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FOXKs promote Wnt/β-catenin signaling by translocating DVL into the nucleus

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SUMMARY

Dishevelled (DVL) proteins serve as crucial regulators that transduce canonical Wnt signals to the GSK3β-destruction complex, resulting in the stabilization of β-catenin. Emerging evidences underscore the nuclear functions of DVLs, which are critical for the Wnt/β-catenin signaling. However, the mechanism underlying DVL nuclear localization remains poorly understood. Here, we discovered two Forkhead box (FOX) transcription factors, FOXK1 and FOXK2, as bona-fide DVL-interacting proteins. FOXK1 and FOXK2 positively regulate Wnt/β-catenin signaling by translocating DVL into the nucleus. Moreover, FOXK1 and FOXK2 protein levels are elevated in human colorectal cancers and correlate with DVL nuclear localization. The conditional expression of Foxk2 in mice induced intestinal hyper-proliferation that featured with enhanced DVL-nuclear localization and up-regulated Wnt/β-catenin signaling. Together, our results not only reveal a mechanism by which DVL is translocated into nucleus, but also suggest unexpected roles of FOXK1 and FOXK2 in regulating Wnt/β-catenin signaling.

INTRODUCTION

The Wnt/β-catenin pathway is an essential signaling pathway that directs cell proliferation, self-renewal, differentiation, tissue homeostasis and embryonic development (Clevers, 2006; Clevers and Nusse, 2012; Huang and He, 2008; Nusse, 2008). Deregulation of the Wnt/β-

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AUTHOR CONTRIBUTIONS

W.W. and J.C. conceived the experiments. W.W. and X.L. performed all the experiments with assistance from A.Z., F.L., M.K.T. and N.L.. M.L. and P.M. performed the axis duplication assay. S.J. and J.P. provided the reagents and significant suggestions on the experiments. J.C. supervised the study. W.W and J.C. wrote the manuscript. All authors commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

catenin pathway contributes to human degenerative disease and tumorigenesis in various tissues (Clevers, 2006; Clevers and Nusse, 2012; Moon et al., 2004; Nusse, 2005). Wnt ligands initiate the signaling pathway by binding to Frizzled and LRP receptors present at the cell-surface and thereby stimulate a series of biological events inside the cell. The core component of the canonical Wnt pathway is the transcriptional co-activator β-catenin, which is regulated at the protein level by proteasome-dependent degradation via the β-TRCP E3 ubiquitin ligase complex (Aberle et al., 1997). The degradation of β-catenin is governed by a cytoplasmic destruction complex, which includes components such as the tumor suppressor proteins, AXIN and APC, and members of two groups of kinases, CK1 and GSK3. Activation of Wnt signaling represses the destruction complex and thereby stabilizes β-catenin, which translocates into the nucleus to assemble a transcriptionally active complex with TCF or LEF to promote the transcription of many Wnt downstream target genes (e.g. *Axin2*, *c-Myc*, *cyclin D1*) (Clevers, 2006; Moon et al., 2004).

Dishevelled (DVL) is a critical scaffold protein that acts between Frizzled receptors and destruction complex. Although the detailed mechanisms remain to be elucidated, activation of the Wnt pathway leads to the phosphorylation of DVL, which then suppresses the downstream destruction complex to stabilize β-catenin (Clevers, 2006; Nusse, 2005), Intriguingly, emerging evidences suggest that DVL also plays a pivotal role in the nucleus by mediating formation of the β-catenin/TCF transcriptional complex in conjunction with c-Jun, which is required for Wnt/β-catenin signaling (Barry et al., 2013; Gan et al., 2008; Habas and Dawid, 2005; Itoh et al., 2005; Torres and Nelson, 2000). In addition, Wnt stimulation promotes DVL nuclear translocation (Gan et al., 2008; Itoh et al., 2005), which appears to act in parallel to its cytoplasmic functions for activation of the Wnt signaling pathway. Moreover, *in vivo* evidence revealed that DVL localizes to the nucleus of intestinal crypt base column (CBCs) and this DVL nuclear localization is enhanced by Wnt signaling during intestine regeneration (Barry et al., 2013). Intriguingly, the Hippo pathway components YAP and TAZ negatively control DVL nuclear functions by sequestering it in cytoplasm through direct protein-protein interactions, and also inhibit CK1 kinase-mediated DVL phosphorylation (Barry et al., 2013; Varelas et al., 2010). These findings indicate that nuclear DVL is critical for activation of the Wnt/β-catenin signaling pathway, while the regulatory mechanism underlying DVL nuclear translocation remains largely unknown.

In this study, we identified two Forkhead box (FOX) transcription factors FOXK1 and FOXK2 as DVL-associated proteins. We demonstrate that FOXK proteins activate Wnt/β-catenin signaling by promoting DVL nuclear translocation, which requires the Wnt signaling-induced DVL phosphorylation. Moreover, we show that FOXK protein expression is significantly increased in human colon cancers and correlates with DVL nuclear localization. Finally, conditional expression of Foxk2 using a transgenic mouse model induced intestinal hyper-proliferation, nuclear translocation of DVL and up-regulation of Wnt/β-catenin signaling in intestine crypts. Together, our results demonstrate a potential oncogenic function of FOXK proteins via their abilities to translocate DVL into the nucleus and activate the Wnt/β-catenin signaling pathway.

RESULTS

FOXK1 and FOXK2 are DVL-binding proteins

In order to identify DVL-associated proteins that may facilitate DVL nuclear translocation, we performed tandem affinity purification using HEK293T cells stably expressing SFB (S protein tag, Flag tag and streptavidin-binding tag) tagged DVL2 or DVL3, as previously described (Wang et al., 2014). Many known DVL-binding proteins were identified by mass spectrometry analysis, which include canonical Wnt pathway components (i.e. AXIN1, AXIN2, GSK3B, and β-TRCP) (Clevers, 2006), non-canonical Wnt pathway components (i.e. VANGL1 and VANGL2) (Torban et al., 2004), an E3 ligase complex component (KLHL12) (Angers et al., 2006), and other reported DVL-binding partners (e.g. WWOX, CK1) (Bouteille et al., 2009; Wallingford and Habas, 2005) (Figure 1A). Interestingly, we also identified two FOX transcription factors, FOXK1 and FOXK2, in our biologically triplicated mass spectrometry analyses (Figure 1A), suggesting that FOXK1 and FOXK2 are potential DVL-binding proteins. We performed reverse purification using FOXK1 or FOXK2 as bait and uncovered all members of the DVL protein family, DVL1, DVL2 and DVL3, as FOXK1- or FOXK2-associated proteins (Figure 1A). The detailed peptide information is listed as a table in Figure 1A. The interactions between DVL and FOXK proteins were further confirmed by co-immunoprecipitation experiments (Figures 1B and 1C). Moreover, the FOXK-DVL interaction is highly specific within the Wnt signaling pathway (Figure 1D) and among the FOX transcription factor family (Figure 1E), which demonstrated the specificity of this interaction. Collectively, these data indicate that FOXK1 and FOXK2 are bona-fide DVL-binding partners.

FOXK1 and FOXK2 promote DVL nuclear translocation

Since FOXK1 and FOXK2 are nuclear proteins, we wondered whether FOXK1 and FOXK2 could regulate DVL subcellular localization. Indeed, overexpression of either FOXK1 or FOXK2 enhanced the nuclear localization of endogenous DVL2, while DVL2 mostly localized to the cytoplasm of un-transfected cells (Figure 1F; Figures S1A–S1C). Consistent with the specific interaction between FOXKs and DVLs (Figure 1E), only FOXK proteins were capable of translocating DVL into the nucleus (Figure 1G). These results indicate that FOXK1 and FOXK2 may regulate DVL nuclear localization.

Mapping of the binding regions required for the FOXK-DVL interaction

To further elucidate the FOXK-binding region on DVL, a series of DVL2 truncation mutants were generated (Figure 1H), where a region comprised of residues 250~355 of DVL2 is required for its interaction with FOXK2 (Figure 1I). This region includes the PDZ domain and a four-amino acid motif, IVLT, which was previously reported required for DVL nuclear import (Itoh et al., 2005) (Figure 1H). Deletion of either the PDZ domain or the IVLT motif disrupted the association of DVL2 with FOXK2 (Figure 1J) as well as the FOXK2-mediated DVL2 nuclear translocation (Figure 1K). Conversely, the FHA domain and its adjacent region (residues 129~171) on FOXK2 are required for its binding to DVL2 (Figures 1L and 1M). This FHA adjacent region is conserved between FOXK1 and FOXK2, where deletion of this region on either FOXK1or FOXK2 disrupted its association with DVL (Figure 1N). Moreover, a hydrophobic motif (Leu137-Phe145-Phe154) within this

region was also required for the binding of FOXKs to DVL2 (Figures 1O and 1P). In addition, the ability of FOXK to promote DVL nuclear translocation correlates with its capability to associate with DVL (Figure 1Q). These data suggested that the PDZ domain-located region (residues 250~355) of DVL and the hydrophobic motif adjacent to the FHA domain of FOXK are required for their interaction.

