

The LIM-only protein FHL2 interacts with β -catenin and promotes differentiation of mouse myoblasts

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FHL2 is a LIM-domain protein expressed in myoblasts but down-regulated in malignant rhabdomyosarcoma cells, suggesting an important role of FHL2 in muscle development. To investigate the importance of FHL2 during myoblast differentiation, we performed a yeast two-hybrid screen using a cDNA library derived from myoblasts induced for differentiation. We identified β -catenin as a novel interaction partner of FHL2 and confirmed the specificity of association by direct in vitro binding tests and coimmunoprecipitation assays from cell lysates. Deletion analysis of both proteins revealed that the NH₂-terminal part of β -catenin is sufficient for binding in yeast, but addition of the first armadillo repeat is necessary for binding FHL2 in mammalian

cells, whereas the presence of all four LIM domains of FHL2 is needed for the interaction. Expression of FHL2 counteracts β -catenin-mediated activation of a TCF/LEF-dependent reporter gene in a dose-dependent and muscle cell-specific manner. After injection into *Xenopus* embryos, FHL2 inhibited the β -catenin-induced axis duplication. C2C12 mouse myoblasts stably expressing FHL2 show increased myogenic differentiation reflected by accelerated myotube formation and expression of muscle-specific proteins. These data imply that FHL2 is a muscle-specific repressor of LEF/TCF target genes and promotes myogenic differentiation by interacting with β -catenin.

Introduction

LIM domains, characterized by a double zinc finger motif, define the expanding family of LIM proteins involved in protein-protein interactions and transcriptional regulation (Beckerle, 1997; Dawid et al., 1998). LIM-only proteins are composed exclusively of LIM domains, whereas LIM-plus proteins combine LIM domains with other functionally active sites (Dawid et al., 1998). Among the LIM-only proteins, a subfamily containing four and a half LIM domains has been described. Of the five members, FHL1, FHL2, and FHL3 are mainly expressed in skeletal and heart muscles (Chu et al., 2000b), whereas FHL4 and ACT are highly expressed in testis (Fimia et al., 1999; Morgan and Madgwick,

1999). Recent studies suggest that FHL proteins may act as co-regulators involved in the modulation of tissue-specific gene expression by interacting with different transcription factors. FHL1 interacts with the mammalian orthologue of the *Drosophila* transcription factor suppressor of hairless (Taniguchi et al., 1998); FHL2 acts as a coactivator of the androgen receptor (Muller et al., 2000); FHL3 and to a lesser extent FHL2, enhance CREB-mediated activation of transcription (Fimia et al., 2000); and ACT functions as a coactivator of c-AMP response element modulator (CREM; Fimia et al., 1999).

FHL2, the first LIM-only protein described with four and a half LIM domains (Genini et al., 1997), was primarily identified as down-regulated in rhabdomyosarcomas LIM-domain protein (DRAL).^{*} Although FHL2/DRAL induces apoptosis in a human rhabdomyosarcoma cell line (Scholl et al., 2000), the function of the protein in normal myoblasts and cardiomyocytes, where it is predominantly expressed (Genini et al., 1997; Chu et al., 2000b), remained unclear because FHL2-deficient mice maintain normal cardiac func-

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^{*}Abbreviations used in this paper: DRAL, down-regulated in rhabdomyosarcomas LIM-domain protein; MyHC, myosin heavy chain.

tion (Chu et al., 2000a). Only the hypertrophic response to β -adrenergic stimulation was reported to be slightly modified in FHL2 knock-out mice (Kong et al., 2001). FHL2 seems to have a dual function. Although it interacts with the cytoplasmic domain of several integrin chains (Wixler et al., 2000), it also acts as a transcriptional coactivator of the androgen receptor (Muller et al., 2000). This duality is also reflected by the subcellular localization of FHL2 in the nucleus as well as in the cytoplasm and at focal contacts (Scholl et al., 2000; Wixler et al., 2000). These findings indicate that FHL2 may mediate signals from the membrane to the nucleus, though no interaction of FHL2 with transcription factors in muscle precursor cells has been reported.

A similar dual function has been shown for β -catenin, a member of the armadillo-repeat protein family that binds to the cytoplasmic domain of cadherins (Kemler, 1993; Hinck et al., 1994) or interacts with TCF transcription factors in the nucleus. The subcellular localization of β -catenin depends on its stability in the cytoplasm. In the absence of Wnt signaling, glycogen synthase kinase 3 β phosphorylates β -catenin, which is subsequently ubiquitinated by β TrCP and degraded via proteasomes (Aberle et al., 1997). On Wnt activation, cytosolic β -catenin is stabilized and translocates to the nucleus, where it associates with HMG box-containing transcription factors of the TCF/LEF family (Behrens et al., 1996; Molenaar et al., 1996; Hsu et al., 1998; Galceran et al., 1999). Although some TCFs can repress transcription by interaction with corepressors in the absence of β -catenin, association with β -catenin causes activation of TCF/LEF target genes (Miller and Moon, 1997; Nusse, 1997; Hecht and Kemler, 2000; Seidensticker and Behrens, 2000).

Vertebrate skeletal muscle cells arise from mesodermal stem cells, which subsequently differentiate into determined myogenic myoblasts. During myogenesis, myoblasts exit the cell cycle, fuse into multinucleated myotubes, and express muscle-specific proteins (Nadal-Ginard, 1978; Brand-Saberi and Christ, 1999). This complex multi-step process can be driven by a variety of myogenic inducers such as growth factors, including Wnts (Munsterberg et al., 1995), cell adhesion molecules, and transcription factors such as myogenic regulatory factors MyoD, Myf5, and myogenin (Zeschneig et al., 1995; Molkentin and Olson, 1996; Yun and Wold, 1996). It has been reported that Wnt1 may regulate Myf5 expression, whereas Wnt7a induces MyoD during embryogenesis, thus stressing the importance of Wnt signaling for muscle development (Cossu and Borello, 1999).

Here, we report that FHL2 interacts with β -catenin in mouse myoblasts and down-regulates β -catenin-mediated activation of a TCF/LEF-dependent reporter gene in these cells. Furthermore, myoblasts overexpressing FHL2 show an increased myogenic differentiation potential. These data imply that FHL2 promotes myogenic differentiation by repressing β -catenin target genes involved in proliferation.

