

the primer pair CD95L1 and CD95L2 (1  $\mu$ M each). Reaction conditions were as follows: one cycle at 94°C for 3 min; 40 cycles at 95°C for 1 min, at 65°C for 1 min 30 s, and at 72°C for 2 min; and one cycle at 72°C for 8 min. Two microliters of a 1:10 dilution of the amplification product served as a template for a second round of PCR amplification with the nested primer pair CD95L3 and CD95L4 (1  $\mu$ M each). Reaction conditions: one cycle for 3 min at 94°C; 40 cycles at 95°C for 1 min, at 55°C for 1 min 30 s, and at 72°C for 2 min; and one cycle at 72°C for 8 min. A portion of the amplification product was blotted on a nylon membrane and hybridized with the  $\gamma$ -<sup>32</sup>P-labeled internal oligonucleotide CD95L5. A single round of PCR amplification was carried out with 2  $\mu$ l of cDNA and the primer pair Act1 and Act2 (1  $\mu$ M each). Reaction conditions were identical to those used in the first-round PCR. Primer sequences: CD95L1, 5'-GTATTTTTCATGGTTCTGGTGG-3'; CD95L2, 5'-ATGAATTC-

TGGTGCCCATG-3'; CD95L3, 5'-AAGCTTCAGCTC-TTCCACCTG-3'; CD95L4, 5'-TAAAGAATAGTAGAT-CATTTT-3'; CD95L5, 5'-AAGTATACTCCGGGGTCCAGT-3'; Act1, 5'-GTGGCCATCTCCTCGAAGTC-3'; and Act2, 5'-GTTTGAGACCTTCAACACCCC-3'.

35. T. Suda and S. Nagata, unpublished data.  
 36. The Fadd dominant-negative molecule [FADD(80–208)] (17) was cloned into the retroviral expression vector pBabe Hygro and transfected into the retroviral packaging cell line GP+E. Recombinant retrovirus was harvested 48 hours later and used to infect S3T3 c-MycER cells in the presence of polybrene (8  $\mu$ g/ml). Control retrovirus was prepared by transfecting the GP+E packaging line with pBabe Hygro vector alone. Cells were selected with Hygromycin (Sigma; 200  $\mu$ g/ml), and resistant cells were pooled.  
 37. The pBabe puro c-MycER construct was transfected into the retroviral packaging cell line BOSC (32). Recombinant retrovirus was harvested 48

hours later and used to infect MEFs prepared from WT, *lpr*, or *gld* embryos in the presence of polybrene (8  $\mu$ g/ml). Twenty-four hours after infection, the medium was replaced and the cells were cultivated in 0.5% FCS in the presence or absence of OHT.

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## A TEL-JAK2 Fusion Protein with Constitutive Kinase Activity in Human Leukemia

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The Janus family of tyrosine kinases (JAK) plays an essential role in development and in coupling cytokine receptors to downstream intracellular signaling events. A t(9;12)(p24;p13) chromosomal translocation in a T cell childhood acute lymphoblastic leukemia patient was characterized and shown to fuse the 3' portion of *JAK2* to the 5' region of *TEL*, a gene encoding a member of the ETS transcription factor family. The TEL-JAK2 fusion protein includes the catalytic domain of *JAK2* and the TEL-specific oligomerization domain. TEL-induced oligomerization of TEL-JAK2 resulted in the constitutive activation of its tyrosine kinase activity and conferred cytokine-independent proliferation to the interleukin-3–dependent Ba/F3 hematopoietic cell line.