FOXK1 and FOXK2 positively regulate Wnt/β-catenin signaling by translocating DVL into the nucleus

Previous studies suggested that nuclear DVL enhances Wnt signaling by stabilizing the β-catenin/TCF transcription complex, which is required for Wnt/β-catenin signaling (Gan et al., 2008; Itoh et al., 2005). Since FOXK1 and FOXK2 promote DVL nuclear translocation, we wondered whether FOXK1 and FOXK2 are involved in the regulation of Wnt/β-catenin signaling. Indeed, loss of FOXK1 or FOXK2 in 293T cells suppressed LiCl-induced transcription of the Wnt target genes, i.e. *AXIN2*, while double knockdown of FOXK1 and FOXK2 further suppressed it (Figures 2A and 2B; Figure S2A). Similar results were also observed in HT29 colon cancer cells (Figures 2A and 2B; Figure S2B). Moreover, loss of FOXK suppressed the TOPFLASH luciferase activity (Figure 2C). These data indicate that FOXK1 and FOXK2 are required for Wnt/β-catenin signaling.

Additionally, overexpression of FOXK1 or FOXK2 promoted LiCl-induced TOPFLASH luciferase activity and this stimulation was inhibited by the β-catenin/TCF complex inhibitor iCRT-14 (Figure 2D), which further support the positive roles of FOXK1 and FOXK2 in Wnt/β-catenin signaling regulation. Notably, this regulation is independent of FOXK1 and FOXK2's DNA binding activity, since their DNA-binding mutants were still able to bind and translocate DVL into the nucleus (Figures S2C and S2D), and facilitate LiCl-induced TOPFLASH luciferase activity (Figure 2E). However, either mutation of the FOXK2 hydrophobic residues or deletion of the FHA domain-adjacent region abolished the ability of FOXK2 to promote LiCl-induced TOPFLASH luciferase activity (Figure 2F), suggesting association with DVL protein is required for the FOXK-mediated positive regulation of Wnt/β-catenin signaling. Importantly, knockdown DVL suppressed FOXK-induced TOPFALSH luciferase activity (Figures 2G and 2H), indicating this FOXK-mediated Wnt/β-catenin activation depends on DVL.

Since FOXK1 and FOXK2 promote the nuclear translocation of DVL (Figure 1F), we next asked whether depletion of FOXK1 and FOXK2 would inhibit the nuclear localization of DVL. Consistent with previous findings, Wnt3A treatment increased the nuclear fractionation of DVL2 (Gan et al., 2008; Itoh et al., 2005) (Figure 2I), however, we could hardly detect nuclear DVL2 in either basal or Wnt3A-treated FOXK1 and FOXK2 double knockdown cells (Figure 2I). Moreover, inhibition of the nuclear export machinery by treatment with leptomycin B (LMB) increased nuclear localization of DVL2; however, this was not the case in FOXK1 and FOXK2 double knockdown cells (Figures 2J and 2K). Furthermore, artificially translocating DVL into the nucleus by fusing DVL2 with an additional nuclear localization signal (NLS) (Figure 2L) was sufficient to promote LiCl-induced TOPFLASH luciferase activity (Figure 2M) and partially rescued the suppression of Wnt signaling in 293T and HT29 cells (Figures 2N-2Q). These data together support our

conclusion that FOXK1 and FOXK2 are required for the nuclear translocation of DVL, which then activates Wnt/β-catenin signaling in the nucleus.

To gain further insight into the role of FOXK in the Wnt/β-catenin signaling pathway, we *used Xenopus laevis* embryos for axis-duplication assays (Funayama et al., 1995). Only FOXK2, but not FOXK1, can be identified in *Xenopus laevis*. The effect *of Xenopus* FOXK2 (xFoxk2) on Wnt signaling was monitored by secondary axis formation following injection of *in vitro*-transcribed messenger RNAs (mRNAs). First, we examined the association between FOXK2 and DVL2 in *Xenopus laevis*. As shown in Figure 3A, while wild-type xFoxk2 and its DNA-binding mutant associated with *Xenopus* DVL2 (xDvl2), mutation of the FHA-domain adjacent region in xFoxk2 (residues 92~134) or one of the noted hydrophobic residues (L100A in xFoxk2) disrupted the binding of xFoxk2 with xDvl2, indicating that the DVL-FOXK interaction is conserved in *Xenopus laevis*. Second, *xβ-catenin* mRNA, which was titrated to a sub-phenotypic level as previously described (Jung et al., 2013), was co-injected with *xFoxk2* mRNA into ventrovegetal blastomeres. Co-injection of *xFoxk2* with *xβ-catenin* led to the formation of duplicated axis, but this was not observed when *β-galactosidase* control mRNA was co-injected with *xβ-catenin* (Figures 3B and 3C). Interestingly, mRNA encoding the *xFoxk2-L100A* mutant (Figure 3A) failed inducing axis duplications when co-injected with *xβ-catenin* mRNA (Figures 3C and 3D), suggesting that association with xDvl is required for this activity of xFoxk2. Notably, the fact that ventrovegetal injection of *xFoxk2* alone failed to induce axis duplication (data not shown) indicated that active β-catenin is required for the positive roles of xFoxk2 in Wnt signaling. This finding is consistent with previous results that FOXK1 and FOXK2 only promoted Wnt/β-catenin signaling in HEK293T cells treated with LiCl (Figure 2D). These data suggest that Foxk2 activates Wnt/β-catenin signaling during early embryogenesis.

In human colorectal cancers (CRC), activation of Wnt/β-catenin signaling is usually associated with elevated β-catenin levels and activity due to mutations of the APC tumor suppressor or of β-catenin itself (Clevers and Nusse, 2012). We showed that loss of FOXK1 and FOXK2 suppressed Wnt signaling in HT29 colon cancer cells (Figure 2B and 2C), suggesting a potential positive role of FOXK1 and FOXK2 in CRCs. Indeed, loss of FOXK1 and FOXK2 in two independent colon cancer cell lines (HT29 and DLD-1) inhibited cell proliferation and xenograft tumor growth (Figures 3E-3I). Further immunohistologic analysis showed that tumors driven from FOXK1 and FOXK2-deficient HT29 or DLD-1 cells exhibited decreased proliferation (Ki67 staining) and down-regulated Wnt/β-catenin signaling (e.g. CD44 staining) (Figure 3J). Reconstitution of wild-type FOXK1 or FOXK2 rescued cell proliferation and xenograft tumor growth in FOXK1 and FOXK2-deficient DLD-1 cells, but this was not the case in cells reconstituted with FOXK2-L137A mutant (Figures 3G-3I). To address the role of nuclear DVL in this process, NLS-DVL2 (Figure 2L) was expressed in FOXK1 and FOXK2-deficient DLD-1 cells (Figure 3K). In consistent with previous cell line studies (Figures 2N-2Q), this artificial NLS-DVL2 expression in FOXK1 and FOXK2-deficient DLD-1 cells was also able to promote TOPFLASH luciferase activity (Figure 3L) and xenograft tumor growth (Figures 3M and 3N), indicating that the oncogenic functions of FOXK proteins in colon cancer cells may be at least in part mediated by their abilities to promote DVL nuclear translocation. Interestingly, DVL2 or β-catenin expression

(Figure 3K) failed to rescue the TOPFLASH luciferase activity (Figure 3L) or xenograft tumor growth (Figures 3M and 3N) in FOXK1/2-deficient DLD-1 cells, suggesting that FOXK-dependent activation of Wnt signaling is mainly medicated by DVL nuclear localization.

The interaction between DVL and FOXK is regulated by DVL phosphorylation

Next, we investigate the mechanism underlying the DVL-FOXK complex formation. Previous study showed Wnt3A treatment increased the nuclear localization of DVL (Itoh et al., 2005). Consistent with this early finding, we showed that the interaction between DVL2 and FOXK1 or FOXK2 was increased following Wnt3A treatment (Figure 4A). Wnt3A treatment induced phosphorylation of DVL, which was marked by slower migration of DVL in SDS-PAGE (Figure 4A). Interestingly, we found that the phosphorylation of DVL2 is required for its binding to FOXK2, since DVL2 treatment with λ protein phosphatase disrupted its interaction with FOXK2, while the same treatment of FOXK2 did not affect FOXK2-DVL2 interaction (Figure 4B). Moreover, we found that all the hydrophobic site mutations on FOXK2 also disrupted the binding of FOXK2 to SDS3, which is a known FOXK-associated phospho-protein that binds to FOXK via the FOXK FHA domain (Shi et al., 2012) (Figure 4C). This indicates that the previously identified hydrophobic motif on FOXK may function together with its adjacent FHA domain. Together, these data suggest that Wnt3A-induced DVL phosphorylation promote the DVL-FOXK interaction.