Results

Identification of β -catenin as a novel interaction partner of FHL2

A yeast two-hybrid screen of a cDNA library derived from mouse myoblasts induced to differentiate into myotubes was

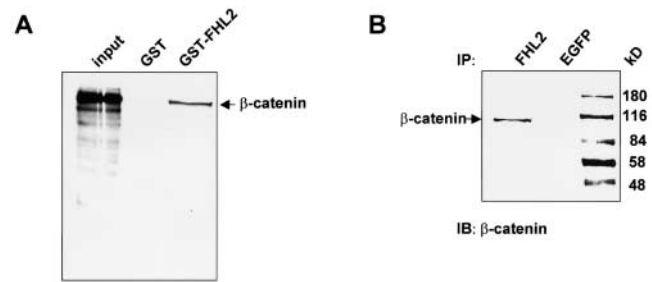


Figure 1. Interaction between FHL2 and β -catenin in vitro and in vivo. (A) Recombinant GST or GST-FHL2 fusion protein was incubated with [35 S]methionine-labeled β -catenin. After extensive washing, proteins retained by GST or GST-FHL2 were analyzed together with 10% of the input material by SDS-PAGE and fluorography. (B) C2C12 cell lysates were immunoprecipitated (IP) with rabbit pAbs against FHL2 or EGFP (negative control) as indicated and analyzed by SDS-PAGE and immunoblotting (IB) using the anti- β -catenin mAb 14. The arrows show coprecipitated β -catenin.

performed with FHL2 as bait. Of 40 isolated clones, four harbored inserts corresponding to cDNA-encoding mouse β -catenin. These inserts contained most of the β -catenin coding region, with the shortest clone lacking the first 24 amino acids. None of the β -catenin clones showed an interaction with the GAL4 DNA binding domain alone or with a control bait protein (unpublished data). Consistent with the yeast two-hybrid data, bacterially expressed GST-FHL2 efficiently interacted with in vitro-translated 35 S-radiolabeled β -catenin (Fig. 1 A). Moreover, using FHL2-specific pAbs, we were able to coimmunoprecipitate the endogenous β -catenin from lysates of exponentially growing C2C12 mouse myoblasts (Fig. 1 B). Together, these results show that the interaction of FHL2 with β -catenin is direct and occurs in yeast as well as in mammalian cells.

In addition to FHL2, we analyzed the interaction pattern of FHL1 and FHL3 proteins with β -catenin and γ -catenin (plakoglobin). Yeast two-hybrid interaction tests showed that only FHL2 was capable of binding β -catenin, but not the related LIM-only proteins, FHL1 or FHL3. In addition to β -catenin, FHL2 also bound the armadillo repeat protein (γ -catenin), but not the vinculin-like α -catenin (Fig. 2 A). The specificity of the FHL2- β -catenin interaction was confirmed by coprecipitation experiments after myc-tagged β -catenin and GST-tagged FHL proteins were coexpressed in HEK 293 cells. High levels of β -catenin were only present in GST-FHL2 complexes (Fig. 2 B; lane 4, first panel), although a very weak interaction was also noticed with FHL1. Reciprocally, only the GST-tagged FHL2 protein was found in immunocomplexes precipitated with myc-specific antibodies (Fig. 2 B; lane 4, third panel). These data clearly confirm the specificity of FHL2- β -catenin association.

Identification of protein domains important for the β -catenin-FHL2 interaction

To understand the molecular basis of the FHL2- β -catenin association, we wished to identify the interaction domains on both proteins. For this purpose, we created a series of deletion mutants for β -catenin and FHL2 (Figs. 3 and 4). Our deletion mutant analysis revealed the importance of the β -catenin NH₂ terminus for binding to FHL2. In yeast interaction as-

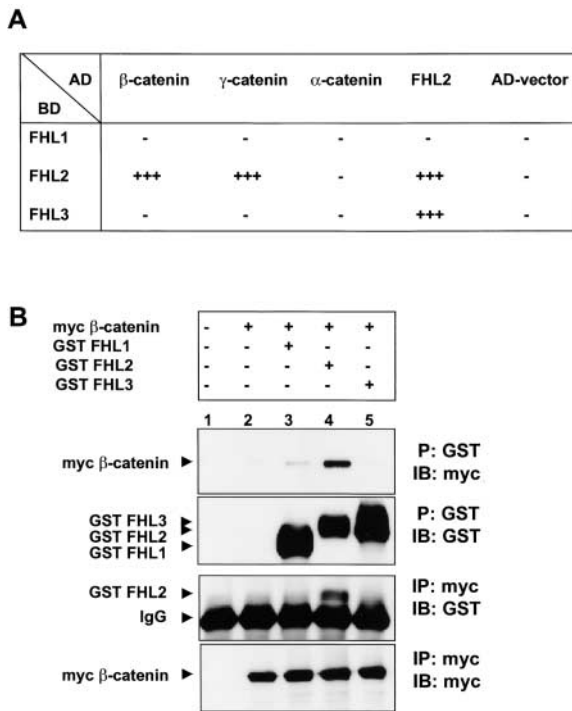


Figure 2. Specificity of interaction of FHL proteins with α -catenin, β -catenin, and γ -catenin. (A) Yeast Y190 cells were cotransformed with GAL4-DNA-binding domain (BD) and GAL4-activation domain (AD) chimeric constructs. The interaction was evaluated using a β -Gal filter assay as described in Materials and methods. The interactions of FHL proteins with AD-FHL2 or the empty AD-vector represent positive or negative controls. (B) HEK 293 cells were transiently transfected with cDNA constructs as indicated. After 34 h, RIPA cell lysates were divided into two parts and proteins were either precipitated (P) with glutathione-conjugated Sepharose beads for GST-tagged proteins or immunoprecipitated (IP) with the antibody 9E10 for myc-tagged proteins. The coprecipitated proteins were analyzed in immunoblots (IB) with appropriate antibodies. After the first immunodetection, the blots were stripped and redeveloped with antibodies against GST- or myc-tag to ascertain the amount of precipitated proteins.

says, the NH₂ terminus alone (aa 1–132) still showed binding, whereas the armadillo repeat region alone or the COOH-terminal half of β -catenin failed to do so (Fig. 3 A). Consistent with the yeast two-hybrid data, full-length β -catenin and a fragment containing the first armadillo repeat in addition to the NH₂ terminus could be coprecipitated equally well with FHL2 after coexpression in HEK 293 cells (Fig. 3 B). Because we could not observe any interaction of FHL2 with the NH₂ terminus of β -catenin alone, our data indicate that the NH₂-terminal part of β -catenin is sufficient for protein association in yeast; but in mammalian cells, addition of the first armadillo repeat is necessary for strong binding to FHL2.

