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# Interplay of Fli-I and FLAP1 for regulation of β-catenin dependent transcription

**Young-Ho Lee and Michael R. Stallcup***

Department of Biochemistry and Molecular Biology, University of Southern California, 1333 San Pablo Avenue, MCA 51A, Los Angeles, CA 90089, USA

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## ABSTRACT

**β-catenin mediates Wnt/wingless signaling and transcriptional activation by lymphocyte enhancer binding factor 1/T cell factor (LEF1/TCF) proteins with the assistance of multiple coregulators, including positive cofactors like p300/CBP and negative cofactors like HDACs. We previously demonstrated that a developmentally essential protein, Flightless-I (Fli-I), serves as a coactivator for nuclear receptor-mediated transcription. To further understand the action mechanism of Fli-I, we investigated the functional roles of Fli-I and Fli-I leucine rich repeat associated protein 1 (FLAP1) in transcriptional activation by β-catenin and LEF1/TCF. β-catenin-dependent transcription was activated by exogenous FLAP1 but inhibited by Fli-I. Reduction of endogenous FLAP1 levels compromised transcriptional activation by LEF1/TCF, β-catenin and the p160 coactivator GRIP1. FLAP1 interacted directly with β-catenin, GRIP1 and p300 and enhanced their activity. Furthermore, FLAP1 was strongly synergistic with p300 in supporting transcriptional activation by β-catenin and LEF1/TCF, but Fli-I disrupted the synergy of FLAP1 with p300 and β-catenin. Thus the opposing effects of Fli-I and FLAP1 may be a key regulatory mechanism for β-catenin and LEF1/TCF-mediated transcription and thus for Wnt signaling, and some mutations of Fli-I may result in developmental defects, such as the flightless phenotype of Drosophila, by causing dysregulation of the Wnt/β-catenin pathway.**

## INTRODUCTION

β-catenin is an essential factor in various developmental and pathological processes of animals from Drosophila to humans (1,2). β-catenin has important roles in regulating cell–cell contacts and actin cytoskeleton configuration (3). β-catenin is also involved in the Wnt/wingless signaling pathway and acts as a coactivator for the lymphocyte enhancer binding factor 1/T cell factor (LEF1/TCF) family of transcriptional activator proteins (1). Binding of Wnt ligand to a Frizzled receptor leads to the activation of Disheveled protein and the inhibition of kinase activity of the glycogen synthase kinase-3B/Axin/adenomatous polyposis coli complex. This prevents phosphorylation of β-catenin and thus results in stabilization of β-catenin in the cytoplasm. The accumulated β-catenin protein translocates into the nucleus, where it binds to and enhances transcriptional activation by LEF1/TCF (4). The β-catenin-LEF1/TCF complex regulates the expression of the c-Myc and cyclin D1 genes among others. β-catenin and LEF1/TCF dependent gene expression is regulated by the interplay of various coregulators. Positive transcription regulators for β-catenin and LEF1/TCF include p300/CBP (5–7), BRG1 (8), the p160 coactivator GRIP1 (9,10) and CARM1 (11). Negative regulators of β-catenin and LEF1/TCF include HDACs, CtBP, Groucho and Chibby (4,12–16).

Interestingly, the β-catenin mediated pathway has crosstalk with nuclear receptor (NR) dependent pathways. β-catenin interacts directly with androgen receptor (AR) and acts as a coactivator for AR-dependent transcription (10,17,18). Some common coactivators, including β-catenin and p300, mediate transcriptional activation by LEF1/TCF and NRs (5–7,9–11). These coactivators enhance transcription activation by remodeling chromatin and by direct interaction with other components of the transcription machinery. Many coactivators form complexes that synergistically enhance transcriptional activation. For example, the three p160 coactivators (SRC1, GRIP1/TIF2, pCIP/ACTR/AIB1/RAC3/TRAM1) interact with other coactivators like the protein acetyltransferase p300 and coactivator associated arginine methyltransferase 1 (CARM1) to regulate histone acetylation and methylation. The C-terminal activation domain (AD) 2 of GRIP1 binds to CARM1 and the adjacent domain AD1 binds to p300/CBP (19). In addition, the N-terminal AD3 domain of GRIP1 interacts with Fli-I (Flightless-I) and other coactivators (18,20,21). Many of these components cooperate synergistically as coactivators for various DNA-binding transcription factors. For example, CARM1 and p300 synergistically enhance the activity of NR, β-catenin, p53, NFkB

*To whom correspondence should be addressed. Tel: +1 323 442 1145; Fax: +1 323 442 1224; Email: stallcup@usc.edu

and other transcription factors (11,22–24). Similarly, CARM1 and Fli-I show synergy in the activation of NR-dependent transcription (20)

Previously, we identified Fli-I as a CARM1 binding protein and as a coactivator for NR-dependent transcription (20). Fli-I was originally characterized as a developmentally essential protein in Drosophila (25). Severe mutations or homozygous knock-out of the gene encoding Fli-I lead to impaired cellularization and gastrulation of Drosophila embryos and early embryonic death in mice (26). Even mild mutations of Fli-I in Drosophila cause defects in the development of flight muscles and a loss-of-flight phenotype. The human Fli-I gene is located in a region of chromosome 17p which is associated with Smith-Magenis syndrome, a genetic disease causing developmental and behavioral abnormalities (27). In spite of the developmental significance of Fli-I, its biochemical roles remain to be further elucidated. Fli-I has a highly conserved protein structure among Drosophila, mouse and human (25), with a leucine rich repeat (LRR) motif at the N-terminus and a gelsolin-like domain in the C-terminal region. The C-terminal region of human Fli-I has 31% identity and 52% similarity to human gelsolin, which is a member of an actin-binding protein family. The gelsolin-like domain of Fli-I interacts with actin and the actin-like protein BAF53 (Brg1 associated factor 53), which are both components of the Swi/Snf complex (20,28). In cultured cells Fli-I can be in the nucleus or associated with actin in the cytoskeleton, depending on the serum levels and growth conditions, suggesting multiple roles for Fli-I in transcription and cytoskeleton regulation (29). As with the LRR-motifs of other proteins, the LRR domain of Fli-I consists of 16 tandem LRRs. Proteins with LRR domains have diverse cellular localization and functions such as transcription and signal transduction (30). LRR domains often function in protein–protein interactions. To understand the roles of Fli-I LRR, two independent groups identified interacting proteins by yeast two-hybrid screening (31,32). These include mouse Fli-I LRR associating protein 1 (FLAP1) and human LRR Fli-I interacting proteins 1 and 2 (LRRFIP1 and 2). A related protein, the human immunodeficiency virus TAR RNA interacting protein (TRIP), was isolated by another group of investigators (33). Interestingly, all of these proteins, FLAP1, LRRFIP1 and 2 and TRIP have similar sequences and predicted structure rich in alpha-helices and coiled-coils. However, the biochemical functions of these proteins remain obscure.

In this study, we investigated the functional roles of Fli-I and FLAP1 in regulating transcriptional activation by β-catenin and LEF1/TCF. FLAP1 is a key activator, cooperating synergistically with p300 to mediate transcriptional activation by β-catenin and LEF1/TCF. This synergy of FLAP1 and p300 was negatively regulated by Fli-I. This study suggests that the interplay of Fli-I and FLAP1 may be one of the important regulation mechanisms of the β-catenin dependent, Wnt signaling pathway.

## MATERIALS AND METHODS

### Plasmids

A FLAP1 expression vector, pCMV-FLAP1 was obtained from Dr Helen L. Yin (University of Texas Southwestern Medical Center) (32). The other constructs were described in previous publications as follows: coactivator expression vectors: pSG5-HA.GRIP1, pSG5-HA.CARM1, pCMV-p300, pSG5.HA (24); Fli-I expression vectors: pcDNA-Fli-I, pSG5-flag.LRR and pSG65-flag.Gelsolin (20); GST-protein expression vectors: pGEX4T-KIX, pGEX4T-GBD (34); pGEX4T-GRIP1N, pGEX4T-GRIP1M, pGEX4T-GRIP1C, pGEX4T-ER (24); pGEX-β-catenin, pGEX-LEF1, TOP-flash, pGL3OT-luc, pSG5-HA.β-catenin, pSG5-HA.LEF1, pM-β-catenin (9,11); pM-GRIP1N, pM-GRIP1M and pM-GRIP1C (19).

### Transfection

293T cells were transfected with plasmids by using F1 Targefect reagent (Targeting Systems) according to the manufacturer's protocol as described previously (20,24). Luciferase activity was measured after 40 h. Results shown are the mean and deviations from mean of two transfected cultures. Luciferase assay data shown are representative of at least three independent experiments.

### GST-pulldown assay

The procedure for GST-pulldown assays was described previously (20,34) and used GST fusion proteins produced in *Escherichia coli* BL21 cells. The transformed *E.coli* cells were induced by IPTG and lysed by sonication. GST-fused proteins were collected by incubation with glutathione Sepharose beads.

### Co-immunoprecipitation and immunoblot

The co-immunoprecipitation assay was described previously (20,24). One microgram of anti-flag antibody (Sigma) and protein A/G agarose were incubated with cell extracts. The immunoprecipitated fractions were washed and analyzed by immunoblot analysis with anti-HA antibody (Roche).

### siRNA

siRNA assays were described previously (20,34). siRNAs for FLAP1 (designed from the human LRRFIP1 sequence in GenBank accession no. NM_004735) are as follows. siFLAP1A: (sense) AGCCGGGAGAUCGACUGUUdTdT, (anti-sense) AACAGUCGAUCUCCCGGCUdTdT; siFLAP1B: (sense) GAAACACAUCGGCUUCUGAdTdT, (anti-sense) UCAGAAGCCGAUGUGUUUCdTdT.

Sequences of siRNAs for Fli-I, AIB1 and CARM1 were described previously (20). Transfections were performed by using oligofectamin or lipofectamine 2000 (invitrogen). MCF-7 and 293T cells were used for this assay. FLAP1 antibody (BD Transduction laboratories) was used in immunoblots for detecting endogenous FLAP1 in MCF7 cells.

## RESULTS

### FLAP1 and Fli-I regulate transcription mediated by β-catenin and LEF1

LEF1/TCF-mediated transcription was examined by transfection of the pGL3OT-Luc or TOPflash-luc reporter plasmid (regulated by binding sites for LEF1/TCF) into mammalian

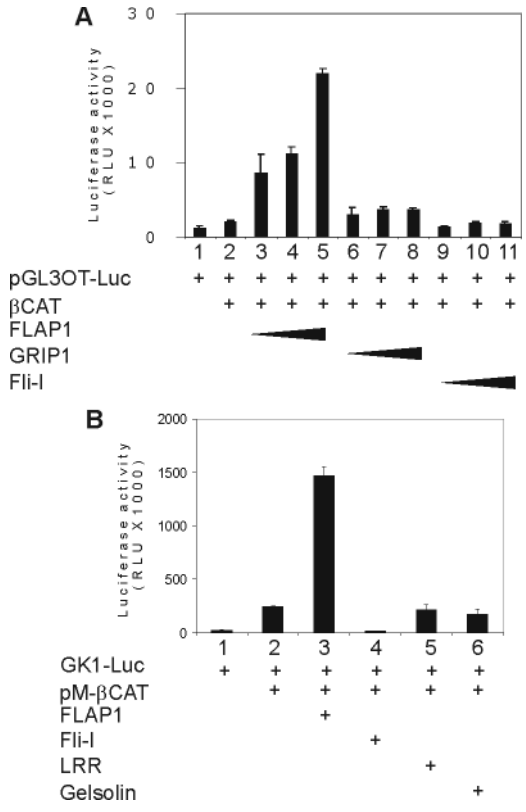



**Figure 1.** FLAP1 and Fli-I regulate β-catenin and LEF1/TCF-mediated transcription. (**A**) Reporter plasmid pGL3OT-luc (0.2 μg) was transfected into 293T cells in 12-well dishes along with pSG5-βCAT (0.2 μg) and pCMV-FLAP1, pSG5.HA-GRIP1 or pcDNA-Fli-I (0.2–0.6 μg) as indicated. (**B**) Reporter plasmid GK1-Luc (0.2 μg) was transfected into 293T cells with 0.2 μg of each of the following plasmids, as indicated: pM-β-catenin, pCMV-FLAP1, pcDNA-Fli-I, pSG5-Fli-LRR, pSG5-Fli-Gelsolin.

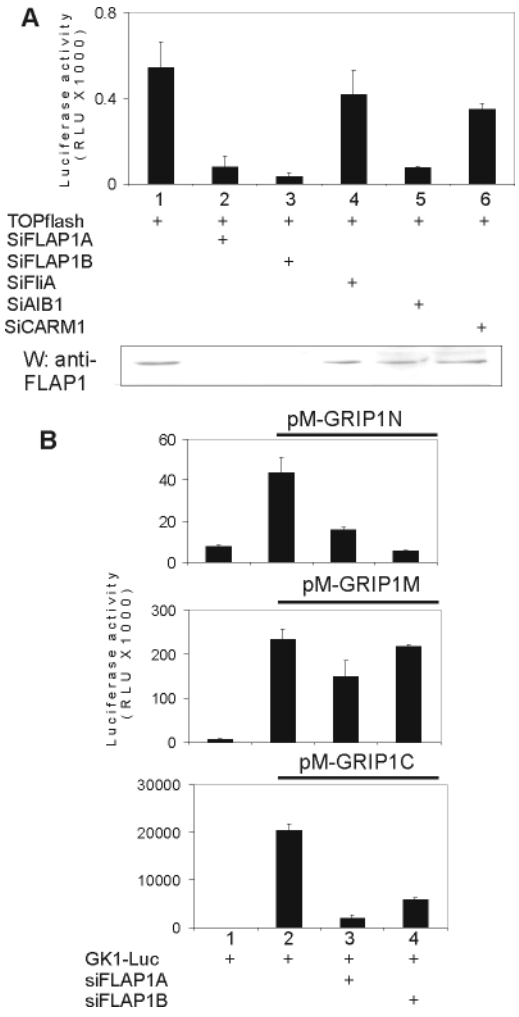



**Figure 2.** Inhibition of LEF1/TCF-mediated and GRIP1-mediated transcription by siRNAs for FLAP1. (**A**) TOPflash reporter plasmid (0.5 μg) was transfected into MCF-7 cells in 12-well dishes along with 50 pmol of siRNA for FLAP1, Fli-I, AIB1 or CARM1. Luciferase activity was measured at 40 h (top panel) and part of each cell extract was analyzed by immunoblot with 1 μg of anti-FLAP1 antibody to monitor the endogenous level of FLAP1 protein (bottom panel). (**B**) GK1 reporter plasmid (0.2 μg) was transfected into 293T cells along with pM-GRIP1N, pM-GRIP1M or pM-GRIP1C (0.2 μg) and 50 pmol of siRNAs for FLAP1.

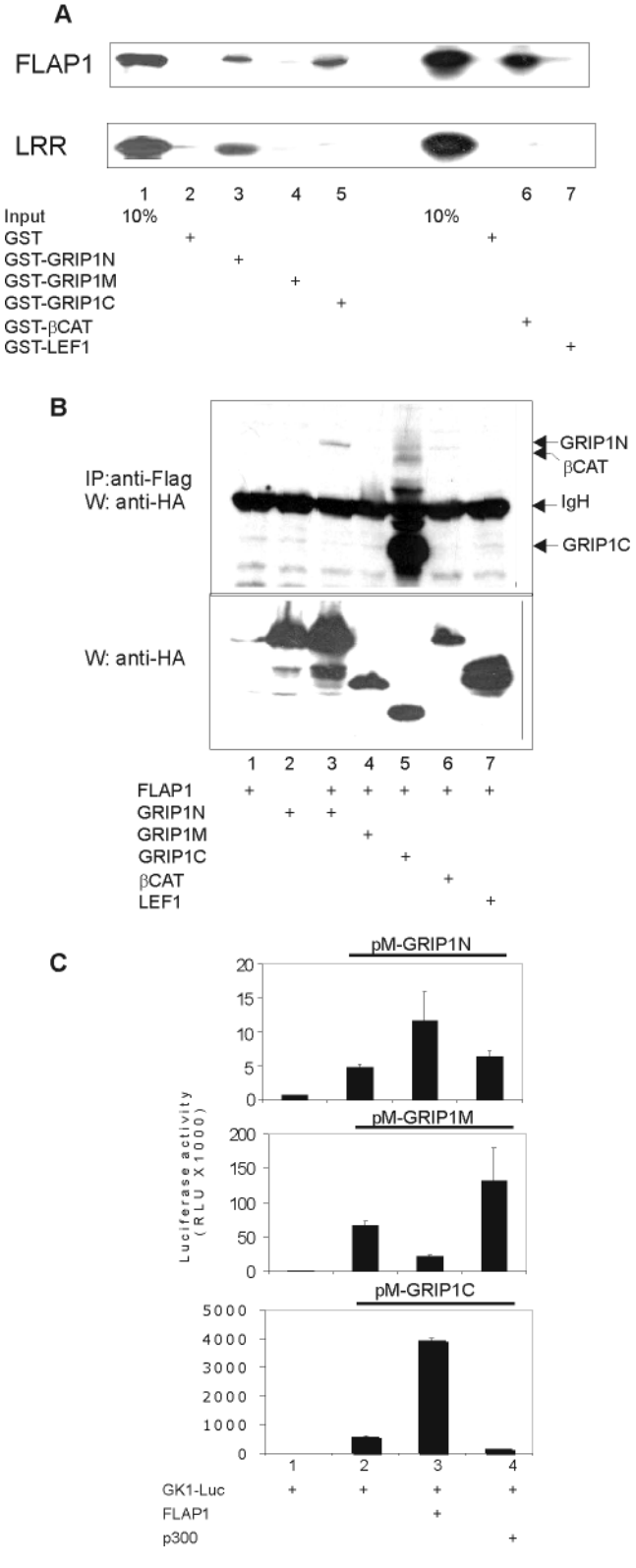
cells. Over-expression of β-catenin activated the TOPflash reporter in 293T cells (Figure 1A, bar 2) and transfection of increasing amounts of FLAP1 expression plasmid led to a dramatic activation of LEF1/TCF-dependent transcription (bars 3–5). In contrast, over-expression of Fli-I did not affect transcription activation by β-catenin and LEF1/TCF (bars 9–11). GRIP1 also cooperated with β-catenin to enhance LEF1/TCF-dependent transcription (bars 6–8) as previously shown (9,10).

To test the effects of FLAP1 and Fli-I on β-catenin activity in a one hybrid assay, a plasmid encoding GAL4 DBD fused to β-catenin was transfected along with the GK1-luc reporter plasmid, which is controlled by binding sites for GAL4 DBD. GAL4 DBD fused to β-catenin enhanced expression of the GK1-luc reporter gene (Figure 1B, bar 2) and co-expression of FLAP1 further enhanced β-catenin-mediated transcriptional activation (bar 3). Surprisingly, over-expressed Fli-I strongly inhibited β-catenin activity (bar 4). However, the LRR and gelsolin fragments of Fli-I caused little if any inhibition of β-catenin-mediated transcription (bars 5 and 6), even though these Fli-I fragments were expressed at higher levels than full length Fli-I (20). This result suggests that both LRR and gelsolin domains are required for the inhibitory effect of Fli-I on β-catenin activity. Thus, FLAP1 enhanced LEF1/TCF-mediated transcription (Figure 1A) and β-catenin dependent transcription (Figure 1B), while full-length Fli-I acted as a negative regulator of β-catenin-dependent transcription (Figure 1B).

## siRNAs for FLAP1 inhibit LEF1/TCF mediated transcription

Two siRNAs for FLAP1 (designed according to the sequence of the human ortholog LRRFIP1) efficiently reduced the level of endogenous FLAP1/LRRFIP1 in MCF-7 cells (Figure 2A, bottom panel). However, siRNAs for Fli-I, AIB1 and CARM1 did not affect the endogenous protein level of FLAP1. The siRNAs for Fli-I, AIB1 and CARM1 were previously demonstrated to cause reduced levels of the corresponding endogenous proteins (20). The roles of endogenous FLAP1 and Fli-I in transcription mediated by β-catenin and LEF1/TCF were first tested in MCF-7 cells, which have elevated levels of Wnt (35,36). The

LEF1/TCF-dependent TOPflash reporter plasmid had high basal activity in MCF-7 cells, but reduction of endogenous FLAP1 by siRNAs reduced TOPflash expression (Figure 2A, upper panel, bars 1–3). siRNA for AIB1 also suppressed TOPflash expression, but siRNAs for CARM1 and Fli-I had little effect (bars 4–6). Similar results were obtained in 293T cells or when a different LEF1/TCF-dependent reporter plasmid, pGL3OT-luc, was used (data not shown). These results suggest that endogenous FLAP1 is an essential coactivator for LEF1/TCF mediated transcription.




## Interaction of FLAP1 with β-catenin and other coactivators

Endogenous and exogenous FLAP1 has coactivator activity for β-catenin mediated, LEF1/TCF dependent-transcription (Figures 1 and 2). To define molecular interactions that may contribute to these activities of FLAP1, GST-pulldown assays were performed (Figure 3A). FLAP1 synthesized *in vitro* bound to GST-fused β-catenin, but not to GST-LEF1 and GST controls. FLAP1 also interacted with N- and C-terminus, but not with the middle region, of GRIP1. In contrast, the N-terminal LRR fragment of Fli-I bound to the GRIP1 N-terminal region but not to any of the other GST fusion proteins tested, as shown previously (20). Roughly equivalent levels of all the GST fusion proteins were loaded onto the glutathione-Sepharose beads, except that somewhat lower levels of GST-GRIP1M were used (Supplementary Figure 6A).