Given that the FOXK2-binding region on DVL2 was identified (Figures 1H and 1I), we wished to further define the phosphorylation sites within this region that are required for the DVL2-FOXK2 interaction. There are 18 potential phosphorylation sites in this region (Figure 4D), where T350 is not required (data not shown). We mutated them one by one or in groups if these sites resided adjacent to each other, and found that four grouped phosphorylation sites (M1-M4) are required for the binding of DVL2 to FOXK2 (Figure 4E). Furthermore, mutating all the 11 sites in these four groups abolished the association of DVL2 with FOXK2 (Figure 4F), whereas individual mutation of each S or T site only partially decreased the binding of DVL2 to FOXK2 (Figure 4F). Of note, 10 of 11 sites identified here are evolutionarily conserved in all three DVL proteins, where two of them (T267 and T269) locate within the PDZ domain of DVLs. These data indicate that multiple phosphorylation sites on DVL are required for its binding to FOXK proteins.

As for the kinases required for the phosphorylation of DVL, we searched the candidate kinases in the prey lists obtained from our previous DVL2 and DVL3 mass spectrometry analysis. Members from GSK kinase family, CK1 kinase family and MARK kinase family were identified in both DVL2 and DVL3 protein complexes. While treatment with inhibitor for CK1 or MARK (39621) alone partially suppressed the interaction between DVL and FOXK (Figure 4G), combinational treatment with both CK1 inhibitor and 39621 further inhibited the FOXK-DVL association (Figure 4G), suggesting that both CK1 and MARK may contribute to DVL phosphorylation and its association with FOXK. Of note, the CK1 family of kinases have been shown previously to promote DVL phosphorylation and one of the members CK1ε is activated by Wnt ligand treatment through DDX3 (Cruciat et al., 2013). Overexpression of CK1ε but not its kinase dead mutant promoted the binding

between DVL and FOXK (data not shown), supporting our conclusion that CK1 kinases may contribute to DVL phosphorylation. In the MARK kinase family, MARK2/PAR1b was reported to directly phosphorylate DVL on two serine sites (DVL2: S252 and S259) (Elbert et al., 2006), which are two of the sites identified by our mutagenesis studies (Figure 4D). This suggests that the MARK kinases may also contribute to DVL phosphorylation and DVL-FOXK interaction. However, it remains unknown how CK1 and MARK kinases are coordinated following Wnt signaling to phosphorylate DVL, and to promote DVL-FOXK complex formation.

FOXK1 and FOXK2 are overexpressed in human colorectal cancers

Through the analysis of public gene expression database (www.oncomine.org), we found that *FOXK1* and *FOXK2* are overexpressed in several types of human cancers including colorectal cancer (CRC) (Figure 5A). The expression of FOXK1 is increased in 4 out of 34 analyses in CRC compared to normal colon tissue using the described thresholds (i.e. $p \leq 10^{-4}$, 2-fold change, gene rank ≤10%). FOXK2 expression is also increased in 1 out of 36 analyses in CRC compared to normal colon tissue using the same thresholds. These data, although not very strong, indicate that FOXK1 and FOXK2 expression may contribute to or facilitate CRC development. Given the critical role of Wnt/β-catenin signaling in CRC, we further examined the correlation of *FOXK1/2* expression and that of the Wnt-target gene *AXIN2* in human CRC. Multiple gene expression datasets indicated that both *FOXK1/2* and *AXIN2* are up-regulated in human colon cancers (Figure 5B). The expressions of FOXK1 and FOXK2 are not controlled by the Wnt/β-catenin signaling (data not shown), indicating that they are not Wnt target genes, but instead their overexpression may contribute to the activation of Wnt/β-catenin signaling in CRC. Using human colon cancer tissue arrays, we confirmed that both FOXK1 and FOXK2 were overexpressed in human colon adenocarcinoma cells compared to normal colon tissues (Figures 5C and 5D). Interestingly, DVL2 mostly localized in the cytoplasm of normal colon tissue cells, while it displayed nuclear localization in colon adenocarcinoma cells (Figures 5C and 5D). Moreover, DVL2 nuclear localization correlated to high expression of FOXK1 or FOXK2 (Figures 5C and 5D). These data further support the role of FOXK-mediated DVL nuclear translocation in Wnt signaling and its involvement in colon cancer formation.

Conditional expression of Foxk2 promotes intestinal hyper-proliferation

Since FOXK1 and FOXK2 have been shown to activate Wnt/β-catenin signaling and they are overexpressed in human colon cancers, we wondered whether mimicking the FOXK overexpression could interfere with intestinal homeostasis using a genetically engineered mouse model. To conditionally express FOXK2, we generated doxycycline (+doxy)-inducible Foxk2 transgenic mice: *iFoxk2* (*TetO-FOXK2-pA*) (Figure 5E). For intestine-specific expression of Foxk2, we generated the *Villin:Cre-Rosa26-LSL-rtTA:iFoxk2* mouse strain, which was also allowed for the doxy-inducible studies (Figure 5E). *Villin-Cre* is specifically expressed in intestinal epithelial cells (IECs) and this enabled the conditional expression of *Foxk2* in IECs through *Rosa26-LSL-rtTA*. Upon doxycycline treatment for 4 months, the mice with IEC-specific Foxk2 expression (*Villin:Cre-Rosa26-LSL-rtTA:iFoxk2*) developed enlarged intestine crypts (Figures 5F and 5G). These enlarged crypts featured increased cell proliferation as demonstrated by Ki67 staining (Figures 5F and 5H). These

phenotypes in crypts are similar to those observed in *R-Spondin* mice (Kim et al., 2005) and *APC* heterozygous mice (Su et al., 1992), indicating that the Wnt/β-catenin signaling is elevated by Foxk2 expression in crypts. Moreover, these crypt cells exhibited increased DVL2-nuclear localization and up-regulation of Wnt/β-catenin target gene *CD44* when compared to crypts in control mice (Figure 5I). In addition, Villus-crypt fractionation indicated that both Foxk1 and Foxk2 are enriched in the crypt fractions (Figure 5J). Conditional Foxk2 expression also enhanced the transcription of the Wnt/β-catenin target genes *Lgr5* and *Axin2* in intestine crypt fractions (Figure 5K). Collectively, these data indicated that Foxk2 overexpression is sufficient to enhance the Wnt/β-catenin signaling and hyper-proliferation in mouse intestine.

DISCUSSION

In this study, we identified two FOX transcription factors FOXK1 and FOXK2 as DVL-interacting proteins, pivotal players in the Wnt signaling pathway. Our results demonstrated that FOXK1 and FOXK2 positively regulate the canonical Wnt pathway by promoting the nuclear translocation of DVL. Moreover, FOXK1 and FOXK2 are potential onco-proteins in human colorectal cancers, as supported by evidence from our transgenic mouse model and analysis of CRC patient samples.

Our current working hypothesis is that FOXK1 and FOXK2 promote the Wnt signaling pathway by facilitating nuclear localization of DVL, which is independent of β-catenin stabilization (Figure S2E). Nuclear DVL has been shown required for Wnt/β-catenin signaling by assembling the β-catenin/TCF/c-Jun transcriptional complex (Gan et al., 2008). DVL nuclear translocation is up-regulated by Wnt signaling (Gan et al., 2008; Itoh et al., 2005). However, the mechanism regulating DVL nuclear translocation was not clear until our study. Here, we show that FOXK1 and FOXK2 are not only sufficient but also required for DVL nuclear translocation. The interaction between FOXK and DVL is critical for this process and this interaction depends on DVL phosphorylation. Wnt signaling enhances the association of FOXK with DVL, which is consistent with its ability to promote DVL nuclear translocation. Moreover, we also show that CK1 and MARK kinases are involved in DVL phosphorylation and the DVL-FOXK interaction, but the detailed mechanisms remain to be elucidated. Notably, the FOXK nuclear localization is critical for its ability to promote DVL nuclear translocation (Figure S2F–S2I) and Wnt signaling activation (Figures S2J).

Of note is that the Hippo signaling pathway has been reported to participate in the regulation of DVL nuclear localization, where cytosolic phospho-YAP/TAZ prevents DVL nuclear entry (Barry et al., 2013; Varelas et al., 2010). On the basis of these reports, FOXK and phospho-YAP/TAZ could serve as the positive and negative regulators, respectively, to govern DVL nuclear localization. Indeed, expression of YAP-S127D mutant (the YAP phosphorylation mimics) suppressed FOXK-induced TOPFLASH luciferase activity (data not shown). On the other hand, overexpression of wild-type FOXK2 but not its L137A mutant reversed the suppression of DLD-1 tumor growth mediated by overexpression of YAP-S127D mutant (data not shown). In these cases, DVL nuclear translocation appears to serve as a critical node for the crosstalk between the Wnt and Hippo pathways, as it is

positively regulated by the Wnt-FOXK axis and negatively controlled by the Hippo-YAP/TAZ axis.

FOXK1 was previously identified as the key regulator for myogenic stem cell proliferation and regenerative capability, which depends on its transcriptional functions (Garry et al., 2000; Hawke et al., 2003; Meeson et al., 2007; Shi and Garry, 2012). FOXK2 works with AP-1 (c-Jun) as a heterotypic transcriptional complex and regulates c-Jun downstream gene transcription (Ji et al., 2012). These studies indicated that FOXK1and FOXK2 are required for stem cell maintenance and progenitor cell proliferation through their transcriptional activities. Here, our results indicate that the interaction of FOXK with DVL may also contribute to FOXK cellular functions, especially in the context of Wnt signaling pathway. Nevertheless, the *in vivo* functions of FOXKs in Wnt pathway and during development need to be further elucidated.

