 1996b). TRAF6 binds to a cytoplasmic domain of CD40, which is distinct from that containing the binding sites for TRAF1, TRAF2, TRAF3, and TRAF5 (Ishida et al. 1996a). TRAF6 binds to a cytoplasmic domain of CD40, which is distinct from that containing the binding sites for TRAF1, TRAF2, TRAF3, and TRAF5 (Ishida et al. 1996a). TRAF6 binds to a cytoplasmic domain of CD40, which is distinct from that containing the binding sites for TRAF1, TRAF2, TRAF3, and TRAF5 (Ishida et al. 1996a).

Another unique feature of TRAF6 is that unlike other TRAFs, it has also been implicated in interleukin-1 (IL-1) signaling (Cao et al. 1996b), leading to the activation of NF-κB. Overexpression of a dominant-negative mutant of TRAF6 in human 293 cells was found to inhibit IL-1-induced NF-κB activation (Cao et al. 1996b). IL-1 is a multifunctional, pro-inflammatory cytokine, which can signal through the IL-1 type 1 receptor (IL-1R1) (Dinarello 1996). Key players involved in this transduction pathway have been identified recently (Huang et al. 1997; Malinin et al. 1997; Muzio et al. 1997; Wesche et al. 1997a), and TRAF6 mediates the signal by interacting with the IL-1-receptor-associated kinase (IRAK) (Cao et al. 1996a; Muzio et al. 1997; Wesche et al. 1997b).

To date, the physiologic roles of two TRAF family members have been determined by gene targeting (Xu et al. 1996; Yeh et al. 1997). Both TRAF2- and TRAF3-deficient mice appear normal at birth but become progressively runted and die prematurely. TRAF2-deficient mice have elevated levels of serum TNF. Ex vivo assays demonstrated that TRAF2−/− cells were highly sensitive to TNF-induced cell death and, in the absence of TRAF2, TNF-mediated JNK/SAPK activation was greatly reduced (Yeh et al. 1997). On the other hand, reconstitution of mice with TRAF3−/− fetal liver cells revealed a requirement for TRAF3 in T-dependent immune responses (Xu et al. 1996).

To investigate the physiologic role of TRAF6, we have generated TRAF6-deficient mice. Analyses of these mutant mice indicate that TRAF6 is essential for both perinatal and postnatal survival. Surprisingly, TRAF6 deficient mice exhibit osteopetrosis. Furthermore, TRAF6-deficient cells showed defective responses when treated with lipopolysaccharide (LPS). Our results not only confirm the crucial role of TRAF6 in IL-1 and CD40 signaling but also reveal an unexpected requirement for TRAF6 in both LPS signaling and bone metabolism.

### Results

#### Generation of TRAF6-deficient mice

The murine trif6 gene was disrupted by introducing a targeted mutation into embryonic stem (ES) cells of 129J background (Fig. 1A). Two independent heterozygous (TRAF6+/−) mutant ES cell lines were microinjected into C57BL/6 (B6) blastocysts, which were subsequently implanted into pseudopregnant CD-1 (ICR) recipients. Male chimeras generated from both cell lines were used to generate TRAF6+/− mice, which were intercrossed to produce TRAF6+/− mice. Homozygous mutants derived from either ES cell line had identical phenotypes. Homologous recombination was confirmed by Southern blot analysis (Fig. 1B). It has been shown previously by Northern blot analysis that TRAF6 is highly expressed in murine kidney (Ishida et al. 1996a). Western blot analysis using a TRAF6-specific antibody showed that TRAF6 protein levels were undetectable in lysates of TRAF6−/− kidney, indicative of a null mutation (Fig. 1C).

**TRAF6 is required for perinatal and postnatal survival**

When the first 327 live pups of heterozygous intercrosses were examined, only 11% were genotyped as TRAF6−/−.
Table 1. Genotypic analysis of offspring derived from TRAF6 heterozygous intercrosses

| Stage        | No. per genotype | Total | Percentages |
|--------------|------------------|-------|-------------|
| E14.5        | +/+ 5            | 15    | 27          |
|              | +/− 6            |       |             |
|              | −/− 4            |       |             |
| E17.5        | +/+ 8            | 32    | 22          |
|              | +/− 17           |       |             |
|              | −/− 7            |       |             |
| 2 weeks old  | +/+ 104          | 327   | 11          |
|              | +/− 187          |       |             |
|              | −/− 36           |       |             |

Embryos of various stages of gestation were obtained by setting up timed breedings between TRAF6+/− males and females (129 × B6 background). E14.5 was defined as 14.5 days after conception. Viable pups (n = 327) were genotyped −2 weeks after birth. Genotypes were determined primarily by PCR analysis. To confirm the validity of the PCR results, genomic Southern blots were performed in parallel for the first several litters.