Chromosomal translocations in human tumors frequently produce fusion genes whose chimeric protein products play an essential role in oncogenesis (1). *TEL* is located on chromosome 12p13 and is frequently involved in chromosomal translocations seen in a variety of human leukemias (2–4). We observed a t(9;12)(p24;p13) translocation in a 4-year-old male with T cell acute lymphoblastic leukemia (ALL) in relapse (Fig. 1A). The involvement of *TEL* was investigated by Southern (DNA) blot analysis with a genomic probe corresponding to intron 5 of *TEL* (5, 6). In addition to the expected germline bands, rearranged frag-

ments were detected in the Eco RI and Bam HI digests of the patient's DNA, suggesting that the chromosome 12 breakpoint localized to *TEL* intron 5 (Fig. 1B). To identify the fusion partner on chromosome 9, we used anchored polymerase chain reaction (PCR) to characterize the fusion transcript in leukemic cells. Sequence analysis of the amplified cDNA clones identified a non-*TEL* sequence fused in frame upstream of *TEL* exon 6. This sequence showed extensive similarity to the genes encoding the JAK family of protein tyrosine kinases, with the greatest similarity to murine *Jak2* (7).

Using these putative *JAK2* cDNA sequences, we isolated lambda phage clones from a normal human genomic library and showed by fluorescent in situ hybridization (FISH) that they hybridized to chromosome 9p24 (8), the chromosomal location of human *JAK2* (9). Partial sequence analysis of these genomic clones allowed us to design *JAK2* oligonucleotides corresponding to sequences on either side of the chromosome 9 breakpoint. These oligonucleotides were used together with *TEL*-specific primers to

analyze RNA from leukemic or control cells by reverse transcriptase (RT)-PCR (10). Both *TEL-JAK2* and *JAK2-TEL* cDNAs were specifically amplified from patient cells but not from control cells (Fig. 1C). The sequence of the amplified human *JAK2* was 90% identical to that of murine *Jak2* at the nucleotide level and 96% identical at the protein level over a stretch of 114 amino acids (Fig. 2A). These results establish that human *JAK2* is the gene fused to *TEL* as a result of the t(9;12) translocation.

*JAK2* is a widely expressed protein tyrosine kinase that associates with the intracellular domains of a number of cytokine receptors and is essential to receptor function (11, 12). *JAK2* shares with other members of the JAK family (such as *JAK1*, *JAK3*, and *TYK2*) seven regions of homology, referred to as JH1 to JH7 (Fig. 2C) (11, 13). JH1 is the catalytic domain, whereas JH2 to JH7 appear to be involved in protein-protein interactions and in the specificity of the different JAK family members (14, 15). In the *TEL-JAK2* fusion, the NH<sub>2</sub>-terminal *TEL* sequences are fused to the kinase JH1 domain (Fig. 2C). *TEL* is a member of the ETS family of transcription factors. In addition to a COOH-terminal ETS domain that is conserved in all ETS proteins, *TEL* specifically contains a 60-amino acid homotypic oligomerization domain at its NH<sub>2</sub>-terminus (16, 17). Fusion of *TEL* and *JAK2* has been observed in two other leukemic patients, which also resulted in a *TEL-JAK2* fusion transcript but not in the expression of the reciprocal *JAK2-TEL* fusion (Fig. 2B) (18). The conserved feature of the fusion proteins encoded by the *TEL-JAK2* transcripts characterized in all three patients is the presence of both the *TEL* oligomerization domain and the *JAK2* catalytic domain (Fig. 2C).