To identify the part of the FHL2 molecule interacting with β -catenin, deletion mutants of FHL2 containing various numbers of LIM domains were tested for their interaction with full-length β -catenin in yeast cells (Fig. 4). Only the full-length FHL2 showed a strong interaction with β -catenin, and deletion of any LIM domain either abolished or markedly reduced the binding to β -catenin. The LIM1-4 mutant lacking the first zinc finger motif was still able to bind β -catenin (this shortened form of FHL2 was also used

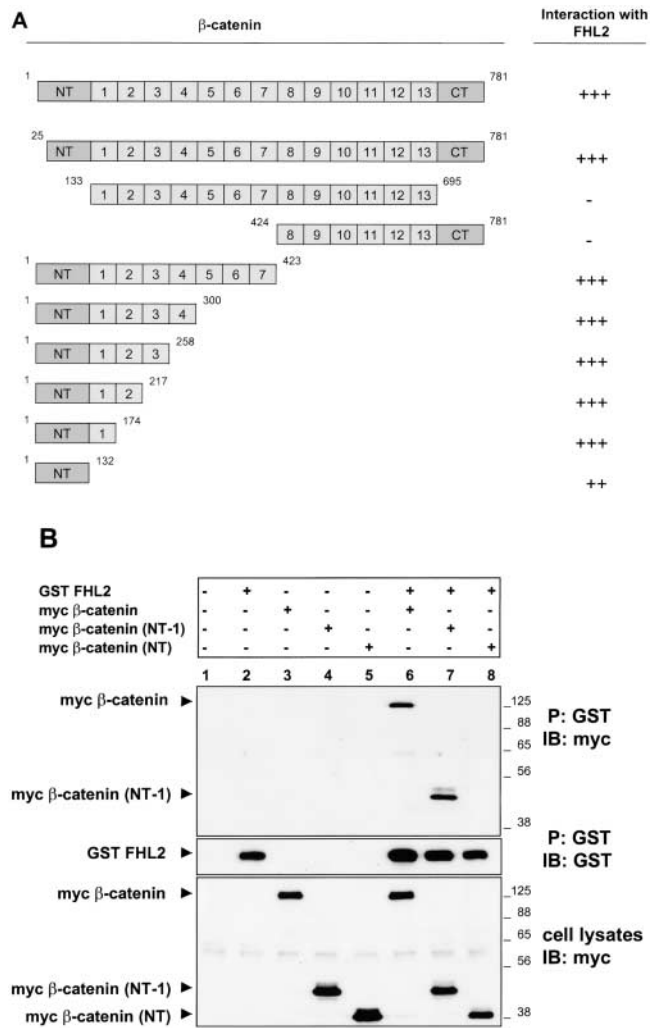


Figure 3. The NH₂ terminus of β -catenin is sufficient for binding to FHL2. (A) Yeast Y190 cells were cotransformed with GAL4-DNA-binding domain (FHL2) and GAL4-activation domain (β -catenin) chimeric constructs, and protein-protein interactions were evaluated by a β -Gal filter assay. The numbers over the diagrams indicate β -catenin amino acids encoded by each construct. The arrangement of armadillo repeats is based on Hulsken et al. (1994). (B) HEK 293 cells were transiently transfected with cDNA constructs as indicated and lysed with RIPA buffer. The presence of myc-tagged β -catenin and its deletion mutants in GST-FHL2 precipitates (P) were detected by immunoblotting (IB) with myc antibody (top blot). To ascertain the amount of precipitated FHL2 protein, the blot was redeveloped with antibodies against GST. The blot on the bottom shows the expression in transfected cells of full-length myc β -catenin and deletion mutants; β -catenin (NT-1) with the NH₂ terminus plus the first armadillo repeat, and β -catenin (NT) with the NH₂ terminus only. 10 μ g of total cell lysates were loaded.

in the original screen of the myoblast cDNA library), whereas the LIM2-4 mutant hardly showed any positive LacZ staining with the few colonies that resulted. The FHL2 deletion mutants showed the same interaction pattern for γ -catenin. Together, these data show that most of the FHL2 molecule, except the NH₂-terminal half LIM domain, is necessary for a strong interaction with β -catenin, whereas the NH₂ terminus plus the first armadillo repeat region of β -catenin molecule is needed for binding to FHL2.

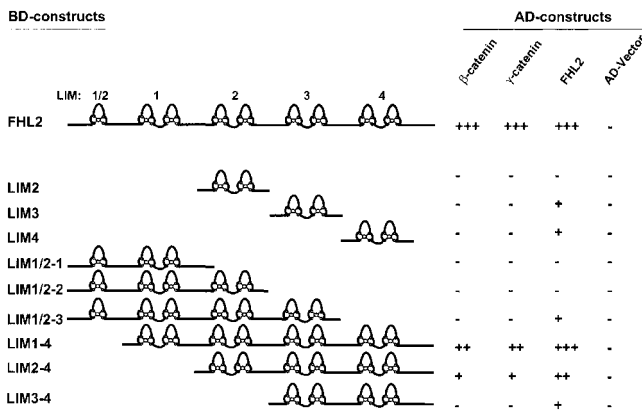


Figure 4. **Identification of the minimal binding site on FHL2 for β -catenin and γ -catenin by yeast two-hybrid assays.** Yeast Y190 cells were cotransformed with GAL4-DNA-binding domain and GAL4-activation domain chimeric constructs and protein-protein interactions were evaluated by a β -Gal filter assay. The known interaction of FHL2 with its own subdomains was used as a positive control, and the empty AD-vector served as a negative control.

FHL2 does not colocalize with membrane-bound β -catenin

To further investigate the interaction between β -catenin and FHL2, we transfected C2C12 mouse myoblasts with EGFP-FHL2 and looked for colocalization with endogenous β -catenin by immunofluorescence microscopy. Endogenous β -catenin showed membrane staining and was concentrated at cell-cell contact sites (Fig. 5, a and d) where it has been shown to interact with cadherins (Ozawa and Kemler, 1992; Redfield et al., 1997). FHL2 was also localized at the cell periphery, but mostly at focal adhesion sites (Fig. 5, b and e), as has been described already in various cells (Scholl et al., 2000; Wixler et al., 2000). Additionally, both proteins showed a cytoplasmic and nuclear staining. An overlay of both images clearly indicates that there is no colocalization of these proteins at the cell periphery (Fig. 5, c and f). The same results were obtained with immunostaining for endogenous FHL2 and β -catenin in cells of epithelial origin. We could see no colocalization of these proteins at the membrane level, regardless of whether cells had dense cell-cell contacts or were growing as single cells (unpublished data). Because there is clearly no colocalization of the two proteins at the membrane, and taking into account that activated

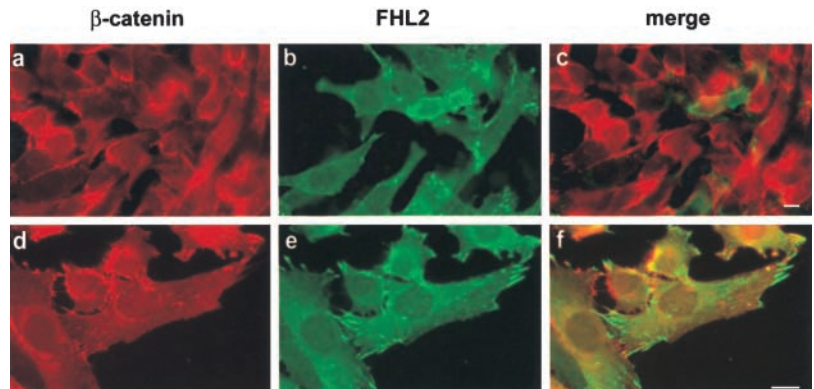
β -catenin (Behrens et al., 1996; Huber et al., 1996; Yost et al., 1996), as well as FHL2 (Scholl et al., 2000), are localized in the nucleus, we hypothesize that their interaction occurs in the nucleus.

FHL2 represses β -catenin-mediated transcriptional activation of TCF/LEF-dependent target genes

Activated β -catenin exerts its function in the nucleus by interacting with members of the TCF/LEF family of transcription factors, thereby stimulating transcription of TCF/LEF-dependent target genes. To test whether expression of FHL2 influences this particular function of β -catenin, we transiently transfected a TCF/ β -catenin-dependent TOPflash reporter construct harboring three TCF/LEF binding sites upstream of the thymidine kinase minimal promoter into I28 myoblasts (Irintchev et al., 1997) together with a plasmid encoding the phosphorylation-refractory β -catenin substitution mutant S33A (Fig. 6 A). As expected, the stabilized β -catenin mutant protein strongly activated transcription of the reporter gene (115-fold). The FOPflash reporter vector containing mutated TCF/LEF binding sites was used as a negative control and was not activated by β -catenin S33A in the same cells (unpublished data). Significantly, when FHL2 was coexpressed in myoblasts together with β -catenin S33A, FHL2 strongly repressed the β -catenin-dependent activation of the reporter gene. Furthermore, the repression effect was FHL2-specific and clearly dose dependent, as expression of the myogenic regulatory transcription factor MyoD had only a slight effect on β -catenin-mediated activation (Fig. 6 A), and transfection of C2C12 cells with increasing amounts of FHL2 cDNA led to increased repression of β -catenin-mediated activation of the reporter gene (Fig. 6 B, bars represent fold repression).

To further describe the FHL2-mediated transcriptional inhibition, we studied whether this effect could also be observed with endogenous β -catenin under conditions of stable high expression of FHL2 protein. For this purpose, we transfected the TOPflash luciferase reporter vector into mock- or FHL2-infected C2C12 cells. In the mock-infected cells, a high basal level of TOPflash reporter gene activity was measured, suggesting a high level of transcriptionally active endogenous β -catenin (Fig. 6 C) because the FOPflash was not active in the same cells (not depicted). Only a slight induction of reporter gene activity was seen when the amount of β -catenin was recombinantly increased, probably due to the

Figure 5. **Subcellular localization of FHL2 and β -catenin.** C2C12 cells cultured on coverslips were transiently transfected with EGFP-FHL2. Cells immunostained with anti- β -catenin mAb and Alexa-conjugated goat anti-mouse polyclonal antibody were analyzed by fluorescence microscopy. Although β -catenin (red) is concentrated at the membrane and at cell-cell contacts, EGFP-FHL2 (green) is concentrated at focal adhesion sites. The merged images show no colocalization of the two proteins at membrane level. Panels a-c and d-f represent pictures taken with 40 \times or 63 \times objectives, respectively. Bars, \sim 10 μ m.



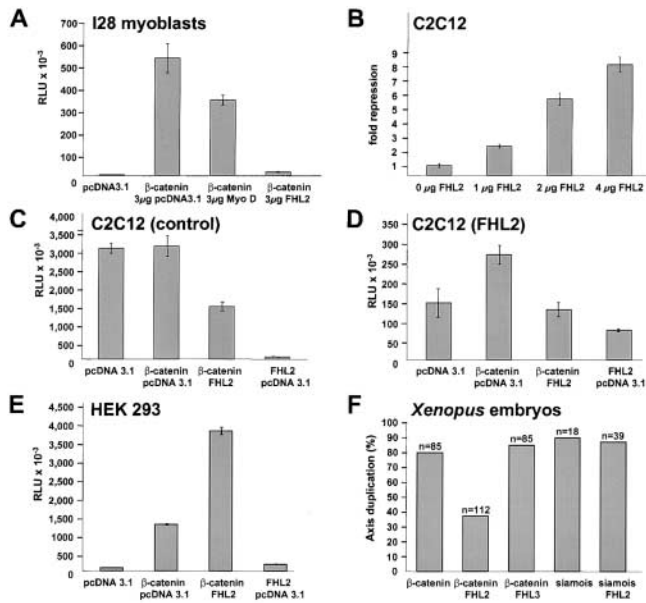


Figure 6. FHL2 influences β -catenin-mediated activation of TCF/LEF-dependent transcription. (A) TOPflash luciferase reporter plasmid was cotransfected with β -catenin S33A and the indicated amounts of FHL2 or MyoD (as a control) cDNAs into I28 mouse myoblast cells. Luciferase activity is given in relative light units (RLU). Basal activity of the TOPflash promoter was measured in the presence of empty expression vector to maintain equivalent amounts of transfected DNA. Results shown are the average of three independent transfections. (B) C2C12 cells were cotransfected with constant amounts of TOPflash plasmid and β -catenin S33A, and with increasing amounts of FHL2 plasmid as indicated. Values indicate fold repression of the β -catenin-induced reporter gene activity. The induction of reporter gene activity by β -catenin alone was arbitrarily taken as unity. (C–E) C2C12 cells stably infected with pBabe vector alone (C), or pBabe myc-FHL2 vector (D), or HEK 293 cells (E) were transfected with TOPflash luciferase reporter plasmid and the indicated expression plasmids. (F) *Xenopus* embryos were injected with different RNAs as indicated at the four-cell stage. Bars represent the percentage of embryos with duplicated axis at stage 19–23. *n*, No. of embryos examined.

high level of endogenous β -catenin, but recombinant FHL2 introduced by transfection strongly repressed the basal activity of the luciferase reporter (Fig. 6 C, 47-fold). However, after coexpression of recombinant β -catenin and FHL2, only a weak repression was observed (Fig. 6 C, twofold). Compared to the control cells, the FHL2-expressing cell line showed a clear reduction of the basal transcription level (Fig. 6 D, 21-fold compared to the control cells [note the different scales of y axes]), and only a marginal effect could be achieved by additional expression of recombinant β -catenin alone. Importantly, this effect was abolished when FHL2 was transfected into these cells in addition to β -catenin (Fig. 6 D).

Together, these data suggest that the observed FHL2-mediated inhibition of β -catenin-induced transcriptional activity in myoblasts was specifically dependent on expression of FHL2. Because FHL2 is known to act as a transcriptional coactivator in various cellular contexts including HEK 293 embryonal kidney cells, we also analyzed the effects of FHL2 on β -catenin-dependent transcription in this cell line (Fig. 6 E). Expression of β -catenin stimulated reporter gene activity, and coexpression of FHL2 proteins synergistically en-

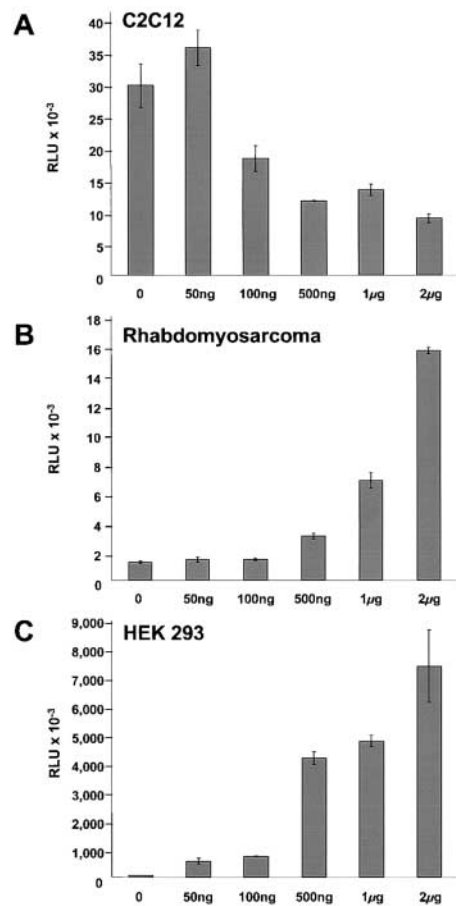


Figure 7. FHL2 exhibits an autonomous repression function restricted to fusion-competent myoblasts. Increasing amounts of GAL-FHL2 plasmid and 1 μ g 5xUAS *tk-luc* reporter plasmid were cotransfected as indicated into C2C12 (A), Rhabdomyosarcoma (B), or HEK 293 cells (C).

hanced the transcriptional activity of β -catenin. Expression of FHL2 alone had only a marginal effect on reporter gene transcription. These data indicate a cell type-dependent regulation of β -catenin function by FHL2, i.e., inhibition in myoblasts (Fig. 6, A–D) and stimulation in HEK 293 cells (Fig. 6 E). In an additional assay using *Xenopus* embryos, ventral overexpression of FHL2 (but not FHL3) was able to block β -catenin-induced axis duplication (Fig. 6 F). This effect was specific with respect to β -catenin, as FHL2 was unable to interfere with axis duplication triggered by siamois, which is a direct downstream target of β -catenin/LEF signaling. Although FHL2 has been described as a transcriptional coactivator (Fimia et al., 2000; Muller et al., 2000), we believe this is the first study implicating FHL2 in transcriptional repression.

FHL2 contains an autonomous transcriptional repression domain

We then investigated whether FHL2 possesses autonomous repression activity regardless of β -catenin interaction. Because no DNA binding site for FHL2 is known, the full-length FHL2 was fused to the GAL4-DNA binding domain and the GAL-FHL2 chimera was tested in transient transfection

tion assays using a luciferase reporter under the control of a multimerized binding site for *GAL4* (*5xUAS tk-luc*). Although GAL-VP16 strongly activates the transcription of the reporter gene (unpublished data), GAL-FHL2 showed a dose-dependent repression of transcriptional activity in C2C12 myoblasts (Fig. 7 A). To study whether the observed FHL2-mediated repression of β -catenin function is restricted only to fusion-competent I28 and C2C12 myogenic cell lines (Fig. 6, A and B), we next examined the transcriptional capacity of GAL-FHL2 in a fusion-incompetent myogenic rhabdomyosarcoma cell line and in HEK 293 human kidney cells. Indeed, the GAL-FHL2 construct, which repressed transcription in the muscle precursor cell line (Fig. 7 A), activated transcription in rhabdomyosarcoma cells as well as in HEK 293 cells (Fig. 7, B and C). These data indicate that FHL2 exhibits an autonomous repression function restricted to fusion-competent myogenic lineages, and additionally confirm the already described autonomous activation function in other cell types (Muller et al., 2000).

FHL2 does not compete with LEF-1 for binding to β -catenin

It is well established that β -catenin mediates induction of target gene transcription by heterodimerizing with members of the TCF/LEF superfamily (for review see Akiyama, 2000). Because TCF/LEF proteins interact with the armadillo repeat region of β -catenin, we expected from our deletion mutant analyses (Fig. 3) that the observed FHL2-mediated inhibition of transcription is not based on interference with LEF/TCF factors. Consistent with this prediction, we could show the formation of an FHL2- β -catenin-LEF-1 ternary protein complex in a GST pull-down assay with bacterially expressed GST-FHL2 and *in vitro*-translated myc- β -catenin and HA-LEF-1 (Fig. 8, lane 8). Furthermore, FHL2 bound β -catenin (as expected), but not LEF-1 when incubated with either of these proteins separately (Fig. 8, lanes 4 and 6), and none of the *in vitro*-translated proteins showed an interaction with GST alone (Fig. 8, lanes 3, 5, and 7). Moreover, in coimmunoprecipitation experiments from transfected HEK 293 cells, LEF-1 did not prevent interaction of FHL2 with β -catenin (unpublished data). Thus,

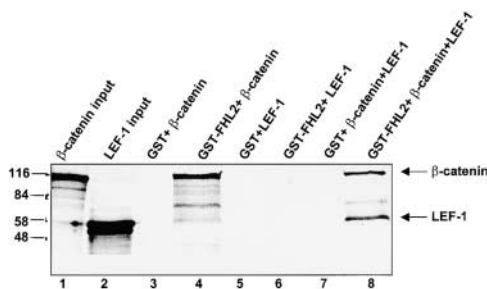


Figure 8. β -Catenin forms a ternary protein complex with FHL2 and LEF-1. Recombinant GST-FHL2 (lanes 4, 6, and 8) or GST (lanes 3, 5, and 7) proteins were incubated with [35 S]methionine-labeled β -catenin (lanes 3 and 4) or [35 S]methionine-labeled LEF-1 (lanes 5 and 6), or with both (lanes 7 and 8). After extensive washing, proteins retained by GST or GST-FHL2 were analyzed together with 10% of the input material (lanes 1 and 2) by SDS-PAGE and fluorography.

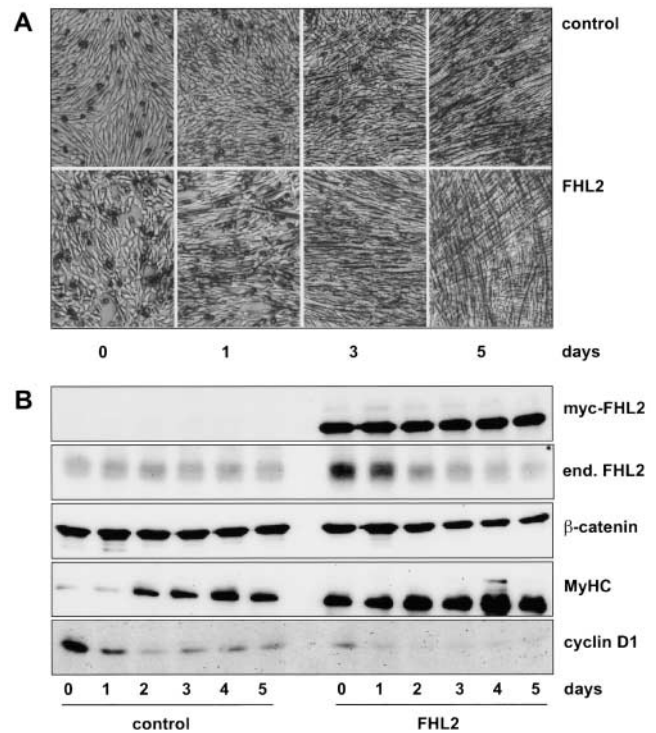


Figure 9. Ectopic expression of FHL2 promotes differentiation of C2C12 myoblasts. (A) C2C12 myoblasts infected with retroviruses containing the pBabe vector alone (control) or pBabe with myc-FHL2 (FHL2) were cultured in growth medium until 90–95% confluency (day 0) and then shifted to differentiation medium (days 1–5). (B) Lysates (20 μ g protein/lane) from cells cultured in growth medium (day 0) or in differentiation medium (days 1–5) were immunoblotted with anti-myc mAb 9E10, anti-FHL2 mAb, anti- β -catenin mAb 14, anti-MyHC mAb NOQ7.5.4D, or anti-cyclin D1 mAb DCS-6.

our data demonstrated that FHL2 does not compete with LEF-1 for binding to β -catenin, and reveals the existence of a ternary complex of these three components *in vitro*.

FHL2 overexpression increases differentiation of myoblasts into myotubes

To show the functional relevance of the FHL2- β -catenin interaction, we stably expressed myc-tagged recombinant FHL2 in C2C12 mouse myoblasts. Cells infected with the myc-vector only and selected for G418 resistance were used as a control. When cultivated in medium with low levels of growth factors, control C2C12 cells underwent myogenesis *in vitro* and differentiated into well-developed myotubes within 5 days (Fig. 9 A, top). The FHL2-overexpressing cells tended to fuse even in growth medium as soon as cell monolayers became confluent, but subsequently died when cultivation in growth medium was continued. However, if transferred to differentiation medium, these cells start to fuse and form myotubes at day 1 after medium change, and fusion began at lower cell densities than it was usually the case with control cells (Fig. 9 A, bottom). The efficiency of myotube formation as well as the longevity of myotubes and the regularity of their shape was also increased. Already, by day 3 after the start of differentiation, we observed many well-developed myotubes organized into parallel-running filamentous bundles. In control cell monolayers, such well-organized

bundles were never seen even by day 5, rather the myotubes formed showed a more amorphous and irregular shape than the FHL2-overexpressing cells.

The expression pattern of the recombinant FHL2 protein as well as that of endogenous β -catenin did not change during the time of cultivation in differentiation medium (Fig. 9 B), which suggests that the observed accelerated myotube formation was due to stable expression of FHL2. Interestingly, ectopic expression of FHL2 molecules leads to increased expression of the endogenous FHL2 protein, which however, decreased during differentiation. The increase of endogenous FHL2 was noticed in all FHL2-overexpressing C2C12 cell lines we generated (unpublished data).

Differentiation processes are usually associated with inhibition of cell proliferation and transcriptional activation of particular genes that are characteristic for the differentiated type of cells or tissue. One of the most prominent markers for differentiated muscle cells is the muscle-specific myosin protein. Therefore, we examined whether the ectopic FHL2 protein influences muscle-specific gene expression. Results presented in Fig. 9 B show that this is indeed the case. Control cells express low levels of myosin heavy chain (MyHC) when kept in growth medium, and induction of the muscle-specific protein is first detectable 48 h after switching the cells to differentiation medium. In sharp contrast, the FHL2 cells express high levels of MyHC even in growth medium, and this high level of the protein is maintained or slightly increased during differentiation.

One of the proteins whose expression is regulated by the β -catenin–TCF complex transcriptional complex is the cell cycle regulation protein, cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999). Western blot analysis showed that after switching the cells into differentiation medium, cyclin D1 expression was rapidly down-regulated in both control and FHL2-overexpressing cells (Fig. 9 B). Interestingly, the expression of cyclin D1 protein was slightly decreased even in logarithmically growing FHL2-C2C12 cells compared to that of mock-infected cells. This observation was in agreement with the slight reduction in proliferative rate of FHL2-C2C12 cells when compared to control cells (unpublished data). Thus, the accelerated myogenesis of FHL2-overexpressing C2C12 myoblasts correlated well with the enhanced down-regulation of cyclin D1. Although the down-regulation of cyclin D1 by FHL2 was reproducible, the extent varied between different cell lines.

Discussion

In this paper, we show that the four and a half LIM-domain protein FHL2 is a novel β -catenin binding protein. Initially identified in a yeast two-hybrid screen, the interaction between FHL2 and β -catenin was confirmed in both direct in vitro pull-down assays as well as in coimmunoprecipitation experiments. Using deletion mutants, we showed that the NH₂ terminus together with the first armadillo repeat of β -catenin is sufficient for its association with FHL2, whereas the four LIM domains of the FHL2 protein were required for binding to β -catenin. Furthermore, β -catenin is able to bind simultaneously to FHL2 and the transcription factor LEF-1, forming a ternary protein complex. When expressed

in myoblasts either alone or together with β -catenin, FHL2 represses LEF/TCF-induced transcriptional activity and promotes myoblast differentiation, triggering myotube formation and expression of muscle-specific genes.

The involvement of FHL proteins in muscle cell function was suggested from observations that FHL1, FHL2, and FHL3 proteins are highly expressed in skeletal and cardiac muscles, and are differentially regulated during myogenesis (Morgan and Madgwick, 1999). Furthermore, FHL2 is down-regulated in rhabdomyosarcoma cells, localized in Z-disks of mature muscles, and like FHL3, is found in focal adhesion complexes of growing myoblasts (Genini et al., 1997; Scholl et al., 2000; this paper). However, despite their structural similarity and the fact that all three FHL proteins are involved in transcriptional regulation (Muller et al., 2002), only FHL2 showed an interaction with β -catenin in our experiments. Thus, although being involved in muscle function or differentiation, the FHL proteins apparently exercise their particular functions in different ways.

The specificity of the FHL2 and β -catenin interaction was confirmed in coimmunoprecipitation assays with GST-tagged FHL proteins as well as after injection of FHL and β -catenin mRNAs into *Xenopus* embryos, where only FHL2 significantly inhibited the β -catenin-mediated duplication of axis formation. Interestingly, FHL2 repressed β -catenin-dependent transcription only in myoblasts that had not lost the capacity to differentiate, whereas in tumor cells, including muscle-derived rhabdomyosarcoma, we usually observed the opposite effect, i.e., activation of β -catenin-mediated transcription. In line with this, transcriptional activation on expression of FHL2 has been reported in a variety of tumor cells by other authors (Fimia et al., 2000; Muller et al., 2002). The fact that FHL2 was also capable of autonomously repressing basal promoter activity in myoblasts when fused to the GAL4-DBD indeed suggests a cell type-specific effect of FHL2 with regard to transcriptional regulation, and also indicates the presence of cell type-specific coregulators for the transcriptional activity of β -catenin.