Previously, Moon and colleagues showed that the C-terminal region of β-catenin interacts with the KIX domain of CBP, a homolog of p300 (7). FLAP1 also interacted with p300 through the KIX domain but did not interact with the GRIP1 binding domain (GBD) of p300 or with GST (Supplementary Figure 6B).

In co-immunoprecipitation assays, FLAP1 associated strongly with the C-terminal fragment of GRIP1 and weakly with the N-terminus of GRIP1 and with β-catenin (Figure 3B, upper panel, lanes 3, 5 and 6). The relatively weak interactions observed may indicate that these proteins associate transiently rather than stably. However, FLAP1 did not interact with LEF1 (lane 7). The HA-tagged GRIP1 fragments, β-catenin and LEF1 were expressed well in 293T cells (Figure 3B, lower panel). Thus, the co-immunoprecipitation results (Figure 3B) were consistent with the GST-pulldown data (Figure 3A), indicating that FLAP1 binds to GRIP1, β-catenin and p300 *in vitro* and/or in mammalian cells. However, FLAP1 does not bind to LEF1, indicating that FLAP1 functions as a secondary coactivator for LEF1/TCF, i.e. the physical and functional interaction of FLAP1 with LEF1/TCF is mediated by one of the other coactivators.

**Figure 3.** Interaction of FLAP1 with β-catenin and other coactivators. (**A**) GST-pulldown assay: *in vitro* translated FLAP1 protein or Fli-I LRR fragment was incubated with purified GST fusion proteins (~1 μg) bound to glutathione-Sepharose beads. Bound protein fractions were analyzed by immunoblot with anti-Flag antibodies. (**B**) Co-immunoprecipitation assay: 2 μg of expression vectors pCMV-flag-FLAP1, pSG5.HA.GRIP1N, pSG5.HA.GRIP1M, pSG5.HA.GRIP1C, pSG5.HA.β-catenin and pSG5.HA.-LEF1 were transfected into 293T cells as indicated. After 40 h, transfected cells were collected and lysed with RIPA buffer. Two percent of cell extracts were loaded into a separate gel for loading control and analyzed by immunoblot with anti-HA antibody (lower panel). One microgram of anti-Flag antibody was added to the remaining extract with protein A/G agarose beads. Anti-HA antibody (0.2 μg of antibody per 1 ml of blocking buffer) was used to detect the co-precipitated proteins by immunoblot (upper panel). (**C**) Mammalian one hybrid assay: 0.2 μg of GK1-Luc, pM-GRIP1N, pM-GRIP1M and pM-GRIP1C were transfected along with 0.2 μg of pCMV-FLAP1 and pCMV-p300 vectors as indicated.

### FLAP1 enhances the activities of two activation domains of GRIP1

p300/CBP binds to and activates the middle domain of GRIP1, which contains AD1 (21). Since FLAP1 binds to both N- and C-termini of GRIP1, we tested the effects of FLAP1 and p300 on the three activation domains of GRIP1 fused to GAL4 DBD. The autonomous transcription activation activities of GRIP1N and GRIP1C (Figure 3C, bars 2) were further enhanced by the expression of FLAP1 (bars 3), but not by p300 (bars 4). In contrast, the autonomous activity of GRIP1M was enhanced by p300, but not by FLAP1. This shows that FLAP1 activates the activation domains in the N- and C-termini of GRIP1, while p300 activates AD1, located in the middle-domain of GRIP1. In support of these conclusions, siRNAs for FLAP1 specifically inhibited transcriptional activation by GRIP1N and GRIP1C, but not GRIP1M, fused to GAL4 DBD (Figure 2B, bars 3 and 4). Endogenous FLAP1 therefore has a selective role in mediating transcriptional activation by GRIP1N and C, but not by GRIP1M. Thus, FLAP1 binds to various coactivators including β-catenin, GRIP1 and p300 and may contribute to the transcription-enhancing activity of β-catenin by direct contact with one or more of these coactivators.

### Synergistic enhancement by FLAP1 and p300 of transcriptional activation by β-catenin and LEF1/TCF or by androgen receptor

FLAP1 and p300 independently enhanced the transcriptional activity of β-catenin tethered to GAL4 DBD (Figure 4A, bars 3 and 4). However, when both FLAP1 and p300 were co-expressed, the β-catenin-dependent transcription was synergistically activated (bar 5). In addition, FLAP1 and p300 independently enhanced LEF1/TCF-dependent transcription in the presence of β-catenin (Figure 4B, bars 4–6). However, the co-expression of FLAP1, p300 and β-catenin caused synergistic enhancement of LEF1/TCF mediated transcription (bar 8). The coactivator synergy of FLAP1 and p300 was not observed without the expression of β-catenin (bar 7), consistent with our finding that FLAP1 does not bind to LEF1/TCF (Figure 3A). The synergy of FLAP1 and p300 was also observed in TOPflash reporter assay system (Figure 4C, bar 5), but not with FOPflash reporter (which has mutant TCF/LEF binding sites) (bar 10). This shows that the synergy of FLAP1 and p300 is dependent upon the binding of LEF1/TCF to DNA.

The synergy of FLAP1 and p300 was also investigated in an AR-mediated transcription system. The mouse mammary tumor virus (MMTV)-luc reporter plasmid (which has binding sites for AR) was transfected into 293T cells along with an AR expression vector and the cells were treated with dihydrotestosterone (DHT). FLAP1 or p300 individually had little effect on AR-mediated transcription (Figure 4D, bars 3 and 4). However, when FLAP1 and p300 were expressed together, AR-mediated transcription was synergistically activated (bars 10 and 11) and this synergy was dependent on the co-expression of either β-catenin or GRIP1 (bar 7). Thus, the combination of FLAP1 and p300 without β-catenin or GRIP1 was not very effective in the activation of AR-mediated transcription. Thus, FLAP1 cooperates synergistically with p300

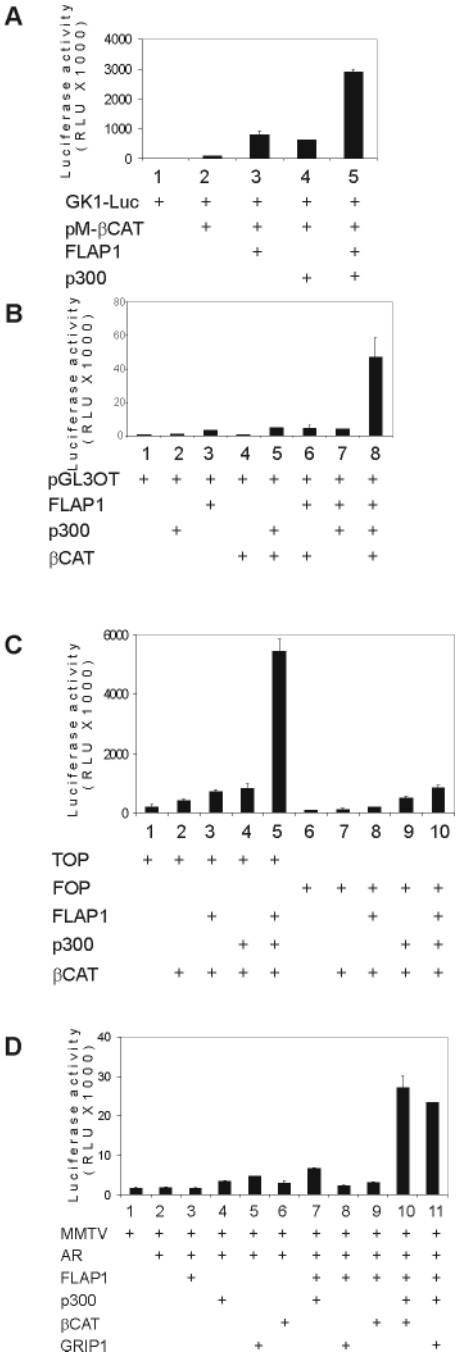



**Figure 4.** Synergy of FLAP1 and p300 for enhancement of transcriptional activation by β-catenin, LEF1/TCF and AR. (**A**) Mammalian one hybrid assay was performed as follows: GK1-Luc, pCMV-FLAP1 and pCMV-p300 (0.2 μg of each) were transfected into 293T cells as indicated. Luciferase activity was measured at 40 h. (**B**) LEF1/TCF-dependent transient transfection assay was performed with pGL3OT-luc reporter plasmid as follows: pGL3OT-luc, pCMV-FLAP1, pCMV-p300, pSG5-bCAT, pSG5.HA-GRIP1 and pcDNA-Fli-I (0.2 μg of each as indicated) were transfected into 293T cells. (**C**) LEF1/TCF-dependent transient transfection assay was performed with TOP/FOP reporter plasmids as follows: TOPflash-luc, FOPflash-luc, pCMV-FLAP1, pCMV-p300 and pSG5-bCAT (0.2 μg of each as indicated) were transfected into 293T cells. (**D**) AR-dependent transient transfection assays were performed as follows: MMTV-luc, pCMV-FLAP1, pCMV-p300, pSG5-β-catenin and pSG5.HA-GRIP1 (0.2 μg of each as indicated) and 10 ng of AR expression vector were transfected into 293T cells. 100 μM of DHT was added after transfection.

as a secondary coactivator for transcriptional activation by LEF1/TCF or AR, by binding to the primary coactivator β-catenin or GRIP1. In spite of its dependence on GRIP1 or β-catenin for its coactivator function with AR and ER (37), we found that FLAP1 did bind to at least some NRs. FLAP1 bound to estrogen receptor (ER) in a ligand-independent manner (Supplementary Figure 6C). However, over-expression of FLAP1 alone was not able to enhance transcriptional activation by either of the two activation domains of ER, AF1 or AF2, which were fused to GAL4 DBD (Supplementary Figure 6D). In contrast, the activities of both ERAF1 and ERAF2 were enhanced by the co-expression of p300.