Role of TRAF6 in bone metabolism and LPS signaling

To further investigate the role of TRAF6 in LPS and cytokine signaling, thioglycollate-elicited peritoneal (PMφ) and naive bone marrow (BMMφ) macrophages derived from TRAF6+/− and wild-type littermates were harvested and treated with either IL-1β, IFNγ, or TNFα in various combinations or with increasing doses of LPS. Treatment of wild-type PMφ macrophages with TNFα (not shown), IL-1β, or IFNγ alone does not induce iNOS [inducible nitric oxide synthase] significantly; however, a dramatic synergistic induction of iNOS activity occurred when the cells were treated with either IL-1β or TNFα in combination with IFNγ [Fig. 4B]. PMφ macrophages derived from TRAF6−/− mice were unresponsive to IL-1β but responded like the wild type to TNFα plus IFNγ [Fig.,]
iNOS was induced in a dose-dependent manner in wild-type BMMΦ macrophages following treatment with increasing concentrations of LPS (Fig. 4C, left). In contrast, TRAF6−/− BMMΦ macrophages were impaired in their ability to induce iNOS, even at high doses of LPS (Fig. 4C, left). This defect was specific to the LPS signaling pathway, because the combination of TNFα plus IFNγ was able to activate iNOS in TRAF6−/− mice. Most TRAF6−/− osteoclasts are withdrawn from the bone surface, whereas osteoclasts in wild type mice are attached to the bone. Magnification 60×. (D) The number of TRAP+ osteoclasts per mm² tissue area in TRAF6−/−, TRAF6+/−, and TRAF6+/+ mice is comparable.

TRAF6 is required for IL-1, CD40, and LPS-induced activation of NF-κB and IL-1-mediated JNK/SAPK activation

Because signaling through IL-1, CD40, or LPS ultimately leads to NF-κB activation, we examined the activation of this transcription factor in various cell types. NF-κB activation was determined in primary embryonic fibroblasts (EFs), splenocytes, and Abelson-transformed pre-B cell lines derived from TRAF6−/− and wild-type mice. NF-κB activation in EF cells was induced by treatment with either IL-1β or TNFα, whereas splenocytes and Abelson-transformed pre-B cells were stimulated with anti-CD40 or LPS. Electrophoretic mobility shift assays (EMSAs) demonstrated that NF-κB activation was impaired in TRAF6−/− EF cells or splenocytes treated with either IL-1β or CD40 but not in mutant cells treated with TNFα (Fig. 5A,B). LPS-induced NF-κB activation in TRAF6−/− Abelson pre-B cells was reduced significantly compared to the wild type (Fig. 5C).

Although overexpression of TRAF6 was shown previously to activate JNK/SAPK (Song et al. 1997), a possible role for TRAF6 in IL-1-mediated JNK/SAPK activation has not been demonstrated clearly (O’Neill and Greene 1998). Here we show an absence of JNK/SAPK activation in TRAF6−/− EF cells treated with IL-1β [Fig. 5D]. Because another stress stimulus, anisomycin, was still able
to activate JNK/SAPK in TRAF6−/− cells (Fig. 5D), TRAF6 appears to be specifically required for the IL-1 pathway of JNK/SAPK activation. Together, these results define crucial roles for TRAF6 in signaling directly related to the activation of both NF-κB and JNK/SAPK.

**Discussion**

A common feature of all TRAF knockout mice generated to date is their failure to thrive normally and survive until adulthood. Apart from this similarity, each TRAF knockout mouse is phenotypically distinct, as demonstrated by the marked differences between TRAF6-, TRAF2- and TRAF3-deficient mice (Xu et al. 1996; Yeh et al. 1997). The results of this study demonstrate that TRAF6 is an important transducer in not only IL-1 and CD40 signaling (as had been suggested by previous ex vivo work) but also in LPS signaling. In addition, we have discovered a novel physiologic role for TRAF6 in the regulation of bone metabolism.