In summary, we have identified FOXK1 and FOXK2 as two binding partners of DVL proteins. FOXK1 and FOXK2 activate Wnt/β-catenin signaling by translocating DVL into the nucleus. FOXK1 and FOXK2 are highly expressed in human colorectal cancers and correlated with DVL nuclear localization, indicating FOXK overexpression may contribute to intestinal tumorigenesis.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids

The information about antibody, plasmid construction, Immunofluorescent staining, tandem affinity purification, reverse transcription in this study is described in the Supplemental Experimental Procedures.

Cell Culture and Transfection

HEK293T, HeLa, HT29, DLD-1, L Wnt3A (CRL-2647) and L cells (CRL-2648) were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 (v/v). Plasmid transfection was performed with the polyethylenimine reagent.

Axis Duplication Assay

Embryo preparation, *in vitro* fertilization and microinjections were performed according to standard methods (Fang et al., 2004). mRNAs for microinjection were generated using in vitro mMessage mMachine (Ambion). *Xenopus β-catenin* (60pg) and the indicated mRNAs were co-injected into one ventral-vegetal blastomere of 4-cell embryos. The secondary axis duplication was counted at embryonic stages 18 through 35.

Xenograft Assays

All mouse experiments followed institutional guidelines, were approved by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center and performed under veterinary supervision. Four-week old female nude mice were kept in a pathogen-free

environment and subcutaneously injected with 2×10^6 cells of HT29 or DLD-1 cells. After 15 days for adaptation, tumor weights were analyzed and immunohistochemistry was performed.

Transgenic Animals

As previously described (Jung et al., 2013), a TetO minimal promoter-*mFoxk2*-BGHpA DNA fragment was injected into the pronucleus of the zygotes to generate transgenic *iFoxk2* mice by Genetically Engineered Mouse Facility (MD Anderson Cancer Center). *iFoxk2* pups from two independent founder strains were utilized for analysis. Foxk2 transgene expression was induced by doxycycline administration (2g/L, 5% sucrose in drinking water) in the successive generations crossed with C57BL/6 mice. All mice were maintained according to the institutional guidelines and Association for Assessment and Accreditation of Laboratory Animal Care International Standards.

Villus-Crypt Fractionation

For villus-crypt isolation, mouse intestine was collected, washed with $Ca^{2+}Mg2^{+}$ free PBS (Gibco) and cut into 1 cm pieces. Incised intestine was incubated with ice cold $Ca^{2+}Mg2^{+}$ free PBS containing 1mM EDTA and 1mM EGTA for 2 min. After gently shaking, supernatant was collected as a sequential fraction by passing through 70μm cell strainer. Then, collected villus-crypt fractions were pelleted and processed for RNA extraction using TRIZOL. Primer sequence information for real-time PCR analysis is: Gapdh-Forward: 5′-TGTTCCTACCCCCAATGTGT-3′; Gapdh-Reverse: 5′-TGTGAGGGAGATGCTCAGTG-3′; Foxk1-Forward: 5′-GGGAGTTCGAGTTCCTGATG-3′; Foxk1-Reverse: 5′-AGAAGTGAGGCTCCTGGAAG-3′; Foxk2-Forward: 5′-TGCAATTAGCCCAGCTACAC-3′; Foxk2-Reverse: 5′-TGGAAGCTGGTGTTGACCTA-3′; Lgr5-Forward: 5′-CCACAGCAACAACATCAGGT-3′; Lgr5-Reverse: 5′-AACAAATTGGATGGGGTTGT-3′; Axin2-Forward: 5′-AAGCCTGGCTCCAGAAGATCACAA-3′; Axin2-Reverse: 5′-TTTGAGCCTTCAGCATCCTCCTGT-3′.

Immunohistochemistry

Colon tissue array were purchased from US Biomax. Samples were deparaffinized and rehydrated. Then, antigens were retrieved by applying Unmask Solution (Vector Laboratories) in a steamer for 30 min. The sections were treated with 1% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. After 1 hour pre-incubation in 10% goat serum to prevent non-specific staining, the samples were incubated with an antibody 4°C overnight. The sections were incubated with SignalStain Boost Detection Reagent for 30 minutes at room temperature. Color was developed with the SignalStain DAB Chromogen diluted solution (all reagents were obtained from Cell Signaling Technology). Sections were counterstained with Mayer hematoxylin. The Pearson chi-square analysis (χ^2) test was used for statistical analysis of the correlation of FOXK1,

FOXK2 or nuclear DVL2 with tissue type (normal versus cancer) and the correlation between FOXK1/2 and nuclear DVL2.

Statistical analysis

We did not exclude any samples or animals from the analysis. Data were analyzed by the Student *t*-test and Pearson chi-square analysis. Standard deviation was used for error estimate. A p value <0.05 was considered as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. 1997; 16:3797–3804. [PubMed: 9233789]

Angers S, Thorpe CJ, Biechele TL, Goldenberg SJ, Zheng N, MacCoss MJ, Moon RT. The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-beta-catenin pathway by targeting Dishevelled for degradation. Nat Cell Biol. 2006; 8:348–357. [PubMed: 16547521]

Barry ER, Morikawa T, Butler BL, Shrestha K, de la Rosa R, Yan KS, Fuchs CS, Magness ST, Smits R, Ogino S, et al. Restriction of intestinal stem cell expansion and the regenerative response by YAP. Nature. 2013; 493:106–110. [PubMed: 23178811]

Bouteille N, Driouch K, Hage PE, Sin S, Formstecher E, Camonis J, Lidereau R, Lallemand F. Inhibition of the Wnt/beta-catenin pathway by the WWOX tumor suppressor protein. Oncogene. 2009; 28:2569–2580. [PubMed: 19465938]

Clevers H. Wnt/beta-catenin signaling in development and disease. Cell. 2006; 127:469–480. [PubMed: 17081971]

Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. Cell. 2012; 149:1192–1205. [PubMed: 22682243]

Cruciat CM, Dolde C, de Groot RE, Ohkawara B, Reinhard C, Korswagen HC, Niehrs C. RNA helicase DDX3 is a regulatory subunit of casein kinase 1 in Wnt-beta-catenin signaling. Science. 2013; 339:1436–1441. [PubMed: 23413191]

Elbert M, Cohen D, Musch A. PAR1b promotes cell-cell adhesion and inhibits dishevelled-mediated transformation of Madin-Darby canine kidney cells. Mol Biol Cell. 2006; 17:3345–3355. [PubMed: 16707567]

Fang X, Ji H, Kim SW, Park JI, Vaught TG, Anastasiadis PZ, Ciesiolka M, McCrea PD. Vertebrate development requires ARVCF and p120 catenins and their interplay with RhoA and Rac. J Cell Biol. 2004; 165:87–98. [PubMed: 15067024]

Funayama N, Fagotto F, McCrea P, Gumbiner BM. Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling. J Cell Biol. 1995; 128:959–968. [PubMed: 7876319]

Gan XQ, Wang JY, Xi Y, Wu ZL, Li YP, Li L. Nuclear Dvl, c-Jun, beta-catenin, and TCF form a complex leading to stabilization of beta-catenin-TCF interaction. J Cell Biol. 2008; 180:1087–1100. [PubMed: 18347071]

Garry DJ, Meeson A, Elterman J, Zhao Y, Yang P, Bassel-Duby R, Williams RS. Myogenic stem cell function is impaired in mice lacking the forkhead/winged helix protein MNF. Proc Natl Acad Sci U S A. 2000; 97:5416–5421. [PubMed: 10792059]

Habas R, Dawid IB. Dishevelled and Wnt signaling: is the nucleus the final frontier? J Biol. 2005; 4:2. [PubMed: 15720723]

Hawke TJ, Jiang N, Garry DJ. Absence of p21CIP rescues myogenic progenitor cell proliferative and regenerative capacity in Foxk1 null mice. J Biol Chem. 2003; 278:4015–4020. [PubMed: 12446708]

Huang H, He X. Wnt/beta-catenin signaling: new (and old) players and new insights. Curr Opin Cell Biol. 2008; 20:119–125. [PubMed: 18339531]

Itoh K, Brott BK, Bae GU, Ratcliffe MJ, Sokol SY. Nuclear localization is required for Dishevelled function in Wnt/beta-catenin signaling. J Biol. 2005; 4:3. [PubMed: 15720724]

Ji Z, Donaldson IJ, Liu J, Hayes A, Zeef LA, Sharrocks AD. The forkhead transcription factor FOXK2 promotes AP-1-mediated transcriptional regulation. Mol Cell Biol. 2012; 32:385–398. [PubMed: 22083952]

Jung HY, Jun S, Lee M, Kim HC, Wang X, Ji H, McCrea PD, Park JI. PAF and EZH2 induce Wnt/beta-catenin signaling hyperactivation. Mol Cell. 2013; 52:193–205. [PubMed: 24055345]

Kim KA, Kakitani M, Zhao J, Oshima T, Tang T, Binnerts M, Liu Y, Boyle B, Park E, Emtage P, et al. Mitogenic influence of human R-spondin1 on the intestinal epithelium. Science. 2005; 309:1256–1259. [PubMed: 16109882]

Meeson AP, Shi X, Alexander MS, Williams RS, Allen RE, Jiang N, Adham IM, Goetsch SC, Hammer RE, Garry DJ. Sox15 and Fhl3 transcriptionally coactivate Foxk1 and regulate myogenic progenitor cells. EMBO J. 2007; 26:1902–1912. [PubMed: 17363903]

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet. 2004; 5:691–701. [PubMed: 15372092]

Nusse R. Wnt signaling in disease and in development. Cell Res. 2005; 15:28–32. [PubMed: 15686623]

Nusse R. Wnt signaling and stem cell control. Cell Res. 2008; 18:523–527. [PubMed: 18392048]

Shi X, Garry DJ. Sin3 interacts with Foxk1 and regulates myogenic progenitors. Mol Cell Biochem. 2012; 366:251–258. [PubMed: 22476904]

Shi X, Seldin DC, Garry DJ. Foxk1 recruits the Sds3 complex and represses gene expression in myogenic progenitors. Biochem J. 2012; 446:349–357. [PubMed: 22716292]

Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science. 1992; 256:668–670. [PubMed: 1350108]

Torban E, Wang HJ, Groulx N, Gros P. Independent mutations in mouse Vangl2 that cause neural tube defects in looptail mice impair interaction with members of the Dishevelled family. J Biol Chem. 2004; 279:52703–52713. [PubMed: 15456783]

Torres MA, Nelson WJ. Colocalization and redistribution of dishevelled and actin during Wnt-induced mesenchymal morphogenesis. J Cell Biol. 2000; 149:1433–1442. [PubMed: 10871283]

Varelas X, Miller BW, Sopko R, Song S, Gregorieff A, Fellouse FA, Sakuma R, Pawson T, Hunziker W, McNeill H, et al. The Hippo pathway regulates Wnt/beta-catenin signaling. Dev Cell. 2010; 18:579–591. [PubMed: 20412773]

Wallingford JB, Habas R. The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity. Development. 2005; 132:4421–4436. [PubMed: 16192308]

Wang W, Li X, Huang J, Feng L, Dolinta KG, Chen J. Defining the protein-protein interaction network of the human hippo pathway. Mol Cell Proteomics. 2014; 13:119–131. [PubMed: 24126142]

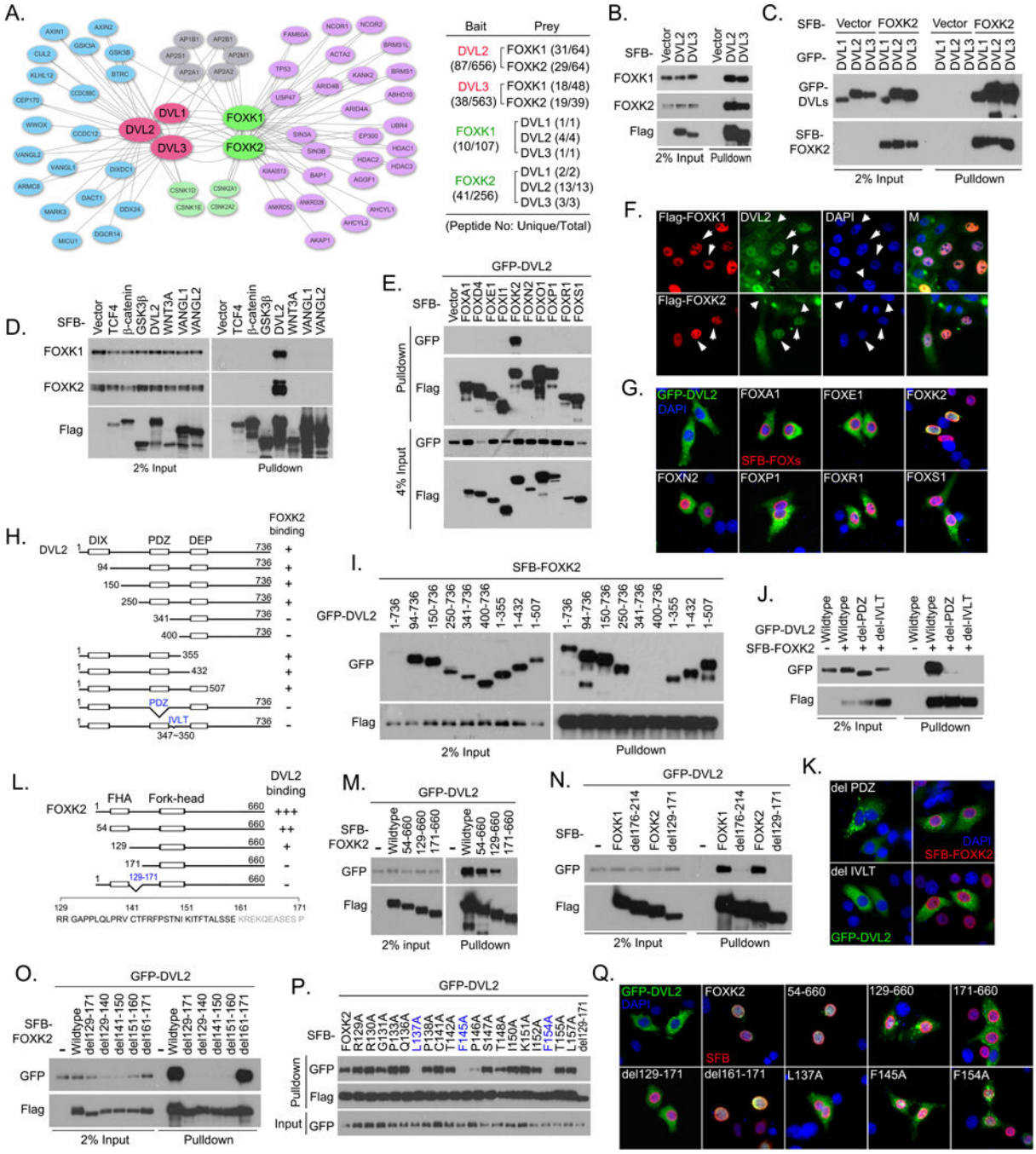


Figure 1. FOXK1 and FOXK2 are DVL-associated proteins and promote DVL nuclear translocation

(A) Identification of DVL-FOXK complex through TAP-MS analysis. HEK293T cells stably expressing SFB (S protein tag, Flag tag and streptavidin-binding tag) tagged DVL2, DVL3, FOXK1 or FOXK2 were used for tandem affinity purification to identify associated protein complexes. The interaction network (with hand-picked preys) within the DVL-FOXK complex was subjected to illustration by Cytoscape. The peptide information identified by the mass spectrometry analysis for the DVL-FOXK complex is summarized in the table. (B) The interaction between DVL and FOXK was confirmed by pulldown-

Western Blot analysis. The SFB-DVL2 and DVL3 purification elutes were analyzed by FOXK1 and FOXK2 antibodies. (C) FOXK associates with all the members of DVL family. GFP tagged DVL1/2/3 was co-expressed with SFB tagged FOXK2 in 293T cells and cell lysates were subjected to pulldown assays with S protein beads. (D–E) The association between FOXK and DVL is specific in Wnt pathway and FOX transcription factor family. (F) FOXK translocates DVL into the nucleus. Endogenous DVL2 was detected in 293T cells transfected with Flag-tagged FOXK1 or FOXK2. Arrows indicated the FOXK positive and negative cells. Nucleus was visualized by DAPI staining. M, merged. (G) The specificity of the FOXK2 mediated DVL2 nuclear translocation. GFP-DVL2 and various SFB tagged FOX transcription factors were co-expressed in HeLa cells. The localization of GFP-DVL2 was detected by immunostaining. (H) Schematic illustration of human DVL2 protein domains and region required for FOXK2 binding. (I) Determination of the region of DVL2 required for the FOXK2 interaction. GFP tagged various DVL2 truncations were co-expressed with SFB-FOXK2 in 293T cells for 24h and cell lysates were subjected to pulldown assays with S protein beads. (J) PDZ domain and IVLT motif on DVL2 are required for FOXK2 binding. GFP tagged DVL2 PDZ or IVLT deletion constructs were co-expressed with SFB-FOXK2 in 293T cells and cell lysates were subjected to pulldown assays. (K) Both the PDZ domain and the IVLT motif on DVL2 are required for FOXK2 mediated nuclear translocation. (L) Schematic illustration of human FOXK2 protein domains and the region required for the DVL2 binding. (M–N) FHA domain and its adjacent region (129~171 amino acids) of FOXK2 are required for the association to DVL2. The conserved region (176~214 amino acids) on FOXK1 is also required for the interaction with DVL2. (O–P) Three hydrophobic amino acids (L137, F145 and F154) on FOXK2 are required for the interaction with DVL2. Various deletions and single site mutations of FOXK2 were co-expressed for 24h with GFP-DVL2 in 293T cells and cell lysates were subjected to pulldown assay. (Q) The FHA domain and the identified three amino acids are required for FOXK2 mediated DVL2 nuclear translocations. GFP-DVL2 and the indicated FOXK2 mutants were co-expressed in HeLa cells. The localization of GFP-DVL2 and SFB-FOXK2 mutants were detected by immunostaining. Please also see the Figure S1.

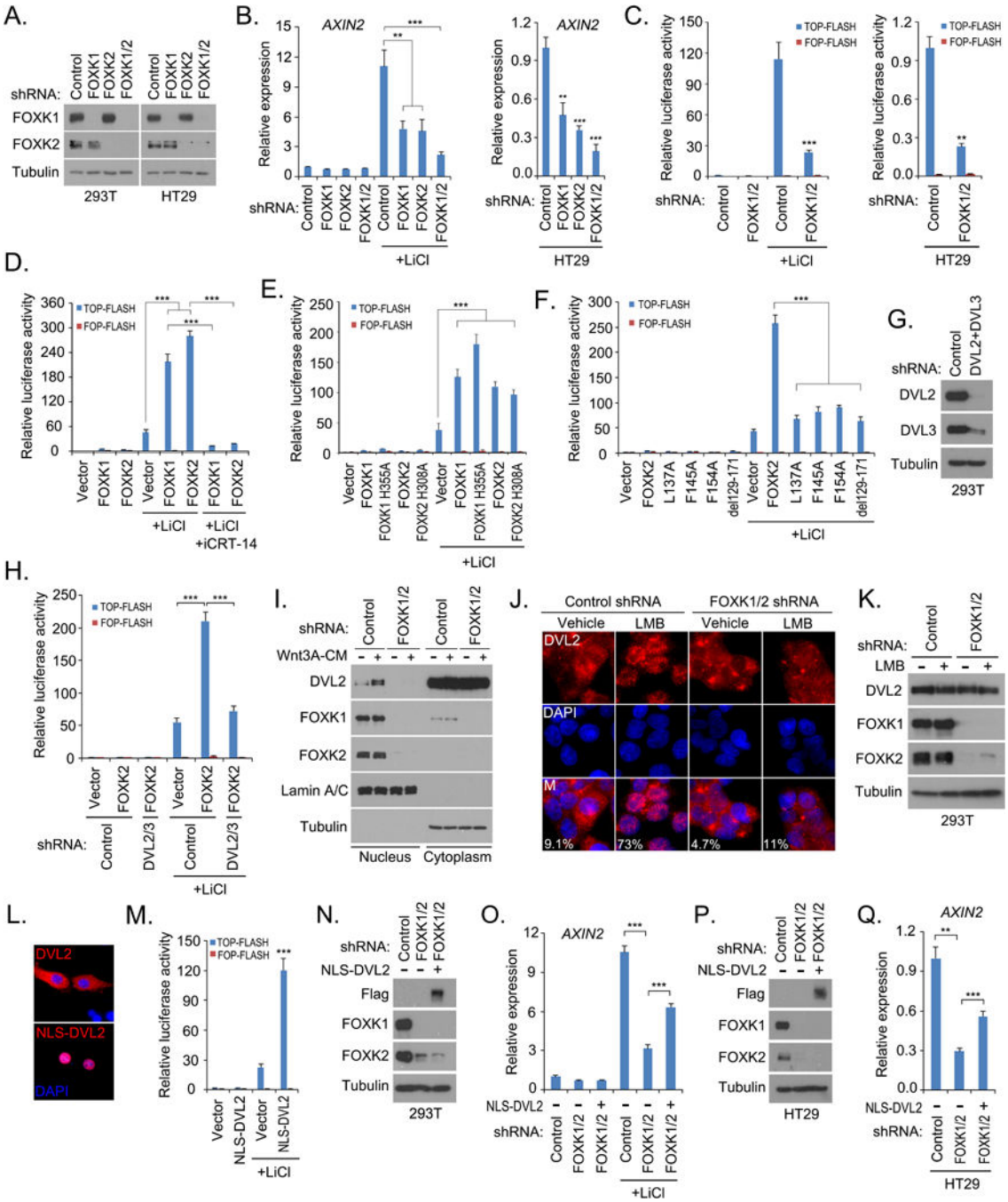


Figure 2. FOXK proteins activate the Wnt pathway by promoting nuclear translocation of DVLs

(A) Knockdown of FOXK1 and FOXK2 in 293T and HT29 cells. Endogenous FOXK1 and FOXK2 expression were detected in the indicated shRNA-transduced cells. (B) Loss of FOXK1 and FOXK2 suppressed β-catenin transcriptional activity. The transcript of *AXIN2* was detected by quantitative PCR in 293T and HT29 cells transduced with the indicated shRNA (n=3, mean±s.d). For 293T cells, Wnt signaling was activated by treatment with 25mM LiCl for 6h. (C) Loss of FOXK1 and FOXK2 suppresses TOPFLASH luciferase reporter activity. TOPFLASH luciferase reporter assay was performed in indicated cells,

where pSV40-Renilla was used as internal control (n=3, mean±s.d). TOP-FLASH and FOP-FLASH indicate pMegaTOPFLASH and pMegaFOPFLASH respectively. (D) FOXK proteins promote the Wnt/β-catenin reporter activity. 293T cells were transiently transfected with the indicated plasmids and treated with 25mM LiCl for 6h with or without iCRT-14 (100μM). Luciferase assay of β-catenin transcriptional activity was performed, where pSV40-Renilla was used as internal control (n=3, mean±s.d). (E) The transcriptional activities for FOXK proteins are dispensable for Wnt/β-catenin pathway activation. β-catenin luciferase reporter assays were performed by transiently transfecting 293T cells with the indicated FOXK plasmids and their DNA-binding mutants (n=3, mean±s.d). Cells were treated with 25mM LiCl for 6h. (F) The association with DVL2 is required for FOXK-mediated Wnt/β-catenin pathway activation. β-catenin luciferase reporter assays were performed by transiently transfecting 293T cells with the indicated FOXK site mutations or internal deletions (n=3, mean±s.d). Cells were treated with 25mM LiCl for 6h. (G–H) FOXK activates Wnt/β-catenin pathway through DVL. TOPFLASH luciferase reporter assays were performed by transiently transfecting FOXK2 plasmid in control-shRNA transduced 293T cells and DVL2/3-shRNA transduced 293T cells (n=3, mean±s.d) (H). The DVL2 and DVL3 proteins were examined in both control and DVL2/3-shRNA transduced 293T cells by Western blot (G). Cells were treated with 25mM LiCl for 6h. (I) Loss of FOXK proteins inhibits Wnt3A induced DVL nuclear translocation. Cellular fractionation was performed of shRNA-transduced 293T cells, which were treated by Wnt3A conditional medium (Wnt3A-CM) for 8h. Indicated proteins were detected by Western Blot. (J–K) Loss of FOXK proteins inhibits DVL nuclear translocation. DVL2 localization was detected by immunostaining in shRNA transduced 293T cells. DVL2 nuclear accumulation was enhanced by treatment of 50ng/ml leptomycin B (LMB) for 12h. The percentage of nuclear DVL2 was quantified in 200 cells. The DVL2 protein expression was examined by Western blot (K). (L–M) Nuclear DVL2 promotes the β-catenin reporter activity. Three tandem nuclear localization sequences (NLS) were fused to DVL2, which translocated DVL2 into the nucleus and activated TOPFLASH luciferase activity (n=3, mean±s.d). 293T cells were treated with 25mM LiCl for 6h. (N–Q) Nuclear DVL2 rescued Wnt/β-catenin signaling in FOXK1 and FOXK2-deficient 293T and HT29 cells. The expression of NLS-DVL2 was examined in FOXK1 and FOXK2-deficient 293T (N) and HT29 cells (P) by Western blot. The transcript of *AXIN2* was detected by quantitative PCR in indicated cells (n=3, mean ±s.d). For 293T cells, Wnt signaling was activated by treatment with 25mM LiCl for 6h. ** $p<0.01$ and *** $p<0.001$ (Student *t*-test). Please also see the Figure S2.