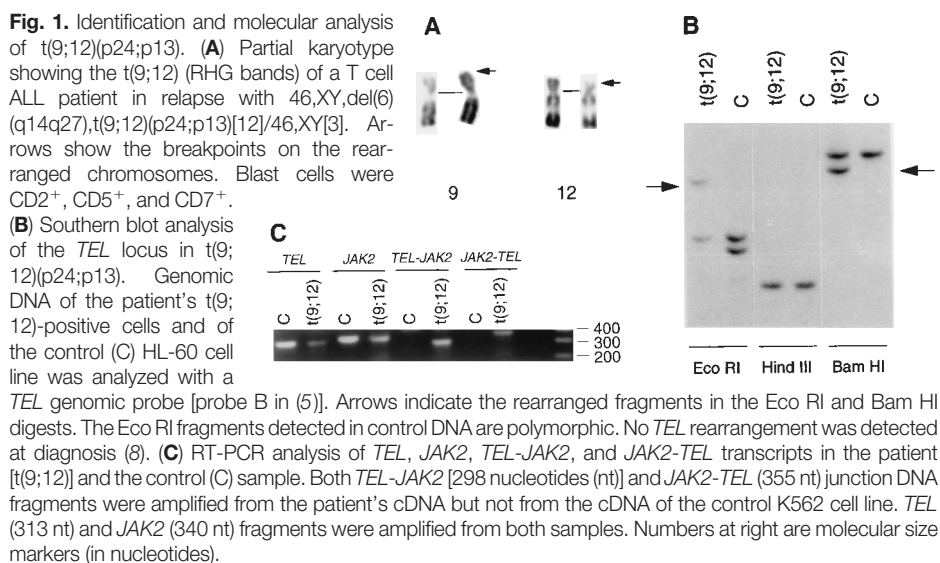
To investigate the oligomerization properties and protein kinase activity of *TEL-JAK2*, we compared the properties of intact *TEL-JAK2*, in which the 336 NH<sub>2</sub>-terminal residues of *TEL* are fused to 318 COOH-

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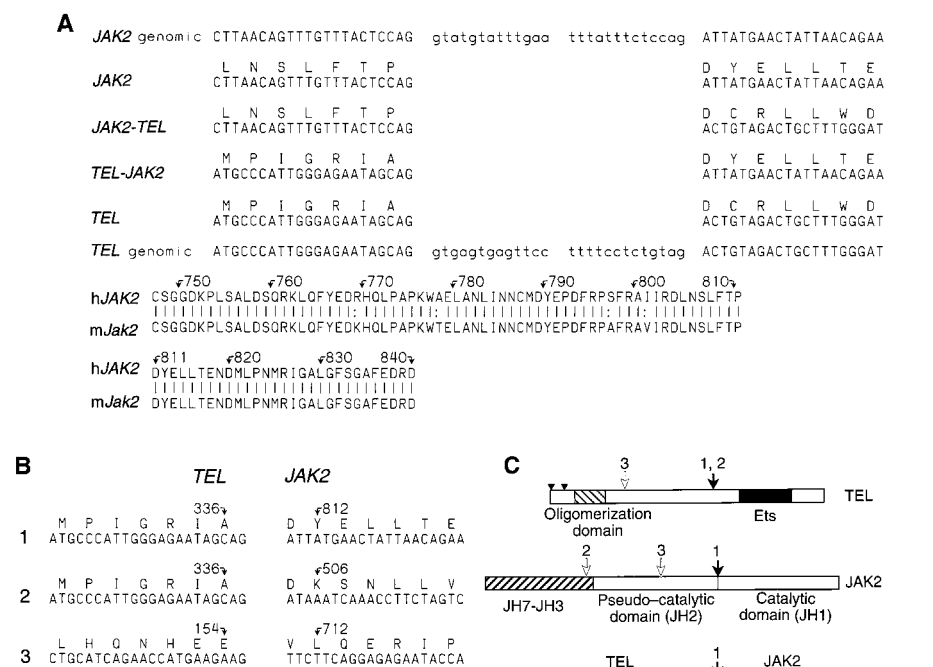


terminal residues of JAK2, with those of two mutants: *TELΔ-JAK2*, in which the *TEL* oligomerization domain (amino acids 40 to 117) was deleted, and *TEL-JAK2* K<sup>-</sup>, a kinase-defective mutant in which a lysine residue essential to the catalytic function of JAK2 (K882) (19) was changed to arginine (Fig. 2C).

Previous experiments have shown that *TEL*-induced oligomerization as it occurs in vivo can be analyzed with in vitro-translated proteins (16, 17). As expected, in vitro-translated *TEL* and *TEL-JAK2* were specifically immunoprecipitated by antibodies directed against the COOH-terminal domains of *TEL* [antibody to C-*TEL* (anti-C-*TEL*)] and JAK2 (anti-JAK2), respectively (Fig. 3A, top). When coexpressed, *TEL* and *TEL-JAK2* formed a complex because they were both immunoprecipitated by either of the antibodies (Fig. 3A, bottom). In vitro association of *TEL* and *TEL-JAK2* required the presence of the *TEL* oligomerization domain, because neither the *TELΔ-JAK2* mutant nor the full-length JAK2 coimmunoprecipitated with *TEL* (Fig. 3A, bottom). These experiments show that the *TEL* oligomerization domain is functional in the *TEL-JAK2* fusion.

To investigate whether *TEL*-induced oligomerization results in activation of *TEL-JAK2* tyrosine kinase activity, we compared the autophosphorylation of JAK2, *TEL-JAK2*, and *TEL-JAK2* mutants. In vitro-translated proteins were analyzed by immunoblotting with a phosphotyrosine-specific antibody (anti-PTyr). For comparison of expression levels, the membranes were stripped and reprobbed with antibodies specific to the NH<sub>2</sub>-terminal domain of *TEL* (anti-N-*TEL*) (Fig. 3B). A high level of tyrosine phosphorylation occurred only with *TEL-JAK2*. The phosphorylation of *TEL-JAK2* reflected its intrinsic tyrosine kinase activity because the *TEL-JAK2* K<sup>-</sup> mutant was not phosphorylated. Constitutive tyrosine kinase activity of *TEL-JAK2* required *TEL*-induced oligomerization because the oligomerization-defective *TELΔ-JAK2* mutant was inactive.

To assay the transforming properties of *TEL-JAK2*, we stably expressed the cDNAs encoding JAK2, *TEL-JAK2*, and the *TEL-JAK2* mutants in the murine Ba/F3 hematopoietic cell line, which is strictly dependent on interleukin-3 (IL-3) for survival and proliferation. Mock-transfected cells and transfectants harboring the pBabeNeo vector alone were used as controls. Analysis of transfectants obtained after G418 selection in the presence of IL-3 showed that expression of exogenous proteins was comparable with that of endogenous JAK2 (Fig. 4A). Consistent with our in vitro data, only the oligomerization-competent *TEL-JAK2*



**Fig. 2.** (A) Nucleotide and deduced amino acid sequences of fused and normal *TEL* and *JAK2*. The fusion occurs within codon 337 of *TEL* (4) and codon 811 of *JAK2* (7). Intronic sequences are shown in lowercase letters. (B) Nucleotide sequence of the RT-PCR-amplified junctions of the *TEL-JAK2* fusion in different leukemic patients (26). Patient 1 is the patient analyzed in this study. The karyotype and the details of the structural analysis of the *TEL-JAK2* fusions in patients 2 and 3 are reported elsewhere (18). No reciprocal *JAK2-TEL* fusion transcript was detected in patients 2 and 3 (18). (C) Schematic structure of *TEL* and *JAK2* proteins, together with the *TEL-JAK2* fusion protein and derivatives used in this study. Arrows indicate the locations of the t(9;12) fusion points in the different patients. Fusion points in patient 1 are shown by black arrows. Fusion points in patients 2 and 3 are shown by open arrows. The *TEL-JAK2* cDNA as characterized in patient 1 was reconstructed to encode the 336 NH<sub>2</sub>-terminal residues of human *TEL* and the 318 COOH-terminal residues of murine *JAK2* by PCR-mediated amplification of the appropriate regions of *TEL* and *JAK2* cDNAs and subsequent subcloning of the products. The nucleotide sequence of the amplified cDNA was determined and found to be devoid of mutations. Arrowheads indicate the two alternative *TEL* initiation codons identified previously (27). Black ovals indicate the hemagglutinin (HA) epitope tags. K → R indicates the mutation of lysine to arginine. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

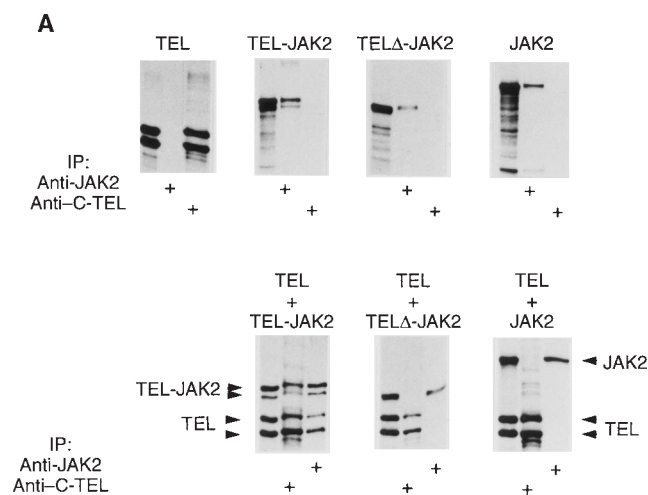
was tyrosine phosphorylated *in vivo* (Fig. 4A). To assay the ability of TEL-JAK2 to confer IL-3-independent proliferation, we deprived Ba/F3 transfectants of IL-3 and seeded the cells in 96-well trays (20). In contrast to control cells, which died by apoptosis under these conditions (0 of 96 wells displaying proliferating cells), the

TEL-JAK2 transfectants showed sustained proliferation (96 of 96 wells with proliferating cells). Similar results were obtained when cell proliferation was monitored by daily cell counting, although the rate of proliferation in the absence of IL-3 was slightly less than that in its presence. TEL-JAK2-induced proliferation required the

constitutive activation of its protein tyrosine kinase activity, because Ba/F3 cells transfected with TEL $\Delta$ -JAK2, TEL-JAK2 K<sup>-</sup>, and wild-type JAK2 did not proliferate in the absence of IL-3 (0 of 96 wells showing proliferating cells).

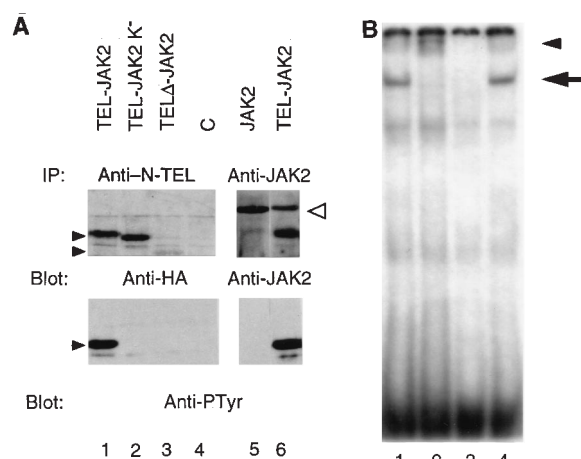
JAK2 couples cytokine receptors to downstream signaling events that control cell survival, proliferation, and differentiation by transiently activating several pathways, including RAS, phosphatidylinositol 3-kinase, and STAT5 (signal transducer and activator of transcription 5). However, the respective role and potential redundancy of these pathways in these cellular responses are presently unclear (11, 13).

An inhibitor study has shown that JAK2 is constitutively activated in human ALL cells (21), but no causal link to leukemogenesis has been established. A JAK pathway has been implicated in a leukemia-like defect in the fruit fly *Drosophila* (22–24). Point mutations in the *hopscotch* locus, which encodes a JAK homolog, cause the hyperactivation of JAK activity and, in turn, phosphorylation and activation of *Drosophila* STAT. The role of STAT proteins in the *Drosophila* leukemia-like defect has also been genetically established (23). Ba/F3 cells expressing TEL-JAK2 show constitutive activation of STAT5 (Fig. 4B), suggesting that alteration of the normal JAK2 signaling pathway participates in the deregulation of cell proliferation. Alternatively, TEL-JAK2 could phosphorylate protein substrates that are not normally involved in JAK2 signaling but are critical to its oncogenic properties.



**Fig. 3.** Oligomerization and tyrosine kinase activity of TEL-JAK2. **(A)** In vitro oligomerization of TEL-JAK2. pBS expression plasmids encoding JAK2, TEL, TEL-JAK2, or TEL $\Delta$ -JAK2 were in vitro translated alone or in combination, as indicated at the top of each panel, with the TNT kit (Pharmacia) in the presence of L-(<sup>35</sup>S)methionine. Translated proteins were analyzed directly (left lanes) or with either anti-JAK2 or anti-C-TEL, as indicated at the bottom of each panel (middle and right lanes). The antibodies used were rabbit polyclonal antibodies raised against amino acids 1110 to 1129 of JAK2 (anti-JAK2; Santa Cruz Biotechnology, Santa Cruz, California) and against the COOH-terminal end of TEL (anti-C-TEL) (27). **(B)** Requirement of oligomerization for activation of TEL-JAK2 tyrosine kinase activity. In vitro-translated TEL-JAK2 and TEL-JAK2 mutants were analyzed by immunoblotting with the phosphotyrosine-specific 4G10 monoclonal antibody (anti-PTyr). After stripping of the membrane, expression levels of the proteins were analyzed with a rabbit antiserum directed to the NH<sub>2</sub>-terminal domain of TEL (anti-N-TEL) (27).

**Fig. 4.** Induction of IL-3-independent proliferation of Ba/F3 cells by TEL-JAK2. **(A)** The cDNAs encoding JAK2, TEL-JAK2, or the TEL-JAK2 mutants were subcloned into the pBabeNeo retroviral expression vector (28). These constructs and the pBabeNeo control were introduced by electroporation into the murine IL-3-dependent lymphoid Ba/F3 cells, and stably transfected cells were selected in the presence of G418 and IL-3. For evaluation of protein expression and phosphorylation, lysates of transfected cells were immunoprecipitated with either anti-N-TEL or anti-JAK2 (IP). After electrophoresis in denaturing polyacrylamide gels, proteins were blotted to nitrocellulose, and the blot was probed with anti-HA, anti-JAK2, or anti-PTyr (blot). TEL-JAK2 and TEL-JAK2 mutants are indicated by solid arrowheads. JAK2 is indicated by an open arrowhead. **(B)** Constitutive STAT5 activation in Ba/F3 cells expressing TEL-JAK2. Electrophoretic mobility-shift assay with a  $\beta$ -casein probe showed a specific STAT5 complex in lane 1 (arrow) (29). Specificity was established by the disappearance of the complex on competition with a 100-fold molar excess of the unlabeled  $\beta$ -casein probe (lane 3), but not after competition with the same molar excess of a nonspecific unlabeled probe (lane 4). Furthermore, addition of anti-STAT5 supershifted the complex (lane 2; the supershift is indicated by an arrowhead).



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## Site of Einkorn Wheat Domestication Identified by DNA Fingerprinting

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The emergence of agriculture in the Near East also involved the domestication of einkorn wheat. Phylogenetic analysis that was based on the allelic frequency at 288 amplified fragment length polymorphism molecular marker loci indicates that a wild group of *Triticum monococcum boeoticum* lines from the Karacadağ mountains (southeast Turkey) is the likely progenitor of cultivated einkorn varieties. Evidence from archeological excavations of early agricultural settlements nearby supports the conclusion that domestication of einkorn wheat began near the Karacadağ mountains.

Wild einkorn wheat, *Triticum monococcum* subsp. *boeoticum*, is the wild relative of the domesticated einkorn wheat *T. m. monococcum* (1–3). In the Near East, primary habitats of *T. m. boeoticum* occur in the northern and eastern parts of the Fertile Crescent (2). Archeological evidence points to this region as the area of einkorn domestication (4); however, it has been impossible to pinpoint the site of domestication (3). We have addressed this question by making two assumptions. The first is that genetic distances within a species can be evaluated by multiple, dominant DNA markers—in our study, amplified fragment length polymorphism (AFLP) fingerprinting (5, 6). The second assumption is that the progenitors of crop plants have not undergone significant genetic changes during the past 10,000 years (4). In the case of wild einkorn wheat, more-

over, the available information indicates for the same period a geographical stability of its primary habitat (3, 4, 7–12). In addition, the domesticated einkorns cultivated in marginal areas (13) have been left untouched by modern plant breeding. Complications were anticipated, however, because wild einkorn has colonized secondary habitats (2) and because a weedy einkorn form (*T. m. aegilopoides*) occurs in the Balkans (14).

In this study we characterized 1362 lines of Einkorn wheats for their agronomic and taxonomic traits. The areas of origin were known for 954 lines. Of these, 338 lines were chosen so as to ensure an even distribution in the area shown in Fig. 1. The 68 *T. m. monococcum* lines were from several countries, and the 9 *T. m. aegilopoides* lines were from the Balkans. The collection sites of the 194 *T. m. boeoticum* lines originating from the Fertile Crescent were known to within  $\pm 5$  km. The 67 *T. m. boeoticum* lines collected outside the Fertile Crescent were from Turkey, the Caucasus mountains, and Lebanon. DNA from these 338 lines was fingerprinted on the basis of the presence versus the absence of 288 AFLP bands (15).

To identify the area where einkorn was domesticated, we assigned 194 lines of *T.*

*m. boeoticum* to nine groups sampled in defined geographical areas of the Fertile Crescent (groups A, B, C, D, E, G, H, I, and L; see Fig. 1). The AFLP results were used to calculate genetic distances among the nine groups, and phylogenetic trees were constructed with different tree-building methods (16) and distance measures (17). All trees had almost identical topologies (18), as exemplified by the tree shown in Fig. 2A. The outcome of this analysis allows two conclusions. The first is that lines sampled within the same area are genetically more closely related than lines sampled in different locations. Indeed, the average genetic distance between lines of the same group is 23.4% smaller than that between the nine *T. m. boeoticum* groups of the Fertile Crescent. The second conclusion is that the group D (originating from the volcanic Karacadağ mountains, southeast Turkey, Diyarbakır district, and consisting of 19 *T. m. boeoticum* lines) is distinctly separated from the remaining groups.

The clustering approach was repeated considering 68 cultivated einkorns and 9 *T. m. aegilopoides* lines. The cultivated lines were from Mediterranean countries (group  $\alpha$ ), Central Europe ( $\beta$ ), the Balkans ( $\gamma$ ), and Turkey ( $\delta$ ). The trees obtained were similar to those shown in Fig. 2, B and C. The cultivated einkorns are closely related among themselves and to *T. m. aegilopoides*. Most importantly, both *T. m. monococcum* and *T. m. aegilopoides* show a close phylogenetic similarity to the *T. m. boeoticum* lines from the Karacadağ region. This finding is supported by the majority rule consensus tree shown in Fig. 2D. This result raises the question whether the Karacadağ lines of *T. m. boeoticum* should be considered the closest relatives of the wild progenitors that gave rise to cultivated einkorn about 10,000 years ago.

Before considering this possibility, we again tested if cultivated einkorn is monophyletic. The phylogenesis of the 388 lines studied (Fig. 2E) indicates that cultivated einkorn is indeed monophyletic. To deter-

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## A TEL-JAK2 Fusion Protein with Constitutive Kinase Activity in Human Leukemia

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