**Osteopetrosis in TRAF6-deficient mice**

Unlike the reported TRAF2 or TRAF3 knockout mice, TRAF6-deficient mice exhibited osteopetrosis. In most other osteopetrotic mice, such as the p50/p52 double mutant (Franzoso et al. 1997), the c-Fos mutant (Wang et al. 1992; Grigoriades et al. 1994), and PU.1-deficient mice (Tondravi et al. 1997), osteopetrosis is attributed to defects in the differentiation of hematopoietic precursors into mature osteoclasts. In contrast, mice deficient for the tyrosine kinase c-src (Soriano et al. 1991) are osteopetrotic because of a defect in osteoclast function rather than development. The osteopetrotic phenotype of TRAF6−/− mice is similar to that of c-src-deficient mice. Both mutants have normal numbers of mature osteoclasts, and in limited contact with the underlying bone. In mouse. The cell is in contact with the mineralized bone, forms an adhesion zone (asterisk), and has a partial and disorganized ruffled border (large arrowhead). Some resorption is evidenced by the dissolved bone material (arrow) and the cytoplasmic vacuolization of the cell. However, on most of the bone surface covered, there is no ruffled border formation and no bone resorption (line of arrowheads). Size bar, 5 µm.

**Figure 3.** Electron microscopy of osteoclasts in TRAF6−/− and TRAF6+/− mice. (A) TRAF6+/− osteoclast seen in a resorption lacuna. The depicted cell exhibits an attachment zone (asterisk), cytoplasmic vacuolization, and ruffled border (arrowhead), features of a normally activated, mineral-resorbing (arrow) osteoclast. (B) This cell illustrates the typical osteoclast in TRAF6−/− mice. There is no evidence of activation or mineral resorption, the cell forms no attachment zone or ruffled border (arrowhead), and is in limited contact with the underlying bone. (C) One of the few activated osteoclasts in a TRAF6−/− mouse. The cell is in contact with the mineralized bone, forms an adhesion zone (asterisk), and has a partial and disorganized ruffled border (large arrowhead). Some resorption is evidenced by the dissolved bone material (arrow) and the cytoplasmic vacuolization of the cell. However, on most of the bone surface covered, there is no ruffled border formation and no bone resorption (line of arrowheads). Size bar, 5 µm.
The role of TRAF6 during osteoclast maturation and activation processes is not known but is likely to involve signal transduction from an osteoclast surface receptor. On the basis of what is known about osteoclast biology, a candidate signaling receptor is RANK (receptor activator of NF-κB), a novel TNFR family member related most closely to CD40 (Anderson et al. 1997). RANK has recently been localized to the surface of osteoclast progenitor cells (Hsu et al. 1999). The ligand for RANK, also known as osteoprotegerin ligand (OPGL), is essential for both osteoclast differentiation and activation (Lacey et al. 1998; Kong et al. 1999). Most recently, it was demonstrated that multiple TRAF proteins, including TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 can interact with the carboxy terminal of RANK (Darnay et al. 1998; Galibert et al. 1998; Wong et al. 1998; Hsu et al. 1999). However, the physiological contribution of each TRAF protein to RANK signaling remains to be elucidated.

Given that TRAF6 and OPGL are believed to act via the same signaling receptor, namely RANK, one would expect both mutant mice to be phenotypically similar. On the contrary, mice deficient in OPGL differ from TRAF6−/− mice, in that the former are completely devoid of osteoclasts [Kong et al. 1999]. There are two possible mechanisms that may account for this discrepancy. First, in addition to acting on RANK, OPGL may act on some heretofore uncharacterized receptor that specifically mediates the generation of osteoclasts independently of TRAF6. Future studies examining the physiological functions of RANK using gene targeting techniques are required to address this issue. Alternatively, it is also possible that RANK is the only receptor for OPGL, but its downstream signaling diverges. In this scenario, the signal[s] leading to osteoclast generation may be compensated by other molecules such as TRAF2 and/or TRAF5, where TRAF6 is indispensable in transducing signals mediating mature osteoclast functions. Future studies aimed at generating mutant mice deficient in multiple TRAF proteins will help answer these questions.

