A genome-wide RNAi screen for Wnt/β-catenin pathway components identifies unexpected roles for TCF transcription factors in cancer

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Communicated by Steven L. McKnight, University of Texas Southwestern Medical Center, Dallas, TX, May 19, 2008 (received for review February 21, 2008)

The Wnt family of secreted proteins coordinate cell fate decision-making in a broad range of developmental and homeostatic contexts. Corruption of Wnt signal transduction pathways frequently results in degenerative diseases and cancer. We have used an iterative genome-wide screening strategy that employs multiple nonredundant RNAi reagents to identify mammalian genes that participate in Wnt/β-catenin pathway response. Among the genes that were assigned high confidence scores are two members of the TCF/LEF family of DNA-binding proteins that control the transcriptional output of the pathway. Surprisingly, we found that the presumed cancer-promoting gene *TCF7L2* functions instead as a transcriptional repressor that restricts colorectal cancer (CRC) cell growth. Mutations in *TCF7L2* identified from cancer genome sequencing efforts abolish its ability to function as a transcriptional regulator and result in increased CRC cell growth. We describe a growth-promoting transcriptional program that is likely activated in CRC tumors with compromised *TCF7L2* function. Taken together, the results from our screen and studies focused on members of the TCF/LEF gene family refine our understanding of how aberrant Wnt pathway activation sustains CRC growth.

cancer genome sequencing | colorectal cancer | functional genomics | TCF/LEF transcription factors | Wnt signal transduction

The Wnt/β-catenin signal transduction pathway is a global regulator of embryonic development (1, 2). In postembryonic animals, Wnt/β-catenin pathway activity sustains homeostatic tissue renewal but can be exploited in cancer to promote deviant cell growth (1–3). For example, pathway activity is necessary for maintenance of stem cells in the intestinal crypts (4) and, when misactivated, gives rise to familial and sporadic CRC (5). Indeed, >90% of CRCs are induced by loss-of-function mutations in the *adenomatosis polyposis coli* (*APC*) gene, which encodes a scaffolding protein that mediates constitutive destruction of the β-catenin transcriptional coactivator in the absence of Wnt ligand (6). Loss of *APC* function results in the accumulation of β-catenin and subsequent activation of a member of the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcriptional effectors, presumably Tcf7l2 [formerly Tcf4 (7–9)].

Despite its importance to cancer, much of our genetic understanding of the Wnt/β-catenin pathway is based on developmental studies in model organisms. The relevance of these findings to human diseases often varies because of differences in gene function in developing and adult animals and the species-dependent sensitivity of tissues to pathway perturbation. For example, mice that have decreased *APC* function develop tumors primarily in the small intestines, whereas, in humans, loss of *APC* frequently results in cancer of the colon (1). Also complicating our understanding of human diseases from model organisms are differences in the redundancy of gene function observed between humans and other animals. For example, a single TCF family member (*Pangolin*) controls both transcriptional repression and activation of Wnt pathway target genes in *Drosophila*, whereas, in mammals, the TCF/LEF gene family consists of four members that are subject to alternative splicing and whose transcriptional roles are isoform dependent (10).

The advent of RNA-mediated interference (RNAi) has enabled rapid testing of gene function, using loss-of-function strategies in human cells derived from diseased tissues, and may allow us to bypass many of the issues associated with the use of model organisms to study human disease processes. Using a screening strategy designed to limit off-targeting effects that are commonly associated with the use of RNAi technology and genome-wide short interfering RNA (siRNA) libraries, we identified novel genes that contribute to mammalian Wnt/β-catenin pathway response and assigned new functions to previously described pathway components in CRC cells. Most importantly, our study reveals a tumor suppressor role for Tcf7l2 in CRC and a novel mechanism for Tcf7l2 action in normal and cancerous Wnt pathway response.

Results

A High-Throughput RNAi Screen for Wnt Pathway Components Using Human Cells. After testing several human cell lines for their responsiveness to Wnt ligand, using an extensively tested Wnt-inducible luciferase reporter construct (SuperTopFlash/STF reporter; *Materials and Methods*), we found that HeLa cells exhibited a robust and faithful response to Wnt3A [supporting information (SI) Fig. S1 *A–C*]. We incorporated this assay into a screening strategy that enabled us to monitor Wnt/β-catenin pathway response and aspects of general cellular functions in cells transiently transfected with chemically synthesized siRNAs and several reporter constructs (Fig. 1*A*). Using this approach, we first screened a human genome-scale siRNA library (Dharmacon) consisting of ≈21,000 pools of siRNAs, each containing four distinct siRNAs targeting a single gene (Fig. 1*B* and Fig. S1 *D* and *E*), an approach shown to be useful in gene discovery studies (11).

Confounding the effective use of genome-scale RNAi screens has been the inability to discriminate between siRNAs that induce off-targeting effects or otherwise grossly affect general cellular functions, from those that target genes-of-interest (12). Directly relevant to this study is the prevalence of false positives resulting from off-targeting observed in a similar screen performed in *Drosophila* to identify Wnt pathway components (12, 13). To limit the influence of off-targeting in our screen, we retested 530 siRNAs of interest identified from the primary screen (Fig. 1*B* and Fig. S2; see Table S1 for screening data), using gene-specific siRNA pools from human and mouse genome-scale siRNA libraries developed by Qiagen in either

Author contributions: W.T., M.D., M.R., and L.L. designed research; W.T., M.D., D.G., C.M., and L.L. performed research; W.T., M.D., and L.L. analyzed data; and W.T., M.D., and L.L. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0804709105/DCSupplemental.

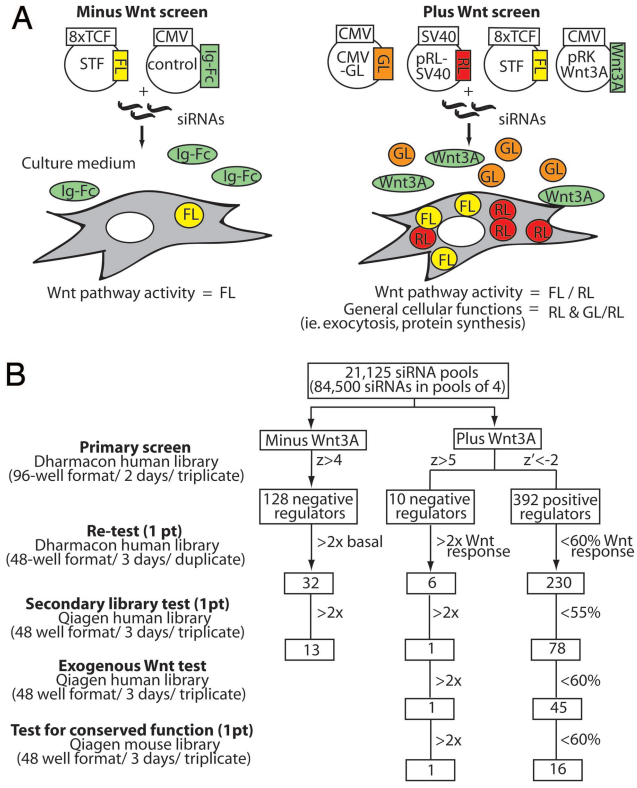

Fig. 1. Overview of the RNAi-based screening strategy to identify components of the Wnt/β-catenin pathway. (*A*) The screening strategy. HeLa cells were cotransfected with siRNA pools targeting a single gene, and various reporter and expression constructs to identify positive and negative regulators of the pathway measured by firefly luciferase (FL) activity generated from the Wnt-responsive SuperTopFlash (STF) reporter construct. Activity of the secreted control *Gaussia* luciferase (GL) protein reflects levels of protein exocytosis. The *Renilla* luciferase (RL) activity also serves as measure of general cellular functions. FL and GL activities were normalized to RL activity in the ''plus Wnt screen.'' Ig-Fc, control Ig protein. (*B*) Summary of primary screen and secondary assay results. A human siRNA library (Dharmacon) was screened in the absence or presence of Wnt-pathway activation, and interesting siRNAs were retested. Subsequent genes of interest were tested again, using nonredundant siRNAs from another human siRNA library (Qiagen). Candidate genes from the positive regulator screen were then screened in the presence of exogenous Wnt3A to eliminate those genes potentially involved in Wnt protein production. Those siRNAs likely affecting cellular response to Wnt3A were tested in mouse NIH 3T3 cells, using a mouse siRNA library (Qiagen). Hits identified from various tests (boxed) were each assigned a single point (1 pt). Format of assays are noted on the left with criteria used to identify hits described in *Materials and Methods*.

HeLa or NIH 3T3 cells (Fig. 1*B*, Fig. S1*F*, Table S2, Table S3, and Table S4). Because these libraries were engineered by using a different algorithm for identifying targeting sequences than that used in the primary screen siRNA library, an observed effect on pathway by at least two siRNA pools are less likely to be due to off-targeting events (Fig. S3). Ultimately, seven known pathway components [*Apc*, *Axin2*, *Tcf7*, *Tcf7l2*, *Ctnnb1* (encoding β-catenin), *Bcl9l*, and *Nlk*] were included among the highest scoring genes, thus confirming the utility of our screening strategy (Table 1).

To assign confidence values to candidate pathway components, we established a scoring system that takes into account the number of different human and mouse siRNA pools targeting the same gene that induce the same effect on pathway response (Fig. 1*B*). Additionally, the "exogenous Wnt test" eliminated those components that likely affected Wnt production. Thus, a maximal score of 3 (*N* in Table 1 and Table S2, Table S3, and Table S4) could be assigned to a gene if it was identified by using three different siRNA pools. All genes assigned a score are listed in Table S2, Table S3, and Table S4, whereas only the highest scoring hits from each screen are listed in Table 1. Because some known pathway components were not identified among the highest confidence hits (Fig. S4), genes with $n < 3$ may also be relevant to pathway response but failed to retest either as a result of differences in the effectiveness of siRNA pools or redundancy of gene function in the cells tested.

We performed epistasis-type experiments using overexpression of a Wnt receptor (*Lrp6*) or the transcriptional activator β-catenin to gain insight into the role of candidate genes within the pathway (Table 1 and Table S5). For example, loss of the novel secreted protein *Tmem43* was fully rescued by expression of Lrp6, which suggests that Tmem43 functions in Wnt response at the cell membrane (Table 1, see also Table S5). We also tested whether the actions of some of our candidate positive regulators within the pathway are limiting by using overexpression studies in our cell-based reporter assay (Table 1, Table S2, and Table S3).

Identification of Wnt/β-catenin pathway components relevant to CRC. Aberrant activation of the Wnt/β-catenin pathway resulting from inactivating mutations in *APC* is present in >90% of sporadic and familial CRCs (6). Less frequently, CRCs are associated with activating mutations in *CTNNB1* (8). We tested the relevance of positive regulators of the Wnt pathway that are predicted to function at the level or downstream of *Ctnnb1* in aberrant pathway response using STF reporter assays in two CRC cell lines that either harbor a mutation in *APC* (DLD-1 cells) or an activating mutation in *CTNNB1* (HCT116 cells; Table 1 and Fig. S5). These genes include *Ctnnb1*, *Bcl9l*, *Tcf7* (which encodes Tcf1), components of the Mediator complex (*Surb7*/*Med21* and *Pcqap*/*Pc2*/*Med15*) that regulate RNA polymerase II activity, and the nuclear import factor karyopherin alpha 3 (*Kpna3*). Components of the Mediator complex have been shown to directly interact with β-catenin and mediate Wnt-dependent transcription of target genes such as *AXIN* (14). Of the genes that reduced aberrant Wnt pathway response in DLD-1 cells by >50% or more (*Ctnnb1*, *Tcf7*, and *Med15*), two specifically reduced DLD-1 growth (*Ctnnb1* and *Tcf7*) in a clonal expansion assay that depends on Wnt pathway activity (9, 15). The identification of *Tcf7* as a positive regulator in CRC is surprising given its suggested role as a tumor suppressor (16) but is consistent with the absence of *TCF7* mutations in CRC tumors (6, 17).

We identified three candidate negative regulators of the Wnt/β-catenin pathway that enhanced aberrant Wnt/β-catenin pathway activity by more than threefold when targeted with siRNAs in DLD-1 and HCT116 cells (*Fwbx10*, *Axin2*, and *Tcf7l2*) (Table 1). *Axin2* is a known suppressor of the Wnt/β-catenin pathway in CRC (18), whereas the F-box protein *Fbwx10* likely contributes to ubiquitin-dependent destruction of β-catenin. However, the negative influence of *Tcf7l2* on STF activity is surprising given its proposed role in promoting transcriptional activation of growth-promoting genes after loss of APC function in CRC (7–9). Notably, loss of *Tcf7l2* results not only in a dramatic increase in STF reporter activity in CRC cells, but also an increase in cell growth potential. When taken together, these observations suggest that *TCF7L2* should be assigned a tumor suppressor role in CRC.

CRC-Associated Mutations in *TCF7L2* Abrogate Its Ability to Restrict CRC Cell Growth. Given the strong association between the Wnt/β-catenin pathway and CRC, we identified potential tumor suppressors by focusing on genes in our candidate pathway suppressor dataset that harbor CRC-associated mutations (6, 17). The most potent pathway suppressor identified in our screen (*APC*), predictably harbored the largest number of CRC-associated mutations as identified from recent cancer genome sequencing results (Table 1). Surprisingly, the same studies also found a significant number of mutations in *TCF7L2*, a gene presumed to be necessary for

Table 1. The role of known and candidate Wnt/β-catenin pathway components in supporting CRC cell growth

		N	OEX	Aberrant Wnt pathway response DLD-1 Ave	DLD-1 SD	HCT116 Ave	HCT116 SD	Cell growth DLD-1 Ave	DLD-1 SD	DLD-1 P-value	HeLa Ave	HeLa SD	HeLa P-value
Positive regulators	**Upstream or at the level of LRP6/CK1γ**												
	COPI components (A,B,D,G)	3	NT										
	MGC3222/TMEM43	3	0										
	Downstream of LRP6/CK1γ and upstream of CTNNB1												
	RPH3A	3	NT										
	LOC124245/NHN1	3	+										
	Downstream or at the level of CTNNB1												
	PCQAP/ MED15	3	+	0.50	0.13	0.44	0.09	0.68	0.06	(<.0001)	0.43	0.07	(<.0001)
	SURB7/MED21	3	0	0.61									
	CDC6	3	0	0.86									
	GNG8	3	NT	0.74	0.16								
	KPNA3	3	0	0.65	0.03								
	CTNNB1	3	0	0.02	0.01	0.02	0.00	0.16	0.02	(<.0001)	1.04	0.14	(0.48)
	TCF7	2	+	0.37	0.02	0.25	0.04	0.53	0.03	(<.0001)	0.97	0.11	(0.55)
	BCL9L	3	NT	0.58	0.13	0.48	0.13						
	NLK	3	NT	0.85	0.19								
Negative regulators	CEBPE	2	0	0.69	0.15								
	TGFB3	2	-	0.94									
	FBXW10	2	NT	3.97	1.06	2.86	0.30	0.89	0.03	(<.005)	1.09	0.07	(0.11)
	GPR65	2	-	1.19	0.19								
	EPC1	2	0	1.28	0.25								
	CHRNA4	2	0	0.82	0.42								
	GIP	2	0	0.78									
	MK2S4	2	-	1.54	0.33								
	RXRB	2	0	0.74									
	TIAM1 [2]	2	0	1.63	0.43								
	CDKL2 [1]	2	-	1.01									
	APC [31]	2	NT	1.77									
	Axin 2 [5]	2	NT	4.02	0.61	2.08	0.54	1.27	0.06	(<.0001)	1.11	0.09	(0.05)
	TCF7L2 [8]	2	-	8.61	1.87	23.27	1.51	1.73	0.05	(<.0001)	1.09	0.03	(0.10)

Positive regulators of pathway were functionally mapped by comparing their requirement in Wnt-mediated pathway response and pathway activation induced by overexpression of Lrp6/Ck1γ or β-catenin in HeLa cells (Table S4 and *Materials and Methods*). *N* = number of siRNAs tested that phenocopy. STF results from overexpression (OEX) studies in HeLa cells were assigned a + if the pathway response was >2 (control = 1), – if it was <0.5, and 0 if neither was true (Tables S1 and S2). Aberrant pathway response was measured with STF reporter for those siRNAs that target genes with functions downstream or at the level of *Ctnnb1*. Results are normalized to control (=1), increased response (>2.0, in green), and decreased response (≤0.5, in red). Effective siRNAs in DLD-1 cells were also tested in HCT116 cells, using STF reporter, and in a clonal density cell growth assay in DLD-1 and HeLa cells (measured using Cell-Titer Glo assay). Only genes with *N* = 3 or 2 are included. All genes assigned a score are described in Tables S1–S3. The number of CRC mutations in each gene is indicated in brackets (6, 17, 18). Values are mean of triplicates ± SD. NT, not tested.

activating growth-promoting target genes subsequent to *APC* loss (Table 1 and Fig. S6).