### Fli-I inhibits the synergy of FLAP1 and p300

Fli-I was previously characterized as a coactivator for NRs and Fli-I functions synergistically with GRIP1 and CARM1 as a coactivator for NRs (20). Since Fli-I also binds to FLAP1 (32) and inhibits β-catenin mediated transcription (Figure 1B), we investigated the effect of Fli-I on the synergy of FLAP1 and p300. As previously shown (Figure 4), FLAP1 and p300 synergistically enhanced transcriptional activation by β-catenin fused to GAL4 DBD (bar 8, Figure 5A). However, the coactivator synergy of FLAP1 and p300 was inhibited severely by co-transfection of increasing amounts of Fli-I expression vectors (lanes 9–13). The LRR and gelsolin-like fragments of Fli-I were less effective inhibitors of β-catenin mediated transcription (bars 5–8, Figure 5B), although they are expressed at higher levels than full length Fli-I (20). This suggests that functions of both the LRR and gelsolin-like domains are required for the inhibitory activity of Fli-I. Thus, although Fli-I cooperates synergistically in GRIP1 coactivator complexes with NRs, Fli-I negatively regulates the activity of β-catenin coactivator complexes. As an initial investigation of the mechanism of this negative regulation, we tested whether Fli-I fragments might inhibit binding of FLAP1 to p300. However, the LRR and Gelsolin-like fragments of Fli-I bound weakly or not at all to the p300 KIX domain (Supplementary Figure 6E and data not shown). We further tested whether the LRR and Gelsolin-like fragments of Fli-I could interfere with the binding of FLAP1 to the p300 KIX domain fused to GST. However, when 5-fold excess levels of *in vitro* translated LRR and Gelsolin-like fragments were mixed and incubated with *in vitro* translated FLAP1 and bead-bound GST-KIX, there was no reduction in binding of FLAP1 to the beads (Supplementary Figure 6F), suggesting that Fli-I does not interfere with the binding of FLAP1 to p300. Thus, the mechanism for the inhibitory effect of Fli-I on the coactivator function of β-catenin, FLAP1 and p300 remains to be determined.

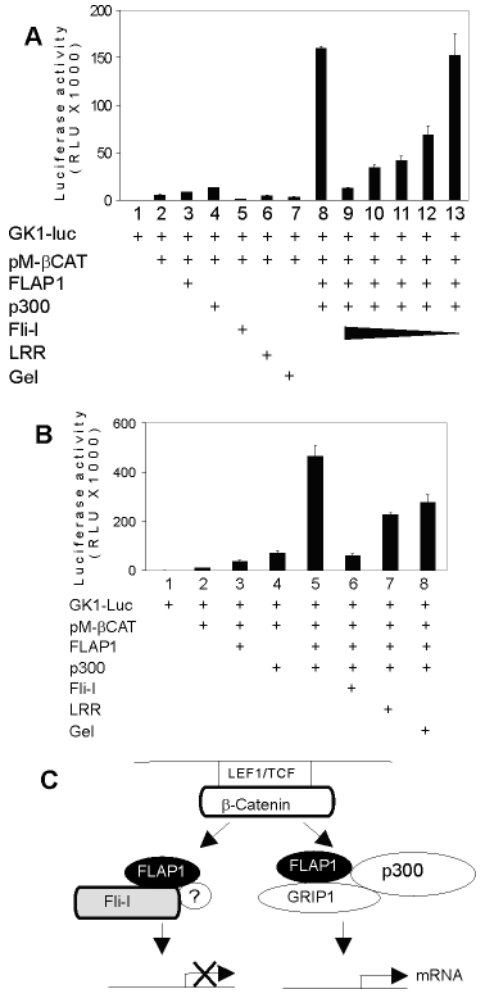



**Figure 5.** Fli-I inhibits the synergy of FLAP1 and p300. (**A**) and (**B**). Mammalian one hybrid assay: GK1-Luc, pM-β-catenin, pCMV-FLAP1, pCMV-p300, pSG5-LRR, pSG5-Gelsolin (0.1 μg of each as indicated), were transfected into 293T cells. Variable amounts of Fli-I expression vectors were used: A, bar 5: 0.1 μg; Bars 9–13: 0.2, 0.1, 0.05, 0.025, 0.01 μg. B, bar 6: 0.1 μg. (**C**) Model: Interplay of Fli-I and FLAP1 for regulating β-catenin mediated gene expression. β-catenin is translocated into the nucleus and interacts with LEF1/TCF to activate transcription. β−catenin recruits other coregulators to modulate LEF1/TCF-mediated transcription. β-catenin interacts with FLAP1, p300 and GRIP1 to activate transcription synergistically. However, elevated levels of Fli-I protein shut down FLAP1-dependent β-catenin/LEF1/TCF mediated transcription activation.

## DISCUSSION

### FLAP1 activates β-catenin/LEF1/TCF-mediated transcription.

FLAP1, its apparent human ortholog LRRFIP1 and another homologous human protein LRRFIP2 were originally identified as proteins that bind to the LRR domain of Fli-I (31,32). Another protein, TRIP, was identified as a TAR RNA binding protein and also has significant homology with FLAP1 (33). However, the functional roles of FLAP1 and the other Fli-LRR interacting molecules are not well characterized. The current study shows that FLAP1 is required for efficient transcriptional activation by β-catenin and LEF1/TCF (Figures 1 and 2). FLAP1 acts as a coactivator by binding to the activation domains of other coactivators like GRIP1 and β-catenin and enhancing their activity (Figure 3).

Recently, LRRFIP2 was shown to be a strong activator for β-catenin dependent transcription (38) and a dominant negative form of LRRFIP2 suppressed Wnt signaling in Xenopus. This is consistent with our data showing an important role for FLAP1 in β-catenin dependent transcription. LRRFIP2 interacts with Dvl (disheveled) and activates the cytoplasmic part of the Wnt/β-catenin pathway (38). However, our study shows that mouse FLAP1 (and therefore

presumably the orthologous human LRRFIP1) binds to nuclear proteins like GRIP1 and p300 and cooperates with these coactivators to enhance transcriptional activation by β-catenin and its associated DNA-binding transcription factors. Thus, it is possible that FLAP1 may activate the Wnt/β-catenin pathway at both the cytoplasmic and nuclear levels. Since FLAP1 and LRRFIP2 are 58% identical in amino acid sequence (by comparison of GenBank accession numbers AAC40072 and AAD41257), FLAP1 may also interact with Dvl and activate the cytoplasmic pathway of β-catenin. On the other hand, it is possible that FLAP1 may have a unique mechanism, which is different from that of LRRFIP2. For example, FLAP1 may primarily act inside the nucleus and LRRFIP2 in the cytoplasm, such that they contribute in a complementary way to regulation of Wnt/β-catenin signaling. Thus, FLAP1, LRRFIP1, LRRFIP2 and TRIP may comprise a family of proteins, which regulate Wnt/β-catenin signaling through a variety of mechanisms.

This study also suggests that FLAP1 cooperates with p300 for the activation of β-catenin dependent, LEF1/TCF mediated and AR-mediated transcription (Figure 5). This is reminiscent of the synergy of CARM1 and p300 or the synergy of CARM1 and Fli-I (11,20,24). The synergy of all these secondary coactivators, including FLAP1, requires the co-expression of a primary coactivator, such as GRIP1 or β-catenin. GRIP1 or β-catenin may act as a bridge factor for stabilizing the coactivator complex, although FLAP1 interacts directly with p300 *in vitro* (Supplementary Figure 6). Alternatively, by associating directly with the DNA bound transcription factor (LEF1/TCF or NR), GRIP1 and β-catenin may provide a platform for recruiting FLAP1 and p300 to the promoter. GRIP1 and β-catenin may have overlapping or complementary roles in promoting the synergistic action of FLAP1 and p300. FLAP1 shows a unique pattern of coactivator binding and transcription activation, which is distinguished from p300 and other coactivators. FLAP1, p300 and CARM1 bind to different ADs of GRIP1 and presumably transmit output signals to different downstream targets in the transcription machinery, thus accounting for their synergistic cooperation. The fact that FLAP1 and p300 together interact with all three ADs of GRIP1 may explain why this combination of coactivators yields such a strong synergy with GRIP1 and β-catenin. Overall, our results suggest that FLAP1 may be an essential partner molecule which cooperates with p300 to mediate the Wnt signaling pathway (Figure 5C).

### Implications of inhibitory roles of Fli-I in β-catenin mediated transcription and Wnt/Wingless signaling

Fli-I was originally identified as an essential protein for the development of Drosophila and mouse. Since Fli-I, like β-catenin, is associated with the actin cytoskeleton in the cytoplasm and shuttles between nucleus and cytoplasm (29), the possible involvement of Fli-I in the β-catenin/LEF1/TCF pathway was previously suggested (20). Here, we demonstrated that, in addition to its previously identified coactivator function with NRs, Fli-I has important roles in the regulation of the β-catenin/LEF1/TCF mediated pathway. Fli-I suppresses β-catenin/LEF1/TCF mediated transcription, whereas FLAP1 has the opposite effect (Figure 5C). Our initial studies on the negative regulation of β-catenin-p300-FLAP1 activity by Fli-I found that Fli-I does not bind well to p300 KIX domain and does not appear to inhibit FLAP1-p300 binding (Supplementary Figure 6E and F). Fli-I may exert its negative influence by squelching the activity of FLAP1 and other essential factors that bind to Fli-I. It is also possible that Fli-I may recruit negative regulators, such as HDACs, CtBP, Groucho and Chibby, to the β-catenin/LEF1/TCF transcription complex. Both the LRR and gelsolin-like domains of Fli-I are required for the negative regulation of β-catenin function (Figures 1 and 5). However, while the LRR domain binds to FLAP1, the inhibitory mechanisms of the gelsolin-like domain are less clear. Some of the interactions of the gelsolin domain, i.e. with BAF53 (a component of the Swi/Snf complex), CARM1, actin and other proteins (20,29), may provide clues to the mechanism by which Fli-I inhibits β-catenin mediated transcription. Since Fli-I acts positively on NR-mediated transcription (20) and negatively on β-catenin/LEF1/TCF mediated function (Figure 5), Fli-I may help to determine the balance between NR and β-catenin/LEF1/TCF activity. Increased nuclear levels of Fli-I would presumably favor NR-mediated transcription, while lowered nuclear levels of Fli-I or increased FLAP1 levels would probably result in the release of FLAP1 and support the activation of β-catenin/LEF1/TCF-mediated transcription through the synergy of FLAP1 and p300. Thus, Fli-I and FLAP1 have antagonistic roles in the regulation of β-catenin/LEF1/TCF mediated transcription and may play key roles in the regulation of the Wnt/Wingless-signaling pathway. In addition, interactions of Fli-I and FLAP1 with other proteins may also be crucial for the regulation of these pathways. It follows that mutations in Fli-I may result in dysregulation of the β-catenin pathway and Wnt/Wingless-signaling and thus may account for the previously reported developmental abnormalities of Fli-I mutants (1,2,25). Furthermore, the opposing roles of FLAP1 and Fli-I may have significance for the process of carcinogenesis. Since β-catenin over-expression is important in many cancers, FLAP1 and Fli-I may play roles as oncogene and tumor suppressor, respectively.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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