The osteoclast-activating role of OPGL has been examined previously in vitro (Fuller et al. 1998; Lacey et al. 1998). Treatment of isolated osteoclasts with OPGL led to a rapid increase in pseudopodial motility and stimulated bone resorption, effects that were specifically inhibited following the addition of the decoy receptor osteoprotegerin (OPG) [Fuller et al. 1998]. Therefore, OPGL–RANK signaling, in addition to playing a role in osteoclast differentiation, is also important for the activation of mature osteoclasts. Our results suggest that TRAF6 could be a signal transducer downstream of RANK that is specifically required for the pathway leading to osteoclast activation.

Requirement for TRAF6 in LPS signaling

Several exciting findings implicate TLR2 [Toll-like receptor 2] and TLR4 in LPS signaling. Both are type 1

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transmembrane proteins with cytoplasmic domains sharing similarities with the intracellular region of the IL-1R1 (Gay and Keith 1991; Medzhitov et al. 1997). TLR2 is activated by LPS in a response that depends on LPS-binding protein and is enhanced by CD14 (Yang et al. 1998). Overexpression of TLR2 confers LPS inducibility of NF-κB activation in mammalian 293 cells, and a dominant-negative mutant of TRAF6 suppresses NF-κB activation in LPS-treated 293 cells transiently expressing TLR2 (Kirschning et al. 1998). Recently, the resistance of two mutant strains of mice (C3H/HeJ and C57BL/10ScCr) to endotoxin has been attributed to defective mutations of TLR4 (Poltorak et al. 1998). Overexpression of dominant-negative TRAF6 also impairs TLR4-induced NF-κB activity (Muzio et al. 1998). Taken together, these results strongly suggest a physiologically important role for TRAF6 in transducing LPS-mediated signals downstream of TLR2 and/or TLR4. Annually, a considerable number of human fatalities occur due to endotoxic shock, the result of activation of the immune system by endotoxin/LPS (Fenton and Golenbock 1998). The elucidation of the signaling molecules in the LPS pathway(s) may lead to the discovery of potential targets for the pharmacological modulation of endotoxic shock.

In conclusion, this study has revealed important new information on the physiologic functions of TRAF6. Through the generation and analysis of TRAF6-deficient mice, we have demonstrated critical roles for TRAF6 in perinatal and postnatal survival and in IL-1 and CD40 signal transduction. We have also identified a novel role for TRAF6 in bone metabolism, in that its presence is required to prevent osteopetrosis. Most importantly, these studies have uncovered an unexpected and prominent role for TRAF6 in LPS signal transduction. Our findings should make a significant contribution to advancing the heretofore limited knowledge of the pathophysiologic mechanisms underlying endotoxic shock.

Materials and methods

Animal husbandry

Mice were housed in a specific-pathogen-free facility according to the ethical and institutional guidelines of the Ontario Cancer Institute. Pathogen status was monitored by standard microbiological and parasitological examinations as well as by histopathological staining for specific pathogens. All mice analyzed in this investigation appeared healthy at the time of use.

Gross anatomical and histological analyses

Groups of TRAF6+/+, TRAF6+/−, and TRAF6−/− mice were necropsied on day 21 or 28 after birth. Radiography was performed using a Faxitron X-ray system (model 43855A, Faxitron X-ray Corp., Buffalo Grove, IL). Bone mineral density was determined in two 0.5-mm cross sections of bone taken at 1.5 and 2.0 mm from the proximal end of the tibia. Both total and trabecular bone mineral density (defined as the innermost 20% of the bone cross section) in the metaphysis were determined (XMICE 5.2, Stratec, Germany). Bone tissue was decalcified using formic acid and embedded in paraffin. Osteoclasts were identified by their expression of the specific marker TRAP. TRAP activity was determined using a method of enzyme histochemistry that results in the specific red staining of TRAP+ cells, that is, osteoclasts (Simonet et al. 1997). Osteoclasts were counted in TRAF6-stained sections with the aid of a graded eyepiece. Measurements were made in an area of ~0.4 mm² just distal to the proximal tibial growth plate in the primary spongiosa. The osteoclast number was determined relative to the tissue area measured.