We investigated the impact of these CRC-associated mutations in *TCF7L2* on its ability to restrict the growth of DLD-1 cells. Of eight CRC mutations in *TCF7L2* previously identified (Fig. S6), we introduced four of these into an expression plasmid encoding the predominantly expressed form of Tcf7l2 in CRC cells, the "E" form (19) (Fig. 2*A* and Fig. S7). Three of these mutations abolish the ability of Tcf7l2 to suppress growth of DLD-1 cells (Fig. 2*B*). Unlike Mut1 and -2, which are likely to be prematurely terminated molecules, Mut4 harbors a single amino acid change in the DNA-binding domain (HMG box) and is readily expressed as a full-length molecule (Fig. 2*C*). Nevertheless, Mut4 fails to suppress DLD-1 cell growth, implying that the effects of overexpressing Tcf7l2 on cell growth depends on its ability to induce changes in the transcription of TCF target genes. The Mut3 protein behaves like a WT Tcf7l2 molecule, suggesting the mutation it harbors is irrelevant in cancer or that our reporter assay does not sufficiently measure all aspects of Tcf7l2 function.

We tested the influence of the same four CRC-associated mutations on the transcriptional activity of Tcf7l2, using the STF reporter. Expression of either Tcf7 or Tcf7l2, respectively, augmented or repressed STF activity in HeLa and DLD-1 cells, consistent with the roles that we had assigned to them (Fig. 2*D*). Of the four Tcf7l2 mutant proteins tested by using this assay, the three that failed to suppress DLD-1 cell growth also failed to repress STF activity suggesting that mutations in Tcf7l2 frequently result in loss of transcriptional repressor function (Fig. 2*E*). Taken together, our data suggest that a cell-growth promoting program is activated subsequent to acquisition of CRC-associated mutations in Tcf7l2.

A Repressor Domain in Tcf7l2. Two distinct regions of Tcf7l2 family members are known to mediate interactions with the transcriptional corepressors Transducin-like enhancer of split protein [TLE or Groucho-related gene (Grg)], and C-terminal binding proteins (Ctbp) (Fig. 3*A*). Whereas the TLE binding domains are present in all TCF/LEF proteins, Ctbp interaction sequences are only present in the long C-terminal tail variants of Tcf7l2 (E forms), and Tcf7l1/Tcf3 (20). Indeed, exchanging the Tcf7 N-terminal sequence that includes the TLE domain for the corresponding sequence from

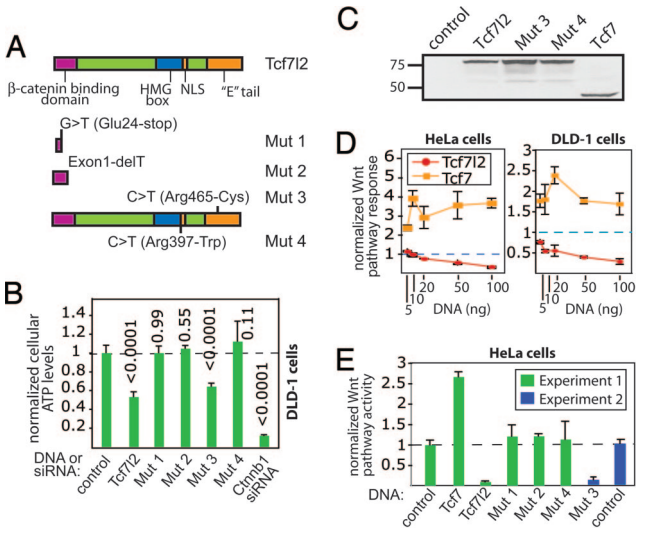

Fig. 2. *TCF7L2* mutations identified in CRC abrogate Tcf7l2 pathway repressor function. (*A*) Predicated changes in Tcf7l2 E variant protein structure resulting from CRC mutations in the *TCF7L2* gene (6, 17). NLS, nuclear localization signal; HMG box, high-mobility group DNA binding domain. (*B*) Effects of mutant Tcf7l2 overexpression on DLD-1 growth. Growth of DLD-1 cells overexpressing wt or mutant Tcf7l2 proteins at clonal density was measured by using the Cell-Titer Glo assay 6 days after transfection. *P* values are provided for each experiment. (*C*) Expression of Tcf7l2 WT and mutant proteins analyzed by Western blot analysis in HEK293T cells. (*D*) Dose-dependent effects on pathway response with overexpression of Tcf7 and Tcf7l2. Transfection of Tcf7 and Tcf7l2 (E form) DNA resulted in a respective increase and decrease of STF activity in either HeLa (with Wnt3A expression) or DLD-1 cells. (*E*) Effects of CRC mutations on Tcf7l2 repressor function. Overexpressed Tcf7l2 and mutant 3 but not mutants 1, 2, or 4 suppress STF activity in HeLa cells.

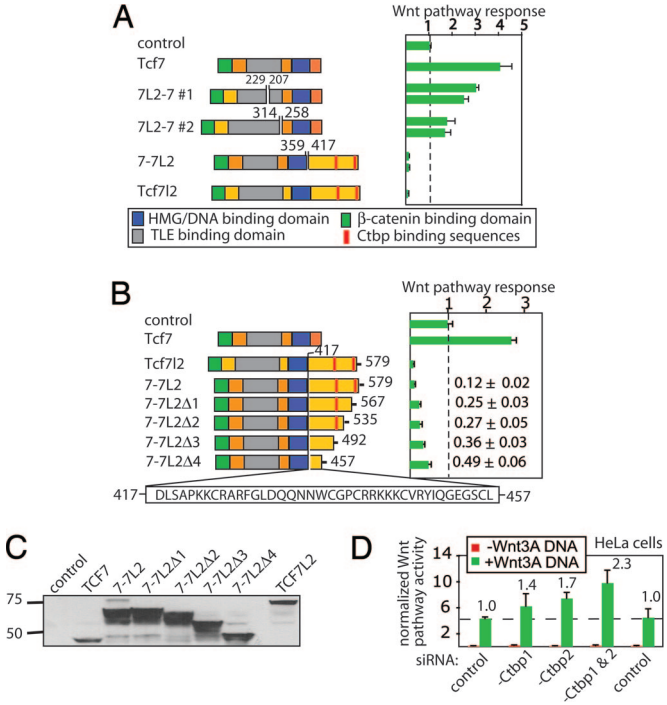

Fig. 3. A repressor sequence in Tcf7l2. (*A*) C-terminal sequences in Tcf7l2 confer repressor function on Tcf7. C-terminal sequences containing the Ctbp binding sites (7–7L2) but not sequences containing the TLE binding domain (7L2–7 #1 and 7L2–7 #2) from Tcf7l2 fused to Tcf7 convert Tcf7 from a transcriptional activator to a repressor as measured by using the STF reporter. In some cases, two separate constructs were tested. (*B*) Protein sequence in addition to Ctbp binding sites contributes to Tcf7l2 repressor function. The effects of serial C-terminal deletions in a Tcf7/7L2 fusion molecule that exhibits pathway repressor activity (7–7L2) were measured in HeLa cells, using the STF reporter. Residue numbering is based on Tcf7l2 sequence. Sequence of the novel repressor domain is shown. Mean of triplicate experiments ± SD are shown. (*C*) Expression of Tcf7 and Tcf7l2 fusion molecules described in *B*. (*D*) Ctbp contributes to repression of STF activity. RNAi of both Ctbp 1 and 2 in HeLa cells results in increased STF activity in response to Wnt protein. The fold increase in pathway response is noted above each triplicate experiment.

Tcf7l2 is insufficient to confer repressor function to the DNA binding sequence of Tcf7 (Fig. 3*A*). However, fusing C-terminal protein sequence from Tcf7l2, which includes the entire E tail, transforms Tcf7 into a repressor of STF reporter activity (7–7L2). A Tcf7 protein that lacks the C-terminal sequence deleted in the Tcf7-Tcf7l2 fusion molecules exhibits WT levels of transcriptional activity (data not shown), arguing against the inadvertent deletion of transcriptional activation determinants in these fusion molecules. Although deletion of both Ctbp binding sites decreased the repressor capability of 7–7L2 (7–7L2Δ 3 and 7–7L2Δ 4), a remaining 41-aa sequence (amino acids 417–457) provided by Tcf7l2 was sufficient to confer some repressor ability on Tcf7 (Fig. 3 *B* and *C*). Interestingly, the sequence we have identified encompasses a DNA binding domain (the "cysteine clamp"), which functions with the HMG domain to confer target gene specificity to TCF family members (21) and is frequently altered in colon cancers with microsatellite-instability (22). Nevertheless, targeting of both *Ctbp1* and *2* with RNAi resulted in an increase in cellular responsiveness to Wnt3A consistent with previous observations that the Ctbp binding sequences in Tcf7l2 contribute to its repressor function (Fig. 3*D*). Thus, we conclude that the cysteine clamp sequence in additional to other protein determinants in Tcf7l2 contribute to its repressor function.

The Functions of Tcf7l2 and Tcf7 in the Wnt Pathway Are Cell-Type Specific. Our genetically based interrogation of Tcf7l2 and Tcf7 function in a variety of human cell lines suggests that these transcriptional effectors often have contrasting regulatory roles within the Wnt pathway (Fig. 4*A*). In the case of Tcf7l2, its transcriptional repressor function in human CRC cells appears to differ from that of an activator as described in the mouse small intestinal tissue (4). We investigated whether or not the basis for this difference is species specificity in Tcf7l2 function. We observed contrasting roles for Tcf7l2 in two different mouse cell lines suggesting that the function of Tcf7l2 is cell-type but not necessarily species specific (Fig. 4*B*). Consistent with the requirement for the E tail in Tcf7l2-mediated repressor function, the predominant form of Tcf7l2 expressed in L-cells is likely the "B" form that lacks this sequence (Fig. 4*C*). The biochemical and genetic evidence presented here supports a repressor role of Tcf7l2 that is not only dictated by the presence of an E tail sequence but likely the presence of effector(s) that act on this sequence. Our assignment of Tcf7 as positive transcriptional regulator of Wnt/β-catenin pathway response in CRC cells is likewise different from its assigned role as a tumor suppressor (16). In contrast to the mouse small intestinal epithelium, where Tcf7 is expressed as a protein that lacks the β-catenin binding domain (16), the major isoform of Tcf7 expressed in several human cell lines appears to contain this domain, consistent with its positive transcriptional role in these cells (Fig. 4 *D* and *E*).

Tcf7l2 Repressor and Positively Acting TCF/LEF Family Members Control Distinct Transcriptional Programs in CRC. Our analysis of Tcf7l2 function, using the STF reporter, suggests that the increase in DLD-1 cell growth upon loss of Tcf7l2 may be attributable to the activation of a growth-promoting transcriptional program. We first used genome-wide expression analysis to identify potential Tcf7l2 target genes (Fig. 5*A*). Next, we determined whether our candidate Tcf7l2 target genes might also be controlled by positively acting TCF family members by comparing expression profiling data from the cell lines lacking Tcf7l2 with those lacking β-catenin, an essential coactivator of TCF-mediated transcription (Fig. 5*A*). Although we detected some overlap in these datasets, none of the genes sensitive to both RNAi-based manipulations were previously identified TCF targets. Indeed we were able to observe transcriptional changes of known or predicted TCF target genes, such as Myc and Lef1, only in cells treated with *Ctnnb1* but not *Tcf7l2* siRNAs despite the effectiveness of both sets of siRNA reagents (Fig. 5 *A* and *B*; see Table S6 and Table S7). The absence of known TCF target genes that exhibit altered expression levels upon loss of Tcf7l2 likely reflects the reliance of previous studies on dominant-negative TCF proteins to assign target genes to individual TCF proteins (23). Given the similarities of TCF/LEF family members in DNA-binding specificity, those strategies likely would broadly identify TCF target genes rather those specific to each TCF/LEF family member.

Our gene expression analysis also revealed that transcriptional levels of known pathway components are not affected by loss of *Tcf7l2* (Fig. 5*B*, Table S6, and Table S7). Some of these observations were also confirmed by using Western blot analysis (Fig. 5*C*). Based on these observations, Tcf7l2 likely does not modulate Wnt pathway activity by altering the expression levels of known Wnt pathway components.

We identified among those genes that were up-regulated upon loss of Tcf7l2, two genes that harbor conserved TCF binding enhancer elements in their respective promoters [hepatocyte growth factor (*HGF*) and the homologue of *Drosophila* morphogenetic factor *headcase* (*HECA*)] (Fig. 5 *D* and *E*). However, none of the genes mutually down-regulated upon loss of *Ctnnb1* or *Tcf7l2* are known or predicted TCF target genes (Fig. 5*A*). Interestingly, *HGF* has been shown to promote cell cycle entry in CRC cells (24). Indeed, loss of *Hgf* or *Heca* induced by RNAi abrogated the growth promoting effects of *Tcf7l2* siRNAs on CRC cells (Fig. 5 *F* and *G*). When taken together, our observations suggest that *HGF* and

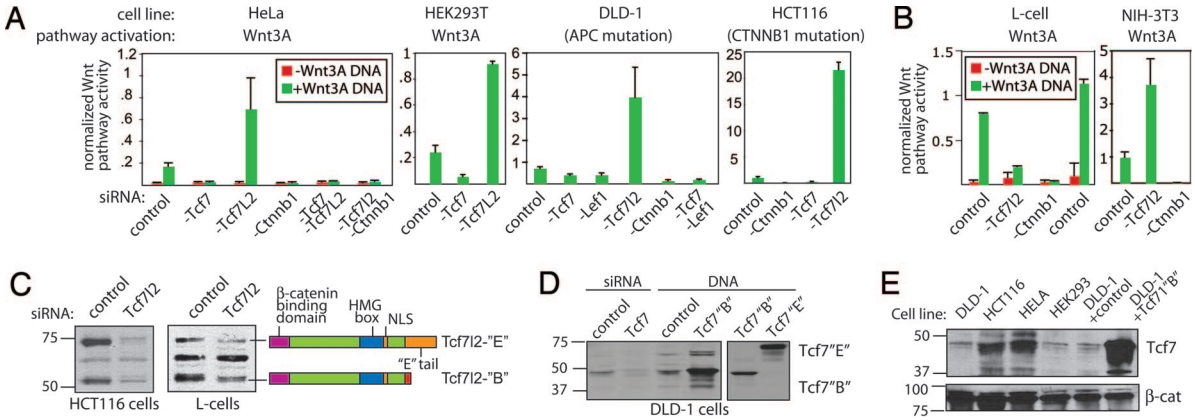

Fig. 4. The function of TCF family members is cell-type-specific. (*A*) Tcf7l2 functions as a transcriptional repressor in many cell types. Effects of decreased *Tcf7l2* function on normal Wnt pathway response were measured by using the STF reporter in HeLa and HEK293T cells expressing Wnt3A. In CRC cells, the pathway is constitutively activated as a result of respective loss- or gain-of-function mutations in *APC* (DLD-1) or *CTNNB1* (HCT116 cells). (*B*) Contrasting transcriptional roles for Tcf7l2 in two different mouse cell lines. A similar experiment described in A but performed in mouse L- or NIH 3T3 cells reveals cell-type-specific functions for Tcf7l2. (*C*) The predominantly expressed forms of Tcf7l2 in CRC cells and L-cells as detected by Western blot analysis in HCT116 cells are respectively the E variant (containing Ctbp binding and the novel repressor sequences) and presumably the B variant (which lacks these repressor sequences). (*D*) Expression of Tcf7 in DLD-1 cells. Tcf7 is predominantly expressed as an isoform that comigrates on SDS/PAGE with the B form that contains a β-catenin binding domain. (*E*) Expression of the Tcf7 B form in different human cells lines detected by Western blot analysis.

HECA are a part of a cellular program controlled by Tcf7l2 that regulates CRC cell growth.

Discussion

Our assignment of *TCF7L2* as a tumor suppressor is based on the following: (*i*) the strong genetic evidence that aberrant Wnt pathway activity is causal to CRC, (*ii*) loss of Tcf7l2 function enhances, whereas gain of Tcf7l2 function suppresses CRC cell growth, and (*iii*) CRC mutations identified in primary tumor samples result in the production of Tcf7l2 proteins that are unable to function as transcriptional repressors of cell growth-promoting genes, such as *HGF* and *HECA*. The roles for *TCF7L2* that we have uncovered in CRCs are different from those described in previous studies. These discrepancies are likely due to the reliance of these prior studies on the use of dominant-negative TCF proteins in lieu of loss-of-function strategies, and possibly the interpretation of phenotypic outcomes of genetically based studies in model organisms. Expression of dominant-negative TCF proteins (proteins lacking β-catenin binding domains) likely suppresses TCF/LEF target gene transcription and growth of CRC cells regardless of their normal cellular

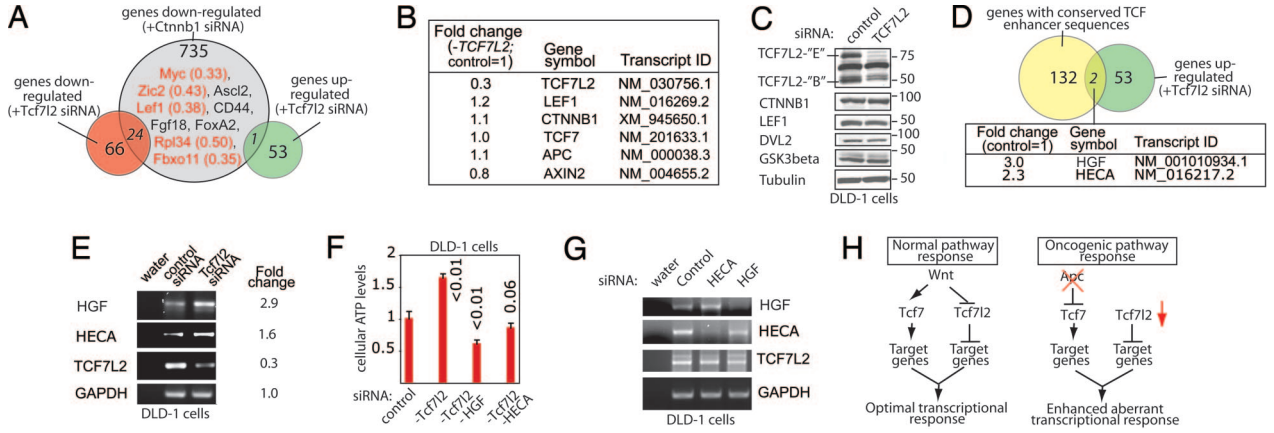

Fig. 5. The cellular program controlled by Tcf7l2 in CRC cells. (*A*) Positively acting TCF molecules and Tcf7l2 repressor control distinct target genes. Gene expression profiling identified genes regulated by positively acting TCF molecules in *Ctnnb1* siRNA-treated cells (gray circle) that were also sensitive to Tcf7l2 loss (red, green circles). Both known [23; see also the Wnt homepage (www.stanford.edu/~rnusse/wntwindow.html)] and predicted (31) TCF target genes were identified by using *Ctnnb1* siRNAs (fraction of control expression shown in parenthesis for some genes; all data are shown in Table S6 and Table S7). However, the expression of these genes was mostly unchanged with loss of Tcf7l2. (*B*) Transcription of several known Wnt pathway components are not altered with loss of Tcf7l2 as determined by gene expression profiling. (*C*) Protein levels of several Wnt pathway components are not altered upon loss of Tcf7l2 as determined by Western blot analysis. Note that cell treatments in *A–C* were simultaneously performed by using identical conditions. (*D*) Identification of genes controlled by Tcf7l2 repressor function in CRC. Genes up-regulated >2-fold upon loss of Tcf7l2 (green) were identified by using expression profiling in DLD-1 cells. Of the genes within this dataset, the HGF and HECA have predicted TCF enhancer sequences in their promoter regions as identified from an *in silico* search for TCF target genes (31). Fold change is an average of results from duplicate microarray experiments. (*E*) Changes in the expression of HGF, HECA, and Tcf7l2, upon introduction of *Tcf7l2* siRNAs in DLD-1 cells, were confirmed by using RT-PCR. (*F*) Loss of HGF or HECA abrogate the cell growth-promoting effects of *Tcf7l2* RNAi. Simultaneous treatment of cells with *Hgf* or *Heca* and *Tcf7l2* siRNAs abrogates the increase in DLD-1 cell growth induced by loss of Tcf7l2 alone as determined by measuring cellular ATP levels. *P* values for each experiment are shown. (*G*) RT-PCR analysis of HGF and HECA expression levels after siRNA treatment. (*H*) Models of normal and aberrant activation of Wnt/β-catenin pathway response in cells that harbor Tcf7l2 repressor function. Optimal induction of Wnt-mediated pathway response in such cells entails simultaneous activation of Tcf7 and inactivation of Tcf7l2. In CRC cells, oncogenic Wnt pathway activity is initiated in most cases by homozygous loss of *APC* function. Additional loss of *TCF7L2* function likely results in greater tumorigenicity.

functions (9). Also, mice lacking *TCF7L2* are able to establish stem cells in the small intestines but cannot maintain them, a defect presumably caused by the loss in expression of Wnt target genes that sustain stem cell self-renewal (4). Based on our observations and recent evidence suggesting that hyperactivation of the Wnt pathway results in loss of stem cells in many tissues (25, 26), the defects observed in *TCF7L2* mice could also be caused by stem cell depletion due to de-repression of *TCF7L2* target genes. Alternatively, our observations could be explained by differences in the function of TCF molecules in the mouse small intestines and human colon. Indeed, mice lacking *TCF7L2* exhibit a loss of homeostatic renewal in the small intestinal epithelium but not in the colon, where most sporadic cancers of the human gastrointestinal tract arise (27).

Using our loss-of-function strategy, we have identified at least two growth-promoting genes that are negatively regulated by Tcf7l2 in CRC cells, *HGF* and *HECA*. Increases of either gene product in the serum (28) or feces (29) of CRC patients correlate with poor prognosis. Our findings predict that chemical inhibitors that inhibit the receptor of HGF, the Met receptor kinase, might be particularly useful in tumors with decreased Tcf7l2 function. Interestingly, HGF is known to activate the Wnt pathway by binding to its receptor (the proto-oncogene Met), which in turn induces β-catenin phosphorylation and nuclear localization (24). Thus, long-lasting consequences of Tcf7l2 loss may also include transcriptional activation of Wnt/β-catenin pathway target genes controlled by positively acting TCF molecules.

In light of our findings, the simplest model for normal Wnt/β-catenin response in cells that harbor Tcf7l2 repressor activity is that simultaneous activation of Tcf7 and inactivation of Tcf7l2 proteins is necessary for optimal transcriptional responses to Wnt proteins (Fig. 5*H*). Although it is unclear at the moment how Wnt proteins might regulate the Tcf7l2 repressor protein, Nemo-like kinase (NLK) may play a role in this regard, because it appears to be a positive mediator of pathway response in at least two cell lines (Table 1) and phosphorylates a consensus sequence found in Tcf7l2 and not Tcf7 (20). In CRC, loss of *APC* and decreased *TCF7L2* function (the mutations analyzed were heterozygous) (Fig. S6) activate two growth-promoting transcriptional programs that result in tumors with more aggressive behavior than that observed in those with only *APC* mutations (Fig. 5*H*). Augmentation of Tcf7l2 function or targeting of growth-promoting genes regulated by Tcf7l2 might be therapeutically useful in CRC and possibly breast cancers, which frequently display decreased expression of Tcf7l2 (30).

Although important cell fate determination pathways such as the Wnt/β-catenin pathway are intensely studied, a molecular understanding of their deviant activity in cancer remains incomplete. Further analysis of these pathways, using genome-scale RNAi coupled with data derived from cancer genome sequencing efforts, should allow rapid identification of candidate cancer genes in a broad range of cancers. Ultimately, this integrated approach should improve our ability to realize mechanism-based therapeutic strategies and to personalize treatment regiments based on detailed knowledge of a tumor's molecular underpinnings.

Materials and Methods

Primary screen in HeLa cells. Approximately 12,000 HeLa cells were plated in 96-well culture plates that contained Effectene (Qiagen) transfection mixes, using all or some the following reagents: (*i*) control or Wnt3A expression construct; (*ii*) siRNAs; and (*iii*) STF, GL, and RL control reporter constructs. Each well received 3 pmol of siRNA. Two days after transfection, culture medium (for GL activity) and lysate (for FL and RL activity) were collected and analyzed for relevant luciferase activities, using either Dual Luciferase kits (Promega) or coelenterazine. The average Wnt induced signal in each screening run (≈800 siRNA pools tested) was at least 20-fold above negative controls. See *SI Materials and Methods* for criteria used to identify hits.

Secondary Screens. Secondary screening assays were performed essentially as described above except in a 48-well format, using Effectene transfection reagent and a 3-day posttransfection incubation period either in duplicate or triplicate. For assays involving conditioned medium, cells were transfected with siRNAs 48 h before exchanging normal for medium conditioned from cells producing Wnt3A or control cells. Cut-offs used to identify hits were based on percentage pathway response compared with controls indicated in Fig. 1*B* and Table S2. For cell growth assays, DLD-1 or HeLa cells were transfected in 96-well format and split two days later to clonal density in 10-cm^2 plates (≈12,000 cells). Four days later, cells were trypsin harvested and cellular ATP levels measured by using Cell Titer Glo assay (Promega).

Cell Lines, siRNA Libraries, DNA Constructs, Antibodies, Gene Expression Profiling, and RT-PCR. See *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Bert Vogelstein for sharing data before publication; Woodring Wright, Andras Roig, Leanne Jones, Rueyling Lin, and members of the L.L. laboratory for useful discussions; Randall Moon, Jorge Laborda, Raphael Kopan, Gail Johnson, and Marian Waterman for constructs; and Leni Jacob and Baozhi Chen for technical assistance. This work was supported by the National Institutes of Health, the American Cancer Society, the Welch Foundation, and an endowment from Virginia Murchison Linthicum.

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Supporting Information

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SI Materials and Methods

Primary Screen in HeLa Cells (Continued). To facilitate identification of interesting genes from the positive and negative regulator screens, three different algorithms were used (one for each screen) that take into consideration the total number of hits to be retested (≈500 combined from all screens), the strength of triplicate experiments, and our ability to identify known pathway components. Thus, the number of hits to be considered for further analysis is based on our ability to identify landmark genes, the reproducibility of results, and practical considerations.

To define hits from the positive regulator screen, a standard score centered on the population mean of each screening run was assigned to the average FL/RL ratio of each triplicate experiment minus the SD (z′ score). A siRNA that generated a FL/RL with z'<-2 was considered for secondary tests, which corresponds to hits with a P value equal or <0.13 based on automated test runs (Fig. S2). We removed from consideration siRNAs that induced changes in either RL or GL/RL [z<-1.75 for RL and z<-1.5 for GL/RL]. Reduction of RL or GL/RL activities indicated compromised cellular functions [exocytosis (GL) or protein synthesis (GL or RL), for example] either through siRNA-mediated off-targeting or specific targeting of housekeeping genes necessary for either (see Table S2 and Table S3). Transfecting a high ratio of RL to GL reporter DNA (10:1) ensured that accumulation of GL activity in cellular lysate because of loss of protein secretion/exocytosis does not confound analysis of the cytoplasmically expressed RL as both RL and GL are measured by using the same substrate. Because of an "edge effect" observed with the GL reporter in some plates, all potential GL hits that also scored as Wnt pathway hits localized to these wells were considered for secondary testing (see Table S2 and Fig. S2). Because siRNAs targeting ribosomal genes would have predictable effects on protein synthesis (1), we also eliminated these from further consideration. The SD of each triplicate experiment in the positive regulator screen was considered in two ways. First, the use of the z' score allows consideration of the SD and not only the average of each triplicate experiment. Second, only those triplicate experiments in which the SD was less than the average SD of triplicates in each screening run were considered (Table S2). With some exceptions, this is approximately equivalent to a SD = 0.2 for a control of value = 1. Overall, ≈1.8% of the siRNAs in the library were considered further as potential positive regulators of pathway.

In the screen for pathway suppressors (minus Wnt), siRNAs assigned a z >4 were considered for secondary testing. This cut-off would identify hits with a P value equal or <0.089 based on pilot automated control studies (Fig. S2). The SD of each experiment was not considered for this screen because of low FL signal that resulted in high SDs. Thus, all averaged values that were equal to or exceeded the cut-off were considered for secondary analysis. Because of the low transfection efficiency in mouse NIH 3T3 cells and the weak basal levels of STF-derived reporter activity, we were unable to establish a reliable assay for testing candidates from the suppressor screen (−Wnt) in these cells. To identify pathway suppressors in the presence of Wnt, all siRNAs associated with a z >5 were considered for secondary analysis. Hits identified in this manner have a P value of 0.0013 or less (Fig. S2). Only those experiments with a SD <30% of the average were considered for secondary testing. In total, ≈0.7% of the siRNAs in the Dharmacon library targeting potentially pathway suppressors were further considered (Table S3 and Table S4).

Mapping Studies. For studies aimed at mapping the function of candidate pathway components, we considered the ratio of pathway response levels measured by induction of pathway either by Lrp6/Ck1γ or β-catenin over-expression, and Wnt3A in the presence of siRNAs against gene candidates. Ratios >2 formed the basis for assigning gene function either upstream or at the level of *Lrp6/Ck1γ*. Ratios <2 were an indication that the gene functioned downstream of *Lrp6/Ck1γ* or downstream/at the level of *Ctnnb1*.

Cell Lines, siRNA Libraries, DNA Constructs, and Antibodies. NIH 3T3 and L-cells were purchased from ATCC. HeLa cells were provided by Phil Beachy. All siRNAs used in primary screens and secondary tests were either from the Dharmacon Human siArray siRNA library or the Qiagen Human Whole Genome siRNA Set Version 1.0, Human Predictive Genome siRNA Set v1.0, Mouse Druggable Genome siRNA Library v1.0, Mouse Whole Genome Supplement siRNA Library, Version 1.0, Mouse Predictive Genome siRNA Set, Version 1.0, siRNA libraries. In all libraries, four siRNAs targeting one gene were pooled in a single well of a 96-well plate. Control siRNAs used in experiments were purchased from Dharmacon (catalog no. D-001210-01-05).

SuperTopFlash reporter is described in ref. 2. Expression constructs were either purchased from Mammalian Genome Collection (MGC) cDNAs from ATCC or Openbiosystems, or Origene. In some cases, expression constructs were generated by inserting cDNAs into pcDNA3.1. Lrp5-myc and Lrp6-myc constructs were provided by G.V.W. Johnson. Expression constructs encoding mutant Tcf7l2 and Tcf7l2-Tcf7 fusion proteins were engineered by using PCR-based mutagenesis. For the 7/7L2 fusion construct, Tcf7 and Tcf7l2 protein sequences were aligned. The point of sequence divergence was identified and used as the point of fusion. The form of Tcf7 used in reporter studies encodes the "B" isoform. Tcf7 "E" form was provided by M. Waterman. CMV-*Gaussia* luciferase was constructed by insertion of the CMV promoter into the pGluc Basic Vector (New England Biolabs). SuperTopFlash/FlopFlash vectors were provided by Randall Moon. The following are the antibodies used in this study and their source: Anti-β-COP (ABR), anti-β-catenin polyclonal and anti-β-tubulin antibody (Sigma), anti-Tcf7 monoclonal (2206S; Cell Signaling Technology; recognizes a sequence adjacent to the HMG domain), and anti-Tcf7l2 monoclonal antibody (Upstate). Notch assays were performed by using a FL reporter construct containing tandem CBF binding sequences (provided by J. Laborda) and an activated Notch protein construct (3).

Gene Expression Profiling. DLD-1 cells were transfected in duplicate using Effectene (Qiagen) with 100 pmol of control or Tcf7l2 siRNA pools (Dharmacon) for 3 days in 6-well dishes. Total RNA was then isolated by using RNeasy Mini kits (Qiagen) and biotinylated cRNA generated by using the Ambion Illumina RNA Amplification kit. cRNA probes were applied to Illumina Human-6 BeadChips containing ≈48K probes. Specific protocol details can be found at (http://microarray.swmed.edu/protocols/illumina.htm). A detection threshold of 0.99 (false detection rate of 1% or lower) was applied to datasets of samples derived from DLD-1 cells treated with control, *Tcf7l2*, or *Ctnnb1* siRNAs

samples using Beadstudio software. The average of duplicate measurements in each dataset for which the average detection threshold was acceptable (Table S6 and Table S7) was used to calculate Tcf7l2- or Ctnnb1-dependent changes in gene expression. Raw data are provided in Table S6 and Table S7. Expression levels of a few genes were also determined by using RT-PCR using the same cDNA samples applied to the Illumina chips for general assessment of the microarray data.

RT-PCR. RT-PCR was performed by using a modified TRIzol RNA extraction method, a cDNA generation kit (New England Biolabs), and standard PCR methods that incorporated the following primers: TCF7L2 Exon 1, 5′-CGGTGGAGGGGATGACCTAG (forward); 5′-CTCGGAATCGGAGGAGCTGT (reverse); HGF, 5′-GGACGCAGCTACAAGGGAACAG (forward), 5′-GGTCCCCCTTCTTCCCCTCGAG (reverse); HECA, 5′-GGAAGGGGTTCACAAGATCA (forward), 5′-GACACTGCTGGACGTTGCTA (reverse); and GAPDH Forward: 5′-GAAGGTGAAGGTCGGAGTC (forward), 5′-GAAGATGGTGATGGGATTTC (reverse).

Quantification of signal intensity was performed by using ImageJ (Version 1.37) software. Briefly, minimum brightness thresholds were increased to remove background signal. Remaining bands were bracketed, plot profiles generated, and area under histograms autotraced.

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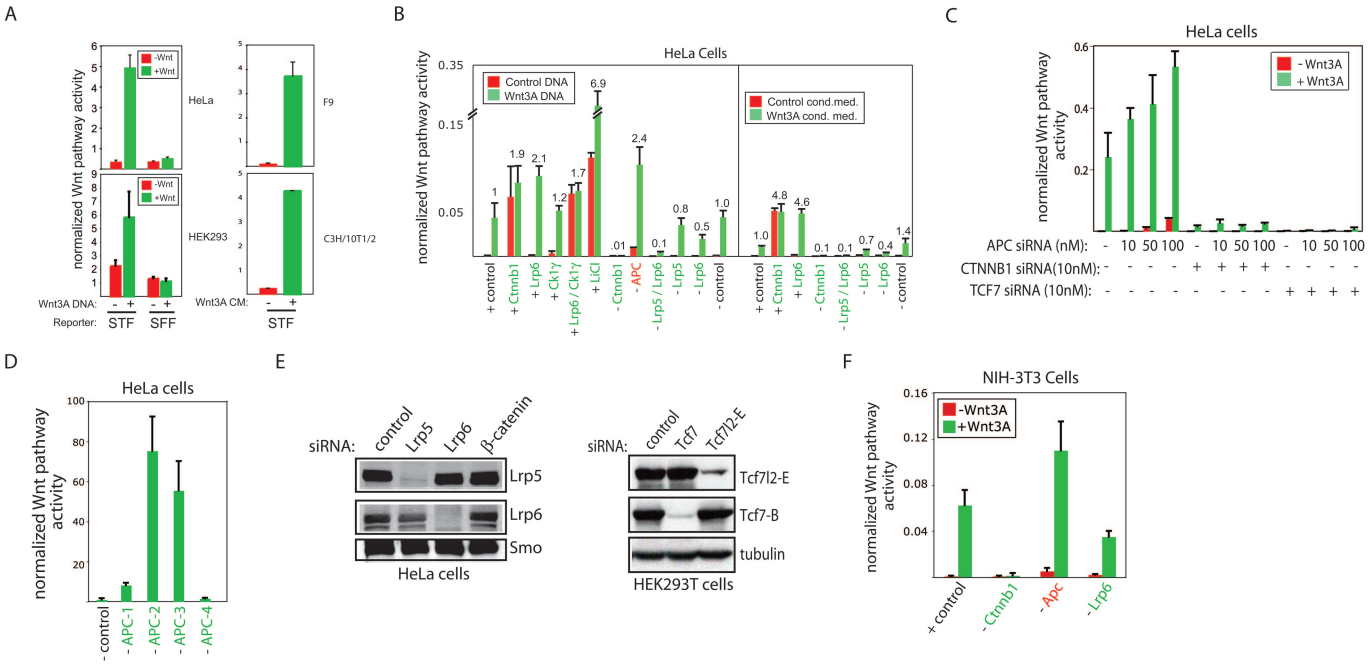
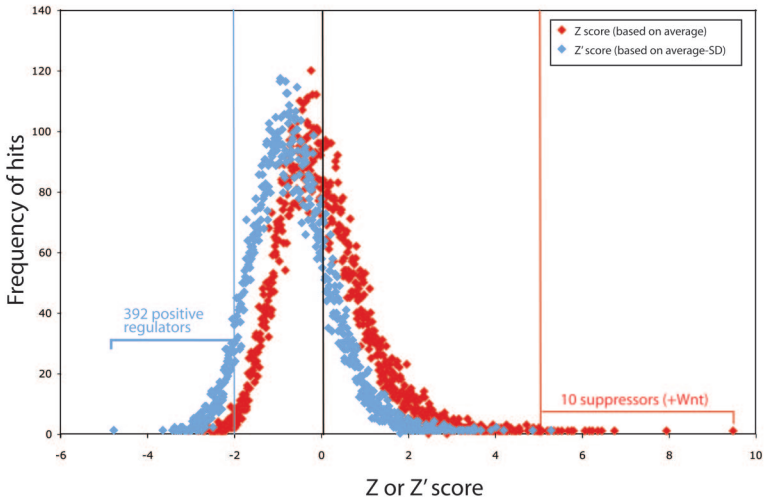


Fig. S1. Characterization of cultured cell-based assays for Wnt/β-catenin pathway response and siRNA reagents. (*A*) Testing of various mammalian cell lines for Wnt/β-catenin pathway response and specificity of the STF reporter. STF or control SuperFopFlash (SFF) reporter, the SV40-RL reporter, and Wnt3A expression construct were transfected into F9, C3H/10T1/2, HeLa, or Hek293T cells. Wnt pathway response is represented as FL activity normalized to RL activity. In both HEK293 and HeLa cells, STF activity is several fold that of SFF activity in the presence of Wnt3A. Data represented as mean ± SD ($n = 3$). (*B*) HeLa cells mediate faithful transcriptional response to Wnt3A protein. HeLa cells were transfected with (*i*) siRNAs or cDNAs that would be predicted to alter functional levels of known Wnt pathway components (respectively designated with ''−'' or ''+'' before gene name), (*ii*) Wnt-responsive (STF) and control RL reporter constructs (see Fig. 1B), and (*iii*) control or Wnt3A expression construct. Two days later, cells were lysed, FL and RL activities measured, and FL normalized to RL activity. Consistent with the genetics, RNAi directed against pathway suppressors such as *Apc* or activators (*Ctnnb1*, *Lrp5*, *Lrp6*) respectively resulted in either predicted gain or loss of pathway activity. Simultaneous suppression of *Lrp5/6* had an additive effect on pathway response consistent with redundant functions in these cells. Addition of LiCl (20 mM), an inhibitor of glycogen synthase kinase 3β (Gsk3β)) activated pathway response by blocking constitutive Gsk3β-mediated phosphorylation and subsequent destruction of β-catenin. Fold induction relative to control in the presence of Wnt is shown above each experimental group. *Left*: cells transfected with control or Wnt3A expression construct. *Right*: cells treated with either control or Wnt3A-containing conditioned medium. Data represented as mean ± SD ($n = 3$). (*C*) To further demonstrate the validity and robustness of our cell-based assay, we used simultaneous targeting of APC, a suppressor of pathway, using increasing amounts of siRNA with constant levels of either *Ctnnb1* or *Tcf7* siRNAs. Regardless of the ratio of APC to *Ctnnb1* or *Tcf7* siRNA introduced, the results faithfully map Ctnnb1 and Tcf7 downstream of APC function. (*D*) Testing individual siRNAs from a pool targeting APC. Two of four siRNAs induce robust pathway activation suggesting the activity of individual siRNAs in each pool targeting a single gene will vary. (*E*) Testing the specificity of several siRNA pools from Dharmacon-produced libraries. Lysates from HeLa or HEK293T cells cotransfected with expression constructs encoding either Lrp5, Lrp6, Tcf7l2 (''E'' from), Tcf7 (''B'' form) or Smo, the transmembrane effector in the Hedgehog pathway, and indicated siRNAs pools from Dharmacon were subjected to Western blot analysis using antibodies specific for a given protein (Tcf7l2, Tcf7, β-catenin, and tubulin) or an anti-Myc epitope monoclonal (Lrp5, Lrp6, and Smo). (*F*) NIH 3T3 cells faithfully respond to Wnt3A protein. Reporter assays involving the indicated siRNAs were performed in NIH 3T3 cells essentially as described above for HeLa cells. All siRNAs had predicted affects on Wnt pathway response in these cells. Data represented as mean ± SD ($n = 3$).