None of the so far identified interaction partners of FHL2, such as integrin chains (Wixler et al., 2000), the androgen receptor (Muller et al., 2000), presenilin2 (Tanahashi and Tabira, 2000), or CDC47 (Chan et al., 2000), seem to be directly involved in myoblast differentiation. Our observation that FHL2 interacts with β -catenin and modulates the β -catenin-mediated transcriptional activity sheds new light on the role of FHL2 in muscle cells. β -Catenin was originally identified as a component of the cadherin-based cell–cell adhesion complex, but is also a transcriptional effector of the Wg/Wnt pathway. In the presence of Wg/Wnts, β -catenin is activated and carried to the nucleus where it activates target genes by interacting with members of the TCF/LEF family of transcription factors. We could show that the interaction of FHL2 with β -catenin represses the β -catenin-mediated activation of target gene transcription in proliferating myoblasts, promoting myogenic differentiation. Consistent with our observations, β -catenin has been implicated in the control of skeletal myogenesis (Goichberg et al., 2001; Petropoulos and Skerjanc, 2002), but its effect on myogenic differentiation appears complex. Although translocation of β -catenin from the nucleus to adherens

junctions is important for myogenic differentiation, and overexpression of β -catenin reduces myogenin expression in myoblasts (Goichberg et al., 2001), expression of activated β -catenin in pluripotent P19 embryonic carcinoma cells is sufficient to induce the transcription of muscle-specific genes (Petropoulos and Skerjanc, 2002). Our work showed that FHL2 interferes with transcriptional activation mediated by β -catenin, indicating a delicate balance of β -catenin activity for proper muscle cell differentiation.

Our interaction studies in yeast identified the NH₂ terminus of β -catenin as being sufficient for an interaction with FHL2, whereas in mammalian cells the first armadillo repeat in addition to the NH₂ terminus was necessary for binding. In contrast, the full FHL2 molecule, except the first half LIM domain, was required for strong binding to β -catenin, suggesting that although LIM domains can fold independently, the suprastructure formed by the four LIM domains of FHL2 is required for binding β -catenin. Interestingly, FHL2 deletion mutants showed the same interaction pattern for γ -catenin, but its specific binding site for FHL2 has yet to be determined.

As FHL2 is an adaptor protein without known enzymatic activity, it might regulate β -catenin function either by changing its conformation or by competing for binding with other interacting proteins. It is well established that β -catenin is positively and negatively regulated by numerous proteins. Because transcriptionally active β -catenin heterodimerizes with members of the TCF/LEF family of transcription factors, negative regulators often compete with TCF/LEF proteins for binding to β -catenin, thereby preventing the formation of the transcriptionally active TCF/LEF- β -catenin complex (Tago et al., 2000). However, our results imply that FHL2, when bound to β -catenin, does not prevent association of the latter with LEF/TCFs. Indeed, formation of a ternary protein complex was confirmed in direct binding assays with recombinant FHL2 and in vitro-translated β -catenin and LEF-1. The same is true for mammalian cells, where FHL2 does not interfere with the β -catenin-LEF-1 interaction (unpublished data).

Several β -catenin-interacting proteins that bind to the NH₂ terminus or to the NH₂ terminus plus the first armadillo repeat have been described recently (for review see Zhurinsky et al., 2000). Among them are α -catenin, β -TrCP, IQGAP, and the regulators of transcription (NEMO-like kinase, CBP/p300 histone acetyltransferase, TATA binding protein, and Pontin52). An intriguing possibility is that FHL2 interferes with the binding of NEMO-like kinase, pontin52, or CBP/p300, which negatively or positively regulate the transcriptional activation of target genes by the β -catenin-TCF complex (for review see Zhurinsky et al., 2000). Indeed, depending on the cell type, our experiments showed either an inhibition or activation of LEF/TCF-driven transcription. It is of interest that FHL2 has been characterized as a coactivator for the transcription factor CREB/CREM, which in turn is regulated by CBP/p300 (Fimia et al., 2000). It is also an intriguing possibility that FHL2 recruits transcriptional repressors or activators to the β -catenin-TCF complex. Our experiments with GAL4-FHL2 fusion protein indicated that FHL2 functions autonomously as a repressor or activator of transcription, depending on the cellular context.

An important step in myoblast differentiation is the down-regulation of *cyclin D1* gene expression (Skapek et al., 1995), which represents one of the transcriptional target genes of the β -catenin/TCF protein complex. It is interesting that a clear shift towards enhanced myotube formation and enhanced expression of MyHC was noticeable on FHL2 overexpression, and that this was also accompanied by a down-regulation of cyclin D1 protein. A lower expression of cyclin D1 was already noticed in proliferating FHL2-overexpressing cells. Whether these effects are a result of FHL2- β -catenin interactions remains to be elucidated. Nevertheless, the observed down-regulation of cyclin D1 and β -catenin target gene transcription by FHL2 and the promotion of myogenesis might fit with the proposed role of cyclin D1-responsive cdk's as negative regulators of myogenic differentiation via inhibition of MyoD function (Wei and Paterson, 2001). Conceivably, FHL2 might modulate the down-regulation of cyclin D1 by repressing its transcriptional activator, β -catenin, and thus indirectly facilitate myogenic differentiation.

Together, our results show a new mode of β -catenin regulation and could explain the putative tumor suppressor potential of FHL2/DRAL (Genini et al., 1997). These findings now raise additional important questions about the underlying mechanisms. Possible factors that might support the opposing effects of FHL2/ β -catenin-mediated transcription activity have to be identified as well as the relationship of this interaction to the Wnt signaling pathway.

Materials and methods

DNA constructs

For the yeast two-hybrid screen, a cDNA fragment coding for aa 35–279 of murine FHL2 was inserted into the DNA-binding domain vector pBD-GAL4 Cam (Stratagene). cDNA fragments encoding the complete sequence of FHL1, FHL2, FHL3, or their deletion mutants were cloned in-frame into pACT2 or pAS2-1 vectors (CLONTECH Laboratories, Inc.) as described previously (Wixler et al., 2000). Deletion mutants of β -catenin representing either the NH₂ terminus only or the NH₂ terminus plus one, two, or three armadillo repeats were generated by PCR and cloned in-frame into the pACT2 vector. The pGAD424-GAL4-AD fusion constructs of α -catenin, β -catenin, and plakoglobin, as well as the other deletion mutants of β -catenin, are described elsewhere (Hulsken et al., 1994; Behrens et al., 1996).

For expression in mammalian cells, the cDNA fragment coding for aa 3–279 of murine FHL2 was cloned into the pcDNA3.1/His A vector (Invitrogen) or into the pEGFP-C3 vector (CLONTECH Laboratories, Inc.). The GAL-FHL2 construct was obtained by cloning the FHL2 insert into the pABgal 94 linker (provided by Aria Baniahmad, University of Marburg, Marburg, Germany; Baniahmad et al., 1995). The GST-fusion constructs of FHL1, FHL2, or FHL3 were obtained by cloning into the pEBG vector (Wixler et al., 2000). The FHL1 cDNA was provided by Po-Hsien Chu (University of California, San Diego, La Jolla, CA). The wt or myc-tagged β -catenin S33A mutants (the s33A β -catenin mutant was obtained from Jörg Stappert, Max-Planck Institute for Immunobiology, Freiburg, Germany; Aberle et al., 1997) were cloned into the pcDNA3.1 plasmid (Invitrogen). The myc-tagged β -catenin deletion mutants were generated by cloning the appropriate inserts from pACT2 vector into the pCS2 + MT expression vector. The MyoD expression plasmid as well as the HA-tagged LEF-1 constructs were described elsewhere (Lassar et al., 1986; Behrens et al., 1996). For generation of FHL2 retrovirus stocks, the myc-tagged FHL2 insert was cloned into the pBabe-neo vector. After transfection of GPE + 86 virus-producer cells with pBabe vector alone or pBabe-myc-FHL2, the cells were selected for G418 resistance, and supernatants from confluent monolayers were used as retroviral stocks. All constructs were verified by sequencing before use.

Yeast two-hybrid screen

A mouse cDNA library constructed from I28 skeletal muscle cells after 30-h induction for myogenic differentiation was cloned into the target plasmid

pAD-Gal4 (Stratagene). FHL2 (aa 35–279) in pBD-GAL4 was used as bait and was sequentially transformed into the PJ69-4A yeast strain along with the cDNA library. The yeast two-hybrid screen was performed according to the protocol of Stratagene.

Yeast two-hybrid interaction test

The yeast strain Y190 was cotransformed with the pAS2-1 plasmid containing the GAL4-BD fused with appropriate cDNAs as bait and with pACT2 or pGAD424 plasmids containing cDNAs fused to GAL4-AD as prey. Transformants were grown on SD medium lacking the amino acids leucine, tryptophan, and histidine in the presence of 25 mM 3-amino-1,2,4-triazole. On day 5, the colonies were tested for *LacZ* reporter gene activity in a β -Gal filter assay. The interaction was scored as negative (–) when no blue colonies were visible after 8 h, and scored as weak (+), intermediate (++) , or strong (+++) when blue colonies became visible after 8, 4, or 1 h, respectively.

In vitro pull-down binding assays

LEF-1 and β -catenin were synthesized by in vitro transcription–translation in the presence of [³⁵S]methionine using the TNT™-coupled reticulocyte lysate (Promega). The GST–FHL2 fusion protein or GST alone was expressed in *Escherichia coli* BL21pLysS. Extracts were incubated with glutathione-coated Sepharose affinity beads (Amersham Biosciences) for 20 min at RT. After blocking with 10% milk powder in NENT (100 mM NaCl, 20 mM Tris-HCl, pH 8.8, 1 mM EDTA, and 0.5% NP40), the GST beads were washed two times with NENT and equilibrated with TWB (20 mM Hepes, pH 7.9, 60 mM NaCl, 6 mM MgCl₂, 8.2% glycerol, 0.1 mM EDTA, and 1 mM DTT). The beads were then incubated with [³⁵S]methionine-labeled translation products in TWB at RT for 1 h, followed by extensive washing with NENT to eliminate unspecific binding. Proteins adhering to the beads were analyzed by SDS-PAGE followed by autoradiography or Coomassie staining.

Xenopus injections

RNAs for microinjection into *Xenopus* embryos were prepared using the mMESSAGE mMACHINE™ kit (Ambion). Embryos injected with RNA into both ventral blastomers at the four-cell stage were examined for axis duplication at stages 19–23. Embryos were injected with RNA in the following amounts: β -catenin, 50 pg; FHL2, 200–400 pg; FHL3, 200–400 pg; and siamois, 10 pg.

Cell lines and antibodies

Primary I28 mouse myoblasts were grown in Ham's F10 nutrient mixture supplemented with 20% FCS in 5% CO₂ atmosphere at 37°C (Kaufmann et al., 1999). All other cell lines used in this work were grown in DME supplemented with 10% FCS. To generate a cell line stably expressing the recombinant myc-FHL2 protein, C2C12 cells were infected with retroviruses containing either the pBabe-neo vector alone or the pBabe-neo vector with introduced myc-FHL2. 48 h after infection, the cells were trypsinized and selected further in medium containing 1,000 μ g/ml G418. The expression of myc-FHL2 was analyzed by Western blot procedure using the anti-myc mAb 9E10.

The mouse mAbs used in this work were purchased from BD Biosciences (β -catenin, clone 14; cyclin D1, clone DCS-6), Oncogene Research Products (myc, clone 9E10), or from Sigma-Aldrich (MyHC, clone NOG7.5.4D; GST, clone GST-2). FHL2-specific pAb was raised in rabbit against the full-length protein (aa 3–279). The anti-FHL2 mAb was a gift from Roland Schule (University of Freiburg, Freiburg, Germany). The HPR-labeled secondary antibodies for Western blot analysis were purchased from Jackson ImmunoResearch Laboratories. Alexa Fluor® 594-labeled secondary antibodies were purchased from Molecular Probes, Inc.

Reporter gene assays

I28 cells or C2C12 cells (6×10^5 /60-mm dish) were transfected with various combinations of plasmids using the PolyFect® Transfection reagent (QIAGEN). Generally, 0.5 μ g of reporter gene plasmid DNA (TOPflash, FOPflash; Upstate Biotechnology) (*5xUAS tk-luc*; Baniahmad et al., 1995) was used. The DNA amount of other plasmids varied depending on the experimental protocol, as indicated in the figure legends, but total plasmid DNA per dish was kept constant by adding the appropriate amount of empty expression vector. Each transfection was carried out in triplicate. Luciferase activities were measured 24 h after transfection using the Luciferase Assay System (Promega).

Western blotting and immunoprecipitation

Cells were washed twice with PBS and lysed in RIPA buffer supplemented with 1 mM sodium vanadate, 1 mM PMSF, 5 μ g/ml leupeptin, and 5 μ g/ml

aprotinin at RT for 10 min. The lysates were cleared by centrifugation at 10,000 *g* for 10 min at 4°C. Supernatants were resolved by 10% SDS-PAGE, and after electroblotting onto nitrocellulose membrane, detection of proteins was performed with appropriate antibodies using the ECL detection system (Amersham Biosciences). Coimmunoprecipitations were performed as described previously for M-cadherin and for GST- or myc-tagged FHL2 proteins (Kaufmann et al., 2000; Wixler et al., 2000). Proteins were visualized by Western blots using either the NBT/BCIP (Roche) or ECL detection systems.

Immunofluorescence

Cells grown on coverslips were rinsed in PBS and fixed with 4% PFA in PBS at RT for 10 min. After fixation, cells were permeabilized by incubation with 0.2% Triton X-100 in PBS for 10 min, washed with PBS, and incubated with the relevant antibodies diluted in PBS/10% FCS for 1 h. Primary antibody binding was detected by fluorochrome-conjugated secondary antibodies. Staining in the absence of primary antibodies confirmed the specificity of the immunolabeling. Fluorescence was monitored with a microscope (AxioPhot; Carl Zeiss MicroImaging, Inc.). Pictures were taken with a digital camera (MagnaFire®; Optronics) and Image-Pro® software (Media Cybernetics, Inc.).

This paper is dedicated to the memory of our colleague Franco Tato.

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