Immunoprecipitation and Western blot analysis

Kidneys from TRAF6+/+, TRAF6+/−, and TRAF6−/− littermates were homogenized and lysed in ice-cold lysis buffer containing 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 20 mM EDTA, and the protease inhibitors phenylmethylsulfonyl fluoride [PMSF, 10 µg/ml], leupeptin (1µg/ml), aprotinin (2 µg/ml), and sodium orthovanadate (1 mM). TRAF6 was immunoprecipitated from 1.5 mg of total protein using 25 µl of protein A beads [Pharmacia] and 1 µl of a polyclonal anti-human TRAF6 antiserum that was raised against the full-length TRAF6. Immunoprecipitates and total lysates were fractionated by gel electrophoresis on an 8% Tris-glycine polyacrylamide gel [Novex, San Diego, CA] and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The Western blots were probed with a 1:1000 dilution of the anti-TRAF6 antibody and developed with enhanced chemiluminescence (ECL, Amersham) according to the manufacturer’s instructions. To verify that equal amounts of protein were loaded, blots were reprobed with a 1:100 dilution of an anti-β-actin antibody (Sigma).

Electron microscopy

The lumbar vertebrae were removed at necropsy, split lengthwise, and immersion-fixed in cold 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde and 1.6% paraformaldehyde at pH 7.4. After 24 hr at 4°C, the specimens were transferred to a decalcification solution consisting of 5% EDTA and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer. The decalcification was considered complete after 7 days, and the vertebrae were rinsed in buffer, postfixed in 1% aqueous osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin. Each of the blocks was initially sectioned at 1–2 µm, stained with toluidine blue, and examined by light microscopy. Selected blocks were then trimmed and subjected to ultrathin sectioning. The collected sections were contrast-enhanced with uranyl acetate and lead citrate prior to examination at 100 kV on a Philips CM120 transmission electron microscope.

Cellular proliferation assays

Splenocytes from two healthy, 10-day-old TRAF6+/− or wild-type mice were pooled. Cell suspensions were prepared by passing the spleens through a fine wire mesh. Erythrocytes were lysed using ACK [0.155 M ammonium chloride, 0.1 M disodium EDTA, 0.01 M potassium bicarbonate] for 3 min on ice. T cells were depleted by incubating the suspension for 1 hr at 37°C with a combination of three monoclonal antibodies (Maroun and Julius 1994), anti-Thy1.2 (H103.4.9-2), anti-CD4 (RL-172-4H), and anti-CD8 [3.168], in conjunction with guinea pig complement [Cedarlane]. Mac-1+ cells were depleted by panning using Optilux petri dishes [Falcon] coated with 5µg/ml mouse anti-rat IgG [Jackson Immunoresearch Laboratories] and rat anti-mouse Mac-1 (M1/70, ATCC)]. The cell suspensions were incubated in the coated plates for 1 hr at 4°C, followed by the recovery of Mac-1+ cells (enriched B cells) in three washes.

For proliferation assays, 1 × 10⁴ viable cells per well [96-well,
flat-bottom plate) were cultured in quadruplicate in OptiMEM (GIBCO) supplemented with $5 \times 10^{-5} \text{ M} 2$-mercaptoethanol, NaHCO$_3$ (2.4 grams/liter), streptomycin (5 $\mu$g/ml) and 5% heat-inactivated fetal bovine serum (GIBCO). Cells were treated with 10 $\mu$g/ml rat IgG2a anti-mouse CD40 mAb [PharMingen, 3/23], 10 $\mu$g/ml rat IgG2a isotype control [PharMingen, 11020D], 10 $\mu$g/ml LPS (Difco), or 5 $\mu$g/ml recombinant murine IL-4 (Genzyme) for 2, 3, or 4 days. Plates were pulsed with 1 pCi $[^{3}H]$thymidine for 6 hr and harvested onto glass fiber filters. $[^{3}H]$Thymidine uptake was measured using a scintillation counter [Topcount, Canberra Packard].

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Role of TRAF6 in bone metabolism and LPS signaling

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TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling

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Genes Dev. 1999, 13: