CHAPTER 3

Downregulation of Vertebrate Tel (ETV6) and Drosophila Yan is Facilitated by an Evolutionarily Conserved Mechanism of F-box-Mediated Ubiquitination

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Downregulation of Vertebrate Tel (ETV6) and Drosophila Yan Is Facilitated by an Evolutionarily Conserved Mechanism of F-Box-Mediated Ubiquitination

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The vertebrate Ets transcriptional repressor Tel (ETV6) and its invertebrate orthologue, Yan, are both indispensable for development, and they orchestrate cell growth and differentiation by binding to DNA, thus inhibiting gene expression. To trigger cell differentiation, these barriers to transcriptional activation must be relieved, and it is established that posttranslational modifications, such as phosphorylation and sumoylation, can specifically impair the repressive functions of Tel and Yan and are crucial for modulating their transcriptional activity. To date, however, relatively little is known about the control of Tel and Yan protein degradation. In recent years, there has been a concentrated effort to assign functions to the large number of F-box proteins encoded by both vertebrate and invertebrate genomes. Here, we report the identification and characterization of a previously unreported, evolutionarily conserved F-box protein named Fbl6. We isolated both human and Drosophila melanogaster fbl6 cDNA and show that the encoded Fbl6 protein binds to both Tel and Yan via their SAM domains. We demonstrate that both Tel and Yan are ubiquitinated, a process which is stimulated by Fbl6 and leads to proteasomal degradation. We recently established that the sumoylation of Tel on lysine 11 negatively regulates its repressive function and that the sumoylation of Tel monomers, but not that of Tel oligomers, may sensitize Tel for proteasomal degradation. Here, we found that Fbl6 regulates Tel/Yan protein stability and allows appropriate spatiotemporal control of gene expression by these repressors.

Cell fate is determined by programs of gene expression, which are strictly regulated spatiotemporally by a complex network of interacting molecular mechanisms that control the balance between transcription, translation, and degradation. The Drosophila melanogaster transcription factor Yan and its vertebrate orthologue, Tel (ETV6), are well placed to interrogate these processes. An abundance of molecular and genetic evidence shows that these unique Ets transcription factor repressors are indispensable for normal cell differentiation (15, 19, 22, 27, 35, 36, 45, 46). Importantly, the disruption of normal Tel function leads to neoplasia (10, 11). A finely controlled interplay between posttranscriptional and posttranslational modifications regulates their functions (42). In the case of Tel, the observed heterogeneity of Tel proteins in cells (37, 29, 43) can be accounted for by at least two posttranscriptional mechanisms—the use of an alternative initiation codon (37, 29) and alternative splicing (38; M. G. Roukens and D. A. Baker, unpublished data). Together, these processes can produce Tel isoforms that differentially control Tel function. Yan, on the other hand, appears to be regulated posttranscriptionally by a microRNA, miR7, that might act in a tissue-specific fashion and that limits Yan protein translation by binding to the 3′ untranslated region of yan mRNA (21). Posttransitionally, phosphorylation and sumoylation play pivotal roles in modulating the activities of Tel and Yan, particularly by impairing the repression of transcription by these factors. Specifically, phosphorylation of Yan is a trigger for its downregulation (2, 33, 35, 40, 41), and sumoylation of Tel is PIAS dependent (37) and inhibits the repression of gene expression (5, 37, 49). To date, however, relatively little is known about the control of Tel/Yan protein degradation.

There are vital mechanisms of protein degradation for controlling the timing of the action of proteins and, ultimately, the timing of cellular processes in general. One such crucial mechanism of degradation is mediated by the process of ubiquitination, which impinges on virtually all eukaryotic cellular processes (13). Ubiquitin is a 76-amino-acid polypeptide that can be covalently bonded to target proteins in a number of ways (28). Monoubiquitination has been shown to play an essential role in endocytosis (16) and in the subcellular targeting of proteins (20). Polyubiquitination, in contrast, is almost exclusively associated with protein degradation and turnover, either via the proteasome or through endocytosis and lysosomal sorting (47). Mechanistically, ubiquitination is well defined and involves the concerted action of at least three different catalytic components, namely the E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin ligase) enzymes (48). A crucial aspect of ubiquitination-driven degradation is how substrate specificity is achieved. One way is through the recruitment of F-box proteins. F-box proteins are character-
ized by the presence of an N-terminal F-box domain that is approximately 50 amino acids long (1), and they are broadly classified into three families: the FBW family, which contains WD repeats; the FBL family, which contains leucine-rich repeats; and the FBX family, which consists of F-box proteins with other protein interaction domains. These domains are usually located in the C terminus of the protein, and they associate with substrates and link them to the ubiquitination machinery via the F-box domain (4, 14).

We have performed yeast two-hybrid screens to identify proteins that associate with both Tel and Yan, and by these means, we have uncovered an F-box protein named Fbl6. Our biochemical and genetic analyses of human and Drosophila tissue culture cells, as well as of Drosophila embryos, suggest that F-box-mediated ubiquitination of Tel and Yan promotes their downregulation.

**MATERIALS AND METHODS**

Cell-based ubiquitination assays. Cells were transfected with the appropriate plasmids and then incubated for 6 h with or without the proteasome inhibitor MG132 24 to 36 h posttransfection. His-ubiquitin pull-downs were performed with 50 μl of Ni-nitrotriacetic acid beads (Qiagen) for 3 h at room temperature in 6 ml of 0.5 M guanidinium-HCl, 0.1 M Na2HPO4, 0.1 M NaH2PO4, 0.01 M Tris-HCl (pH 8.0), 20 mM imidazole, and 10 mM β-mercaptoethanol (buffer A). The beads were successively washed twice with 1 ml of each of the following buffers: (i) buffer A plus 0.2% Triton X-100; (ii) 8 M urea, 0.1 M Na2HPO4, 0.1 M NaH2PO4, 0.01 M Tris-HCl (pH 8.0), 20 mM imidazole, 10 mM β-mercaptoethanol, and 0.2% Triton X-100 (buffer B); and (iii) 8 M urea, 0.1 M Na2HPO4, 0.1 M NaH2PO4, 0.01 M Tris-HCl (pH 6.3), 20 mM imidazole, 10 mM β-mercaptoethanol, and 0.2% Triton X-100 (buffer C). Ubiquitinated proteins were eluted in 60 μl of urea sample buffer, which consisted of 37.5% buffer C, 39.3% 3X Laemmli buffer, 20 mM imidazole, and 3.2% β-mercaptoethanol. The samples were boiled and analyzed by Western blotting.

**Immunofluorescence.** Cells were grown on glass coverslips and transfected using Fugene 6 (Roche). Cells were fixed after 24 h with 4% paraformaldehyde for 15 min at room temperature (all of the following steps were done at room temperature) and permeabilized in 0.2% Triton X-100–phosphate-buffered saline for 5 min. Cells were washed with phosphate-buffered saline and blocked with 5% goat serum for 1 h, incubated with primary antibodies for 1 h, washed, and incubated with secondary antibodies for 30 min. For extensive washing, the cells were removed and immunostaining was visualized with a Leica DM5500 B microscope.

**Cell culture, biochemistry, and molecular biology.** A schematic representation of all of the constructs used in this study can be found in Fig. S3 in the supplementary material and also in the relevant figures. Mammalian cell lines were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. Drosophila Schneider cells were cultured at 25°C in Schneider cell medium (Sigma) supplemented with 10% fetal bovine serum. Mammalian cells were transfected with Fugene 6 (Roche) and insect cells with Effectene reagent (Qiagen), according to the manufacturers’ protocol. For transfection in cells, the methods were performed essentially as described previously (17). The following primer sets were deployed for real-time PCR analysis: Telq15 (CCCTGCGCCACTACTACAAC), Telq13 (TGATTTCTTCGCGGTTTTA), Telq25 (CTTTGCCAATCATGATCTCCTCA), and Telq23 (AGGGTCGGAAGATGTTGAAA), hFbl6 (ATGGCTTCACTACCTACTA), hFbl6 (GTAGTCTTGGACTGCTGTG), and hFbl6 (TATAAGGCGCCAGGAACAGG). All methods were performed according to the manufacturers’ protocol. For transfection in cells, the methods were performed essentially as described previously (17). The following primer sets were deployed for real-time PCR analysis: Telq15 (CCCTGCGCCACTACTACAAC), Telq13 (TGATTTCTTCGCGGTTTTA), Telq25 (CTTTGCCAATCATGATCTCCTCA), and Telq23 (AGGGTCGGAAGATGTTGAAA), hFbl6 (ATGGCTTCACTACCTACTA), hFbl6 (GTAGTCTTGGACTGCTGTG), and hFbl6 (TATAAGGCGCCAGGAACAGG). All methods were performed essentially as described previously (17). The following primer sets were deployed for real-time PCR analysis: Telq15 (CCCTGCGCCACTACTACAAC), Telq13 (TGATTTCTTCGCGGTTTTA), Telq25 (CTTTGCCAATCATGATCTCCTCA), and Telq23 (AGGGTCGGAAGATGTTGAAA), hFbl6 (ATGGCTTCACTACCTACTA), hFbl6 (GTAGTCTTGGACTGCTGTG), and hFbl6 (TATAAGGCGCCAGGAACAGG). All methods were performed essentially as described previously (17). The following primer sets were deployed for real-time PCR analysis: Telq15 (CCCTGCGCCACTACTACAAC), Telq13 (TGATTTCTTCGCGGTTTTA), Telq25 (CTTTGCCAATCATGATCTCCTCA), and Telq23 (AGGGTCGGAAGATGTTGAAA), hFbl6 (ATGGCTTCACTACCTACTA), hFbl6 (GTAGTCTTGGACTGCTGTG), and hFbl6 (TATAAGGCGCCAGGAACAGG). All methods were performed essentially as described previously (17).

For the ubiquitination assay, SF9 cells were seeded in Grace’s medium (Invitrogen) at 40 to 60% confluence in 6-cm tissue culture dishes, and cells were lysed 24 to 48 h posttransfection. TelHA, TelFlag, YanHA, and YanFlag constructs were fused in frame with either a hemagglutinin (HA) or a Flag epitope tag and cloned into either the pC2 vector for expression in mammalian cells or the pMT vector (Invitrogen) for expression in insect cells. Glutathione S-transferase (GST)-Tel was cloned in frame with GST in the pGEX-2TK vector. Mutants were generated by PCR. For immunoprecipitations, cells were lysed in 1 ml of either radioimmunoprecipitation assay–sodium dodecyl sulfate buffer (1 mM EDTA, 50 mM Tris [pH 8.0], 150 mM NaCl, 10% glycerol, 1% Triton X-100) or protein lysate buffer (50 mM Tris [pH 7.5], 1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% Na deoxycholate, 150 mM NaCl) with protease inhibitors (phenylmethylsulfonyl fluoride, trypsin, pepstatin A, leucine, and aprotinin) and NaF. Cell lysates were syngred and centrifuged. Immunoprecipitations were carried out with 0.75 μl of either anti-HA polyclonal antibody (Abcam) or anti-V5 monoclonal antibody (Invitrogen). Lysates were boiled for 1 h, after which suitable beads, protein A–Sepharose 4 fast flow (Amersham Pharmacia Biotech) for HA immunoprecipitations or protein G–Sepharose (Sigma–Aldrich) for V5 immunoprecipitations, were added for a further 2 h of incubation. Associated proteins were analyzed by Western blotting. For pull-down assays, Fbl6 proteins were labeled with [35S]methionine by using the TNT coupled reticulocyte in vitro transcription system (Promega) and then incubated with GST-Tel fusions that were immobilized on glutathione–Sepharose beads as previously described (2).
manufacturer’s protocol (Clontech). We confirmed the specificities of interactions between Yan and Drosophila Fbl6 and between Tel and human Fbl6 by transforming yeast with the cDNA of Fbl6, in either the presence or the absence of the ynr bait (for Drosophila fbl6), the tel bait (for human Fbl6), or an unrelated bait. Full-length cDNAs of the human fbl6 gene and of the Drosophila fbl6 gene were isolated by reverse transcriptase (RT)-PCR using total RNA derived from either insect Schneider cells (for Drosophila fbl6) or U2OS human osteosarcoma cells (for human Fbl6).

Antibodies/drugs. The following antibodies (and dilutions, if applicable) were used: anti-V5 antibody (Invitrogen); anti-Flag mouse M2 monoclonal antibody (Sigma-Aldrich); anti-HA:11 mouse monoclonal antibody (Covance); anti-GST rabbit antibody (My Probe), 1:5,000; anti-HA rabbit polyclonal antibody (Abcam), 1:1,000; human anti-Tel rabbit polyclonal antibody, 1:1,000; anti-Yan monoclonal antibody, 1:500; Drosophila anti-SKPA rabbit polyclonal antibody, 1:1,000; and anti-His polyclonal antibody, 1:1,000. For MG132, experiment cells were incubated with 3 μM MG132 (Calbiochem) for 6 h prior to lysis.

RESULTS

Fbl6, a conserved F-box-containing protein, associates biochemically with both human Tel and Drosophila Yan. To uncover evolutionarily conserved pathways that might regulate Yan/Tel function, we performed yeast two-hybrid screens using cover evolutionarily conserved pathways that might regulate Tel. Figure 4 and 5 explore the role of Fbl6 in the regulation of human Tel by human Fbl6, and Fig. 3 describe the regulation of human Tel by human Fbl6, which has been previously classified as LRD1 and LRD2, which have been previously classified as D. A. Baker, unpublished data); however, improved antibodies and mass spectrometry techniques should, in time, resolve this issue. Collectively, these results show that Tel is ubiquitinated and that ubiquitination requires both the Tel SAM domain and the EDBD.

Fbl6 stimulates Tel downregulation and ubiquitination. We next explored the role of Fbl6 in Tel ubiquitination. Several lines of evidence support a role for Fbl6 in promoting Tel downregulation and ubiquitination. First, deletion of the SAM domain, which is essential for Fbl6 binding to Tel (Fig. 1B), abolished Tel ubiquitination (Fig. 2B). Second, ectopic expression of wild-type Fbl6 stimulated Tel ubiquitination, whereas Fbl6 harboring mutations that disrupt the interaction with Tel (Fig. 1C) failed to augment Tel ubiquitination (Fig. 2D). Interestingly, disruption of the F-box domain of Fbl6 also impairs binding to Tel (see Fig. 1C). In cells, both Tel and Fbl6 co-localize in the nucleoplasm, and Fbl6 is also found in the nucleolus (Fig. 1D and see Fig. 2C). Recent reports have highlighted differential regulation of protein function by different isoforms of the same F-box protein that are localized in either the nucleolus or the nucleus (31). It would be interesting as a future study to determine whether Tel is ubiquitinated and whether the ubiquitination of Tel is indeed F-box dependent, one should expect Tel to associate with SKP1, which is a core component of SCF ubiquitin ligases, and one should also expect SKP1 to play a role in regulating steady-state Tel protein levels (Fig. 2E). Third, shRNA-mediated knockdown of Fbl6 both stabilized steady-state endogenous Tel protein levels (Fig. 2E) and inhibited ubiquitin conjugation of Tel (Fig. 2F). If ubiquitination of Tel is indeed F-box dependent, one should expect Tel to associate with SKP1, which is a core component of SCF ubiquitin ligases, and one should also expect SKP1 to play a role in regulating steady-state Tel protein levels. Figure 2E shows that endogenous Tel and endogenous SKP1 were copurified as a complex from tissue culture cells, and it also shows that, similarly to lowering fbl6 levels, the shRNA-mediated knockdown of SKP1 stabilized steady-state endogenous Tel protein levels. In cells, antibodies specific for endogenous Tel detect two differently migrating protein species (29, 43), and the higher-
FIG. 1. (A) Schematic representation of human and Drosophila Fbl6 proteins. Fbl6 is characterized by a conserved, N-terminal F-box domain (1) that is 40 to 50 amino acids long. Two other blocks of conserved sequences, which have previously been defined as leucine-rich regions (LRD1 and LRD2), are represented by black rectangles (4). The alignments of the primary amino acid sequences can be found in Fig. S1 in the supplemental material. (B) The SAM domain of Tel is needed for association with Fbl6 in cells. HA epitope-tagged wild-type Tel, TelSAM, TelEDBD, and Tel harboring substitutions of alanine for arginine in the Ets DNA-binding motif (KMSRALRHYYK) were each coexpressed with V5 epitope-tagged human Fbl6 in 293T cells. Fbl6 complexes were immunopurified from cell lysates using an antibody directed against the V5 epitope, and associated Tel proteins were detected using an antibody directed against the HA epitope. (C) The LRDs of Fbl6 and an intact F-box domain are essential for interacting with Tel. V5 epitope-tagged full-length Fbl6 or the indicated Fbl6 mutants were coexpressed with Tel in 293T cells. Tel complexes were immunopurified from cell lysates by using an antibody directed against the HA epitope, and associated Fbl6 proteins were detected using an antibody directed against the V5 epitope. Fbl6ΔFbox contains a deletion of the F-box domain. Fbl6W and Fbl6LP have point mutations that have been shown to disrupt the F-box domain (37). Fbl6ΔLRD1 and Fbl6ΔLRD2 harbor deletions of the LRD1 and LRD2 sequences, respectively. Fbl6Δ(LRD1 + LRD2) has a deletion of both LRD1 and LRD2. h, human. (D) Fbl6 and Tel are colocalized in the nucleus. Cells were transfected with the indicated constructs and proteins detected with the indicated antibodies. DAPI, 4',6'-diamidino-2-phenylindole.
FIG. 2
E

Total Lysate/ Tel Western
Total Lysate/ Tubulin Western

Control shRNA
fbl6 shRNA #1
fbl6 shRNA #2

F

TelHA + Control shRNA
TelHA + fbl6 shRNA #1
TelHA + fbl6 shRNA #2

His IP/ HA Western
Total Lysate HA Western
His IP/ His Western

V5 Western Tubulin Western

G

Phosphorylated Tel

Tel + Phosphatase

HA Western for Tel
V5 Western for hFbl6

MG132 +MG132

FIG. 2—Continued
molecular-weight form is likely to represent phosphorylated Tel (29). Ectopic expression of Tel also produces two differently migrating proteins, and the higher-molecular-weight Tel form is likely to be phosphorylated Tel because incubation with phosphatase leads to a loss of this band and a concomitant increase in the amount of the faster-migrating, unmodified Tel species (Fig. 2G, upper panel). Figure 2G (lower panel) shows that wild-type Fbl6, but not Fbl6 harboring mutations in either the F-box domain or the leucine-rich regions, strongly promotes the degradation of phosphorylated Tel. This suggests that in cells, phosphorylation of Tel might further sensitize it to Fbl6-mediated downregulation, perhaps by enhancing the affinity of Fbl6 for binding to Tel. Together, these data show that Tel is ubiquitinated and that Fbl6 stimulates Tel downregulation and ubiquitination.

**Tel monomers are efficiently ubiquitinated in cells.** Numerous studies have established that the SAM domain is essential for the monomers of Tel to form homotypic oligomers (17, 33, 37). Moreover, we showed earlier that the SAM domain is also indispensable both for the binding of Fbl6 and for Tel ubiquitination (Fig. 1B and 2B). In light of these findings, we compared the ubiquitination of Tel oligomers and that of Tel monomers. First, we established that Fbl6 can associate with both Tel oligomers and Tel monomers. Figure 3A shows that both wild-type Tel and a Tel mutant that is unable to self-associate (TelA*) (37) efficiently interacted with Fbl6 in vitro, whereas TelΔSAM, which is also monomeric, failed to bind Fbl6. In contrast, in cells, wild-type Tel efficiently coimmunoprecipitated with Fbl6, but TelA*, like TelΔSAM, did not (Fig. 3B). However, the interaction between TelA* and Fbl6, but not that between TelΔSAM and Fbl6, was stabilized in the presence of proteasome inhibitors (Fig. 3B), and furthermore, TelA* was far more readily ubiquitinated than the wild-type Tel (Fig. 3C), suggesting that Tel monomers are more labile than Tel oligomers. We recently reported a similar phenomenon with regard to the sumoylation of Tel (37). In cells, Tel is efficiently sumoylated on lysine 11, whereas TelA* is detectably sumoylated on lysine 11 in cells only in the presence of the proteasome inhibitor MG132. Figure 3C shows that the ubiquitination of TelA*, like wild-type Tel ubiquitination, was also stimulated by Fbl6, in contrast to the ubiquitination of TelΔSAM, which is unable to bind Fbl6. Collectively, these findings revealed that Tel monomers can associate with Fbl6 and are efficiently ubiquitinated.

**Fbl6 associates with Tel2 and stimulates its ubiquitination.** The SAM domain defines a subfamily of Ets transcription factors, including Tel, Tel2, Ets1, and Ets2 from vertebrates as well as Yan and Pointed P2 from Drosophila (23, 34). Since Fbl6 associates with Tel via its SAM domain, we tested whether Fbl6 also interacts with other SAM domain-containing Ets family members. Figure 3F shows that Fbl6 efficiently associates with both Tel and Tel2 but did not copurify detectably with Ets1 or Ets2, both of which express SAM domains, or PU.1, which does not contain a SAM domain. Tel2 strongly resembles Tel, both structurally and functionally (12, 30, 32), and may have resulted from an earlier gene duplication of the ancestral Tel gene. We found that, like Tel, Tel2 is ubiquitinated and that Fbl6 augments Tel2 ubiquitination (Fig. 3F), suggesting that Tel and Tel2 may share a common F-box-dependent degradation pathway. In the future, it will be of interest to determine whether Fbl6 plays a role in regulating the activities of other factors in vivo.

**Drosophila Fbl6 interacts with the Tel orthologue, Yan.** Having established a biochemical and functional interaction between human Tel and Fbl6, we next explored whether this mechanism might be evolutionarily conserved. To that end, we isolated a full-length Drosophila fbl6 (also referred to as CG13213) cDNA from insect Schneider cells. Figure 4A and B show that, like the binding of human Fbl6 to Tel (Fig. 1B and C), Drosophila Fbl6 efficiently associated with Yan, and this interaction depended on the Yan SAM domain (Fig. 4A) and the LRD2 domain of Fbl6 (Fig. 4B). In contrast to Tel-Fbl6 binding, Yan-Fbl6 binding appeared to not require the LRD1 sequence (Fig. 4B).

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**FIG. 2.** (A) Tel ubiquitination. The left panel shows the results of an experiment in which Flag epitope-tagged wild-type Tel was cotransfected into U2OS cells with (+) or without (−) His epitope-tagged ubiquitin and cells were subsequently incubated in the presence (+) or absence (−) of MG132. Ubiquitinated Tel was recovered from cells lysed in guanidinium, by nickel bead purification (hereafter termed a ubiquitination assay). The right panel shows a ubiquitination assay using HA epitope-tagged Tel and either a wild-type His epitope-tagged ubiquitin or a His epitope-tagged version of ubiquitin in which all of the lysine residues have been mutated (K→A) in the presence (+) or absence (−) of MG132. Ubiquitinated Tel was recovered from cells lysed in guanidinium, by nickel bead purification (hereafter termed a ubiquitination assay). (B) The SAM domain and EDBD of Tel are required for its ubiquitination. U2OS cells were transfected with the indicated constructs (described in the legend to Fig. 1B), and a ubiquitination assay was performed as described for panel A. (C) Mutations that disrupt the EDBD of Tel lead to the mislocalization of Tel to the cytoplasm. Cells were transfected with the indicated constructs, and immunohistochemistry was performed with the antibodies shown. (D to G) Fbl6 promotes the ubiquitination of Tel. (D) U2OS cells were transfected with the indicated constructs (see the legend to Fig. 1C), and a ubiquitination assay was performed as described for panel A. (E) shRNA-mediated knockdown of fbl6 and skp1 leads to stabilization of endogenous steady-state Tel protein levels. U2OS cells were infected with lentiviruses delivering the indicated shRNAs, and following 2 days of puromycin selection, stably infected cells were lysed and proteins were subjected to Western blotting. Tubulin was used as a loading control for proteins. Quantitative PCR was used to demonstrate effective knockdown of fbl6 and skp1 transcripts. In both cases, quantitative PCR was also used to show that tel transcript levels were unaffected by any of the shRNA treatments (data not shown). The rightmost panel shows that endogenous Tel and endogenous SKP1 form a complex in tissue culture cells. U2OS cells were lysed in protein lysis buffer (see Materials and Methods), and SKP1 was purified from the lysate with a SKP1-specific antibody. Associated endogenous Tel was detected with a Tel-specific antibody. (F) Short-hairpin RNA (shRNA)-mediated knockdown of Fbl6 inhibits Tel ubiquitination. Cells were transiently transfected with the indicated constructs, and a ubiquitination assay was performed 2 days later. In the absence of an effective antibody specific for human Fbl6, the efficiency of Fbl6 knockdown was assessed separately by targeting V5 epitope-tagged Fbl6 expressed in U2OS cells. A nonspecific small interfering RNA was used as a control (see lower panel). (G) Fbl6 strongly promotes the degradation of phosphorylated Tel. The upper panel shows [35S]methionine-labeled Tel that was immunopurified from U2OS cells, following 3 h of labeling, and then treated with 10 U of calf intestinal phosphatase or left untreated for 30 min at 37°C. In the lower panel, cells were transfected with the indicated constructs, incubated in the presence (+) or absence (−) of MG132, and then lysed in Laemmli sample buffer. Proteins were detected with the indicated antibodies. Western, Western blotting; IP, immunoprecipitation; h, human.
Fbl6 promotes Yan downregulation and ubiquitination. Next, we determined whether Fbl6 influences Yan protein stability. To that end, we established a number of stable Schneider S2 cell lines and monitored their endogenous Yan protein levels. We and others have established that in *Drosophila*, a protein named Mae (modulator of the activity of Ets) orchestrates Yan derepression by binding to Yan, thereby disrupting Yan self-association and binding to DNA and sensitiz-
FIG. 4. (A) The SAM domain of Yan is needed for Yan’s association with Fbl6 in cells. HA epitope-tagged wild-type Yan, Yan expressing a deletion of the SAM domain (YanΔSAM), and Yan harboring a substitution of alanine at position 86 for arginine (YanA*) were each coexpressed with V5 epitope-tagged Drosophila Fbl6 in 293T cells. Fbl6 complexes were immunopurified from cell lysates by using an antibody directed against the V5 epitope, and associated Yan proteins were detected using an anti-Yan monoclonal antibody. (B) The LRD2 of Drosophila Fbl6 is essential for interacting with Yan. V5 epitope-tagged full-length Fbl6 or the indicated Fbl6 mutants were coexpressed with Yan in Drosophila S2 cells. Fbl6 complexes were immunopurified from cell lysates by using an antibody directed against the V5 epitope, and associated Yan proteins were detected using an anti-Yan monoclonal antibody. Fbl6ΔF-box contains a deletion of the F-box domain. Fbl6ΔLRD1 and Fbl6ΔLRD2 harbor deletions of the LRD1 and LRD2 sequences, respectively. Fbl6Δ(LRD1 + LRD2) has a deletion of both the LRD1 and LRD2 sequences. (C) Fbl6 regulates Yan downregulation in Drosophila Schneider (S2) cells. Stable S2 cell lines in which expression of the indicated constructs was placed under the control of the metallothionine promoter were established. Cells were transfected with or without the indicated double-stranded RNA (termed fbl6RNAi [fbl6i] and skpA RNAi [skpAii]), and 2 days later, expression of the indicated constructs was induced with a 600 μM copper sulfate solution. Following a further 24 h of incubation, Western blot analysis was performed with cell lysates by using the indicated antibodies. In the absence of an antibody specific for Drosophila Fbl6, the efficiency of Fbl6 knockdown was assessed by separately targeting V5 epitope-tagged Fbl6 proteins expressed in S2 cells. A nonspecific double-stranded RNA was used as a control (see lower panel). (D) Yan is ubiquitinated, a process which is augmented by Fbl6. The indicated constructs were expressed in insect S9 cells using baculoviruses, and a ubiquitination assay was performed (see the legend to Fig. 2). d, Drosophila.
To further validate these findings, we analyzed the expression that in vivo, Fbl6 participates in the process of Yan downregulation.

Overall, our work suggests that an evolutionarily conserved F-box-protein-dependent degradation pathway participates in the regulation of the activity of both vertebrate Tel and its invertebrate orthologue, Yan. Presumably, this serves to further refine spatiotemporal control of gene expression by these transcriptional repressors.

**DISCUSSION**

The ancestral Ets repressor Yan and its vertebrate orthologue, Tel (ETV6), play pivotal roles in the control of cell differentiation (15, 19, 22, 27, 35, 45, 46). Because these factors directly and negatively regulate gene expression (19, 22, 27, 35), deciphering the mechanisms that regulate their activity is crucial for understanding the spatiotemporal control of cell differentiation. We recently found that sumoylation of an N-terminal lysine residue encoded by Tel (TelK11) serves to inhibit repression of gene expression by Tel (37). We have further explored Tel/Yan posttranslational regulation, and here we report that both Tel and Yan protein downregulation is promoted by an evolutionarily conserved F-box-protein-dependent mechanism. Specifically, we found that both Tel and Yan can be ubiquitinated and that ubiquitination is facilitated by Fbl6, which sensitizes these proteins for degradation. It is, of course, possible that other F-box proteins may also play a role in regulating Tel/Yan activity, perhaps in a tissue-specific fashion, and future studies should clarify this.

**The Tel/Yan SAM domain is required for association with the F-box protein Fbl6.** We found that the SAM domain of both Tel (Fig. 1B) and Yan (Fig. 4A) is required for the binding of Fbl6. The SAM domain defines a subfamily of Ets transcription factors, including Tel, Tel2, Ets1, and Ets2 from vertebrates as well as Yan and Pointed P2 from *Drosophila* (23, 33), raising the possibility that at least some of these factors are also targeted by Fbl6 or a related F-box protein. Indeed, we found that human Fbl6 also associates with the Tel-related Tel2 protein and promotes its ubiquitination (Fig. 3F). Furthermore, we found biochemical interactions between *Drosophila* Fbl6 and the SAM domain-containing proteins Pointed P2 (3, 27) (but not P1, which lacks the SAM domain) and Mae (2, 33, 41) in tissue culture cells (Roukens and Baker, unpublished). However, we were unable to detect associations between Fbl6 and either Ets1 or Ets2, each of which harbors a SAM domain, Tel (ETV6), play pivotal roles in the control of cell differentiation (15, 19, 22, 27, 35, 45, 46).

Our data support the idea that Tel/Yan monomers are especially labile and prone to ubiquitination. Our data suggest that Tel/Yan monomers are more susceptible to ubiquitination and degradation than the oligomeric forms of these proteins are. Apparently, and paradoxically, the biochemical interactions between Tel/Yan monomers and Fbl6 were found to be far weaker than the interactions between Tel/Yan oligomers and Fbl6 (Fig. 3B and 4A). However, these interactions were strongly stabilized in
the presence of proteasome inhibitors (Fig. 3B), suggesting that the failure to find strong associations between these proteins resulted from the instability of the Tel/Yan monomer and of the Fbl6 complex. Consistent with this, we found that Tel monomers were more readily ubiquitinated than wild-type Tel (Fig. 3C). It is worth noting that, in general, wild-type Tel is detected in cells as a phosphorylated or nonphosphorylated protein species and that the phosphorylation of Tel appears to further sensitize it to Fbl6-mediated degradation (Fig. 2G). In contrast, phosphorylated forms of monomeric Tel are evidently especially unstable and are detected only in the presence of proteasome inhibitors (M. G. Roukens, M. Alloul-Ramdhani, and D. A. Baker, unpublished data). It has previously been described that Tel is negatively regulated by extracellular signal-regulated kinase-induced phosphorylation of serine residues 213 and 257 (24). Phosphorylation of these sites could be a trigger for promoting Tel ubiquitination, although in our ubiquitination assays, we were unable to detect an obvious effect of mutating these residues or an additional putative mitogen-activated protein kinase target, serine residue 22 in the N terminus of Tel (M. Alloul-Ramdhani and D. A. Baker, data not shown). Our previous work also supports

FIG. 5. Fbl6 regulates Yan downregulation in vivo. (A) P{GawB}NP0326 flies have strongly diminished levels of fbl6 expression. RT-PCR was performed on cDNA prepared from stage-15 embryos derived from the indicated fly lines. Levels of tubulin transcript were determined as a control for the procedure. (B) The P element of P{GawB}NP0326 flies specifically disrupts fbl6 and not adjacent transcripts. RT-PCR was performed as described for panel A. (C) Yan protein levels are elevated in P{GawB}NP0326 embryos. Embryos were carefully staged to stage 12 and lysed in Laemmli sample buffer. Yan protein levels were determined by Western blotting with a Yan monoclonal antibody, and tubulin protein levels were determined as a loading control. (D) Expression of argos is attenuated in P{GawB}NP0326 embryos. cDNA was prepared from total RNA isolated from differently staged embryos. Levels of argos expression and control tubulin expression were determined by RT-PCR.
the contention that Tel/Yan monomers are relatively labile (37). Monomeric Tel was detectably and very efficiently sumoylated in cells only in the presence of proteasome inhibitors, suggesting that this species of Tel is normally very unstable. The current model of Tel function holds that Tel monomers directly associate via their conserved SAM domains and that the resulting DNA-bound oligomers oppose the transcription-activating apparatus (reviewed in references 34, 42, and 44). Since Fbl6 also interacts with the Tel SAM domain (Fig. 1 and 4), perhaps self-associated Tel oligomers are relatively more resistant to F-box-mediated degradation than are Tel monomers because of reduced accessibility to exposed SAM domains. In the future, it should be of considerable interest to elucidate how Tel sumoylation and ubiquitination are precisely integrated to regulate Tel activity.

**Fbl6 regulates Yan protein stability.** Our work suggests that Fbl6 regulates Yan protein levels in vivo. Drosophila embryos that lack fbl6 expression contain significantly elevated levels of Yan protein. Consistent with this, the levels of expression of a previous studies that delineated the function of these unique evolutionarily conserved pathway of Tel/Yan downregulation. integrated to regulate Tel activity.

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**REFERENCES**


Supplemental TABLE 1. Proteins that interact with both *Drosophila* Yan and human Tel.

Independent yeast-2-hybrid screens were performed using either Yan or Tel as a bait. Yan was used to screen an 18 hr Drosophila embryo library (Clontech) and Tel was used to screen a human bone marrow library (Clontech).

1) Sir2/ CG5216
2) INT6/ CG9677
3) Regulator of chromosome condensation 1 (RCC1)/ CG10480
4) Pin1 (peptidyl prolyl isomerase)/ CG17051
5) (CBP/p300)/ CG15319
6) Fbl6/ CG13213