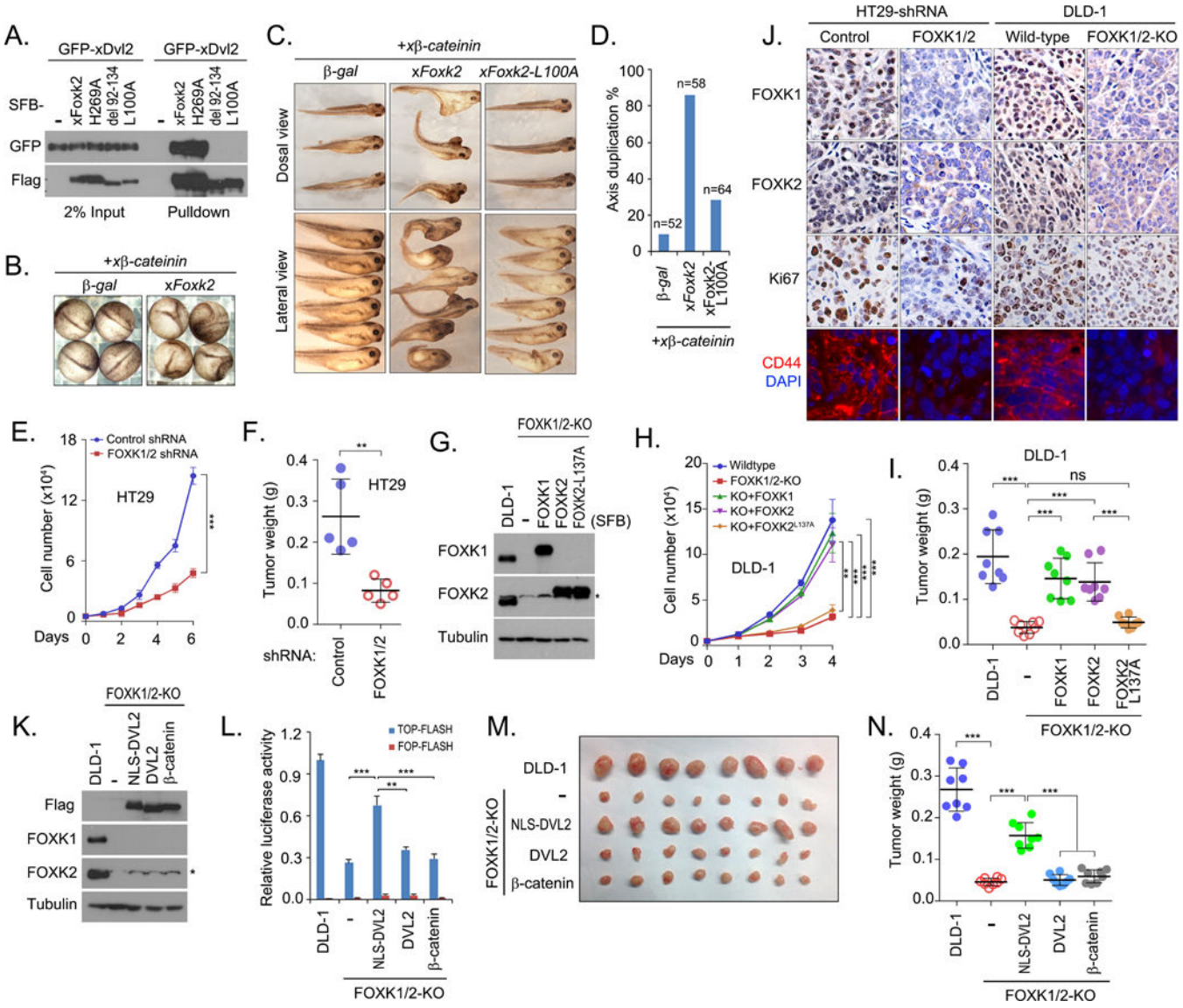


Figure 3. FOXK proteins are required for tumorigenesis of colon cancer cells

(A) The association between FOXK2 and DVL2 is conserved in *Xenopus*. *Xenopus* Dvl2 and the indicated *Xenopus* Foxk2 mutants were co-expressed in 293T cells for 24h and cell lysates were subjected to pulldown assays. (B–D) xFoxk2-induced β-catenin activation leads to axis duplication. *xFoxk2* or *xFoxk2-L100A* mRNA was injected with *xβ-catenin* mRNA into the ventrovegetal blastomeres of *Xenopus. laevis* embryos, where *β-galactosidase* was taken as control. Secondary axes were examined from neurulation (B) through tail-bud stages (C) and quantified (D). (E–F) Loss of FOXK inhibits cell proliferation (n=3, mean ±s.d) and xenograft tumor formation for HT29 colon cancer cells (n=5 mice, mean±s.d). (G–I) Wild-type FOXK1 or FOXK2 but not FOXK2-L137A mutant rescued cell proliferation (n=3, mean±s.d) and xenograft tumor formation (n=8 mice, mean±s.d) in FOXK1/2-Knockout (KO)-DLD-1 cells. The DLD-1 FOXK-KO cells were generated using the CRISPR approach. The FOXK proteins were detected in the indicated cells (G). The cell growth curve (H) and tumor weight (I) were analyzed (mean±s.d). ns, no significant

difference. Star *, none specific band. (J) Proliferation and Wnt/β-catenin signaling were suppressed in FOXK1/2 knockdown (F) or knockout (I) xenograft tumors. Immunohistochemistry was performed with the indicated antibodies in HT29 and DLD-1 xenograft tumors. (K–N) NLS-DVL2 but not DVL2 or β-catenin promoted Wnt signaling in FOXK1 and FOXK2-deficient DLD-1 cells. The indicated proteins were reconstituted in the FOXK1/2-knockout DLD-1 cells and examined by Western blot (K). TOPFLASH luciferase reporter assays were performed in indicated cells (n=3, mean±s.d) (L). Indicated cells were subjected to xenograft study. Xenograft tumors were shown in (M) and the tumor weight was quantified in (N) (n=8 mice, mean±s.d). ** $p<0.01$ and *** $p<0.001$ (Student *t*-test).

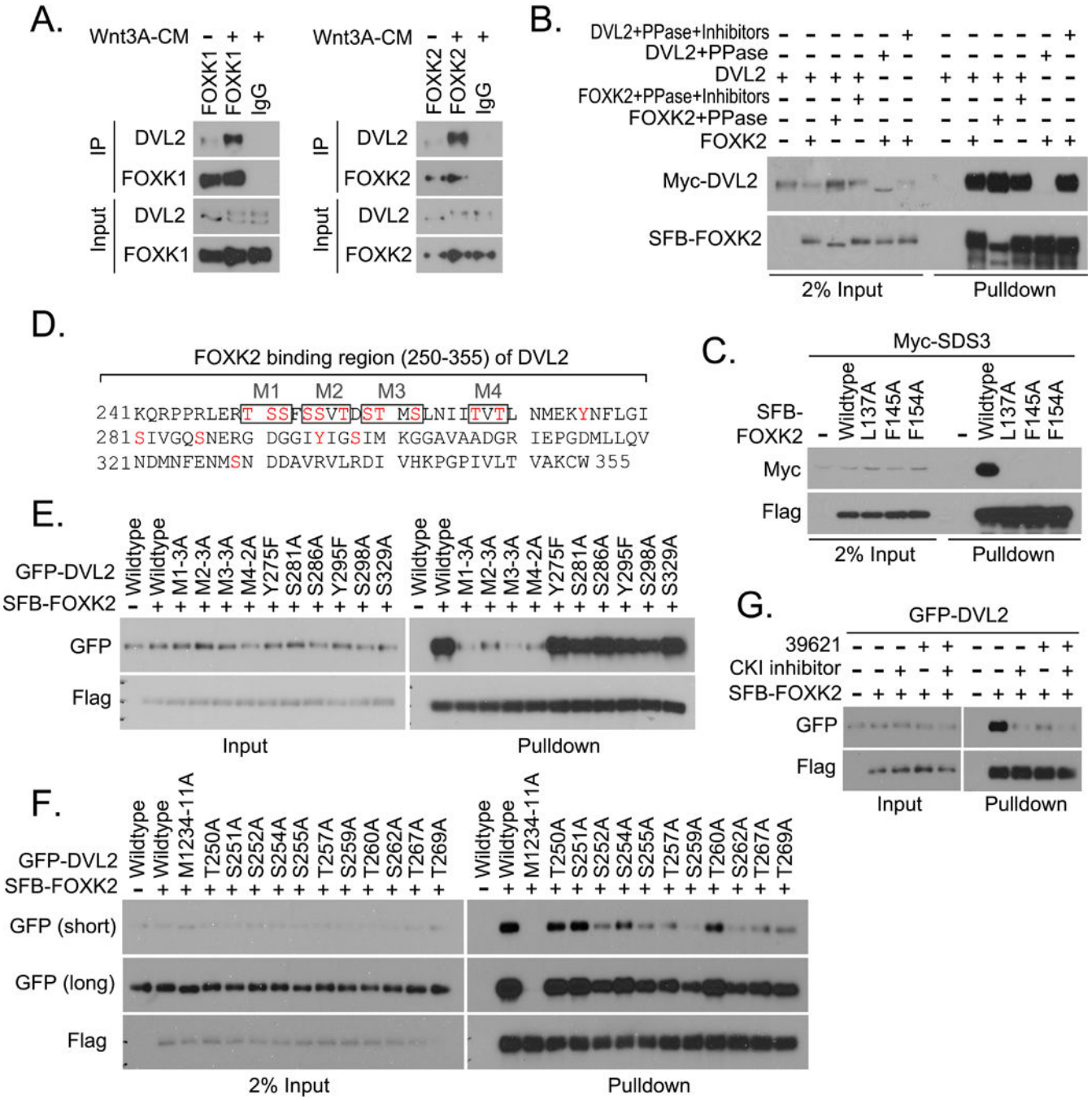
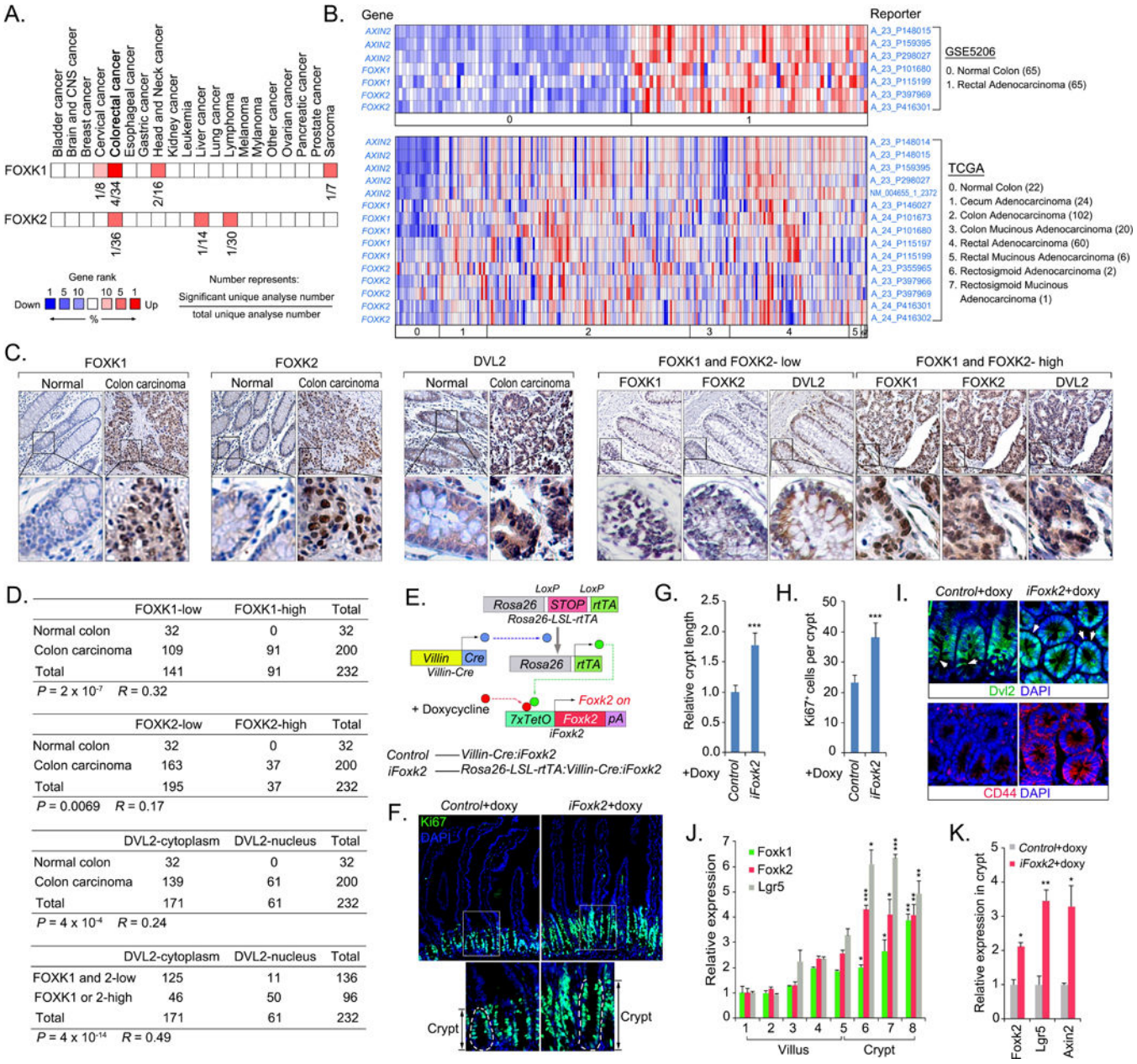


Figure 4. The association between DVL and FOXK is regulated by Wnt signaling

(A) Wnt3A promotes the interaction between DVL2 and FOXK1 or FOXK2. 293T cells were treated by Wnt3A conditional medium (Wnt3A-CM) for 8h and cell lysates were subjected to immunoprecipitation with FOXK1 or FOXK2 antibody, where IgG was taken as control. (B) The phosphorylation of DVL2 is required for its association with FOXK2. 293T cells were transiently transfected with Myc-DVL2 or SFB-FOXK2 and cell lysates were subjected to λ phosphatase treatment (with or without phosphatase inhibitors) at 30°C for 20min. Cell lysates were combined for pulldown assay. (C) Three FOXK2 site mutations

inhibit the association with SDS3 protein. 293T cells were transiently transfected for the indicated proteins and cells lysates were subjected to pulldown assay. (D) Schematic illustration of the amino acid sequence for the FOXK2-binding region on DVL2. Potential phosphorylation sites are labeled in red. Sites close to each other were categorized into four groups (M1, M2, M3 and M4). (E–F) Multi-phosphorylation sites located within the 250~270 amino acid region of DVL2 are required for the association with FOXK2. 293T cells were transiently transfected with the indicated DVL2 mutants and SFB-FOXK2 for 24h and cell lysates were subjected to pulldown assay. (G) CK1 and MARK2/PAR1 kinase are potential kinases regulating the interaction between FOXK and DVL proteins. 293T cells were transfected with GFP-DVL2 and treated with CK1 inhibitor or MARK2/PAR1 inhibitor 39621 alone, or combined for 12h. Cell lysates were subjected to pulldown assay with SFB-FOXK2 cell lysates.



	FOXK1-low	FOXK1-high	Total
Normal colon	32	0	32
Colon carcinoma	109	91	200
Total	141	91	232

$P = 2 \times 10^{-7}$ $R = 0.32$

	FOXK2-low	FOXK2-high	Total
Normal colon	32	0	32
Colon carcinoma	163	37	200
Total	195	37	232

$P = 0.0069$ $R = 0.17$

	DVL2-cytoplasm	DVL2-nucleus	Total
Normal colon	32	0	32
Colon carcinoma	139	61	200
Total	171	61	232

$P = 4 \times 10^{-4}$ $R = 0.24$

	DVL2-cytoplasm	DVL2-nucleus	Total
FOXK1 and 2-low	125	11	136
FOXK1 or 2-high	46	50	96
Total	171	61	232

$P = 4 \times 10^{-14}$ $R = 0.49$

Figure 5. FOXK proteins are pathologically relevant to colorectal cancer formation

(A–B) FOXK1 and FOXK2 expression were analyzed *in silico* in human cancers by using the Oncomine database (www.oncomine.org). The expressions of FOXK1 and FOXK2 were analyzed in various human cancers (A). The co-expression among *AXIN2*, *FOXK1* and *FOXK2* were analyzed in human colorectal cancer datasets (GSE5206 and TCGA) (B). The Oncomine analysis settings used were: 10% gene rank; p-value < 0.0001; fold change > 2. (C–D) FOXK expression and DVL nuclear localization are positively correlated in human colon cancers. Immunohistochemical stainings of FOXK1, FOXK2 and DVL2 were performed in representative normal colon and colon carcinoma samples (C). Brown staining indicates positive immunoreactivity. The indicated regions in the box were enlarged below. Correlation analyses between FOXK1/ FOXK2 levels and DVL2 nuclear localization in

human normal colon and colon carcinoma samples are shown (D). Statistical significance was determined by χ^2 test. R: correlation coefficient. (E) Schematic illustration of generation inducible-mFoxk2 mouse model (*iFoxk2*). Upon doxycycline given in drinking water, *Foxk2* is conditionally expressed in intestinal epithelium by crossing with *Vilin-Cre* and *Rosa26-LSL-rtTA* mouse strains. rtTA, reverse tetracycline transactivator. TetO, tetracycline operator. pA, poly-A tail signal. *Rosa26-LSL-rtTA: iFoxk2* was taken as control group (Foxk2 off) and *Vilin-Cre:Rosa26-LSL-rtTA:iFoxk2* was taken as experimental group (Foxk2 on). Mice were given doxycycline-containing drinking water for 4 months. (F) Foxk2 overexpression promotes the proliferation of mouse intestine. Ki67 staining is shown for representative images. The indicated regions in the box are enlarged below. (G–H) Nearly 50 crypts from control and Foxk2 overexpression mice were randomly chosen for the analysis of crypt length (G) and Ki67 positive cells (n=48 crypts, mean±s.d) (H). (I) Induction of Foxk2 promotes the nuclear translocation of Dvl2 in intestine crypt and up-regulates Wnt/β-catenin signaling. DVL2 and CD44 staining are shown. The indicated regions in the box are enlarged below. Arrows indicate the Dvl2 localization in the crypt. (J–K) Wnt/β-catenin activation was induced in the intestine crypt of mice by conditional Foxk2 expression. Foxk1 and Foxk2 transcripts were analyzed in villus and crypt fractions (n=3, mean±s.d) (J). The transcription of Wnt/β-catenin targeted genes in intestine crypt fractions were analyzed for both control and experimental mice (n=3, mean±s.d) (K). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ (Student *t*-test).