A



B

Positive regulator and Suppressor (+Wnt) screens:

Pilot screen results using 80 control siRNAs:
Mean (FL normalized to RL) = 1
SD= 0.24

Calculated P values for hits from primary screen:
P (z=2; positive regulator screen) = 0.13
P (z=5; suppressor screen; +Wnt) = .0016

Suppressor (-Wnt) screen:

Pilot screen results using 80 control siRNAs:
Mean (FL counts) = 1
SD = 0.75

Calculated P value for hits from primary screen:
P (z=4) = .083

C

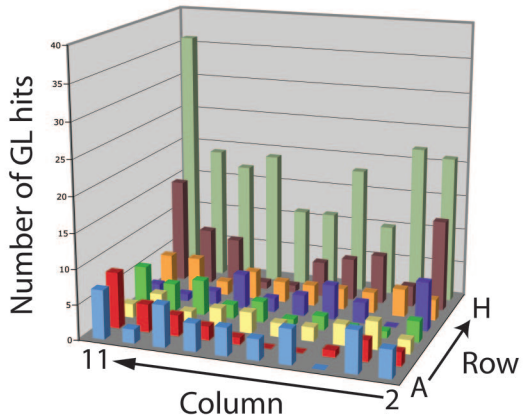


Fig. S2. Overview of screening results. (*A*) Distribution of hits. The frequency of outcomes in the positive regulator and suppressor (+Wnt) screens are plotted based on either z (derived from the mean) or z′ scores (derived from the mean-SD). The number of hits considered for secondary analyses after considering the strength of the triplicate (see Methods) and removal of genes involved in ribosomal regulation are shown. (*B*) Calculation of P values associated with the z scores from the primary screens. P values were calculated from pilot studies using 80 control siRNAs under conditions identical to those used in the primary screens. The high SD associated with the suppressor screen (-Wnt) is due to the nearly background levels of STF reporter signal in the absence of Wnt pathway activity. (*C*) An edge effect associated with the GL reporter. Distribution of hits identified from the use of the GL reporter in a 96-well screening plate are shown. Columns 1 and 12 were not used in the screen. All Wnt pathway hits that also registered as a GL hit in Row H and wells positioned in G_2 and G_{11} were considered for retesting to counter this edge effect.

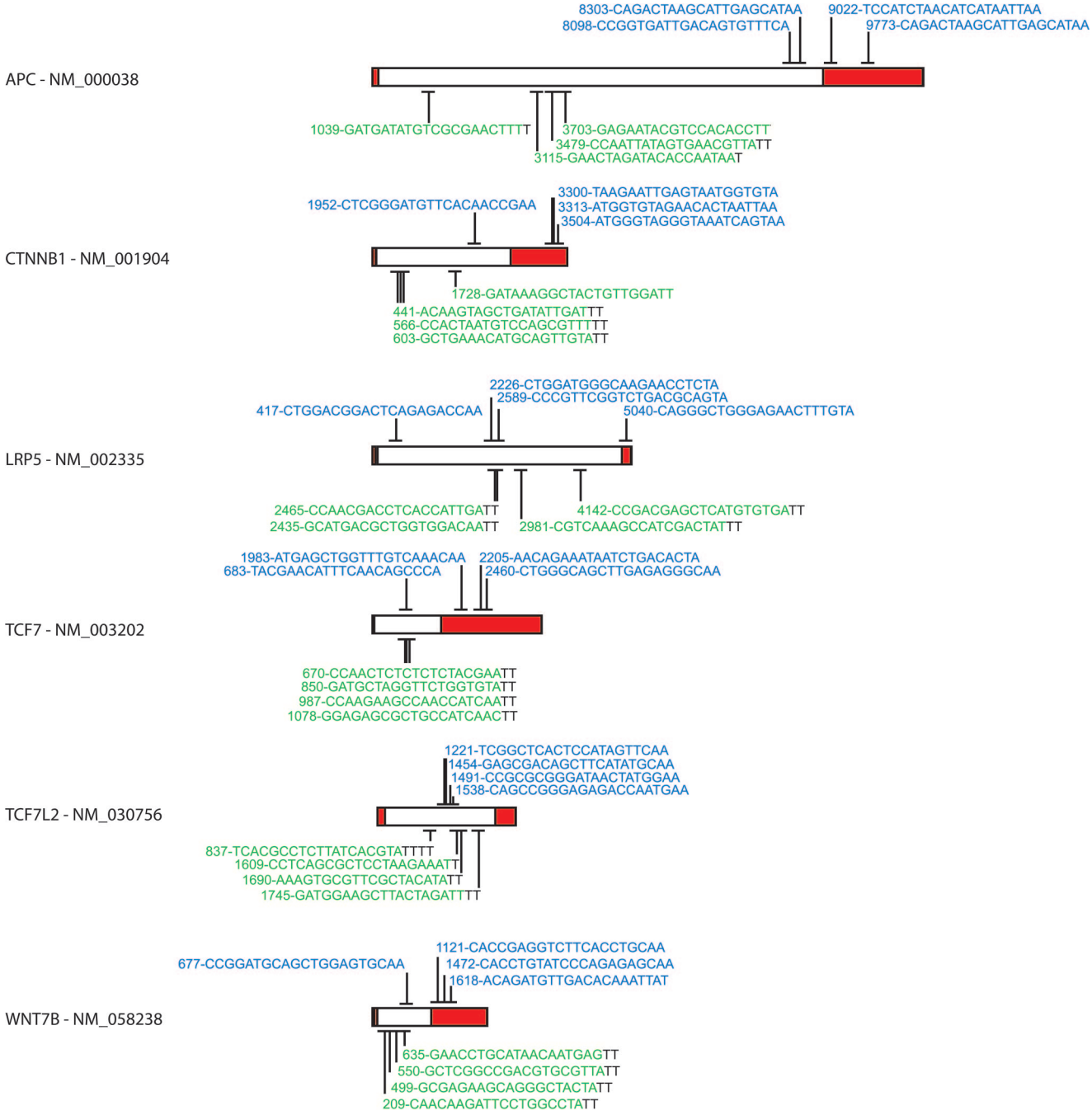


Fig. S3. Comparison of sequences targeted by Dharmacon- and Qiagen-designed siRNAs. Sequences targeted by siRNAs produced by Dharmacon (green) or Qiagen (blue) for several Wnt pathway components were compared (black indicates nucleotides in siRNAs that do not complement target gene sequence). No two siRNAs targeting the same gene share the same sequence suggesting that overall these libraries largely target the same genes using nonredundant sequences. The predominance of siRNAs targeting 3′UTR sequences (red) from the Qiagen but not the Dharmacon library partly accounts for this nonredundancy. Transcript sequences deposited at National Center for Biotechnology Information and used for alignments are noted next to gene name.

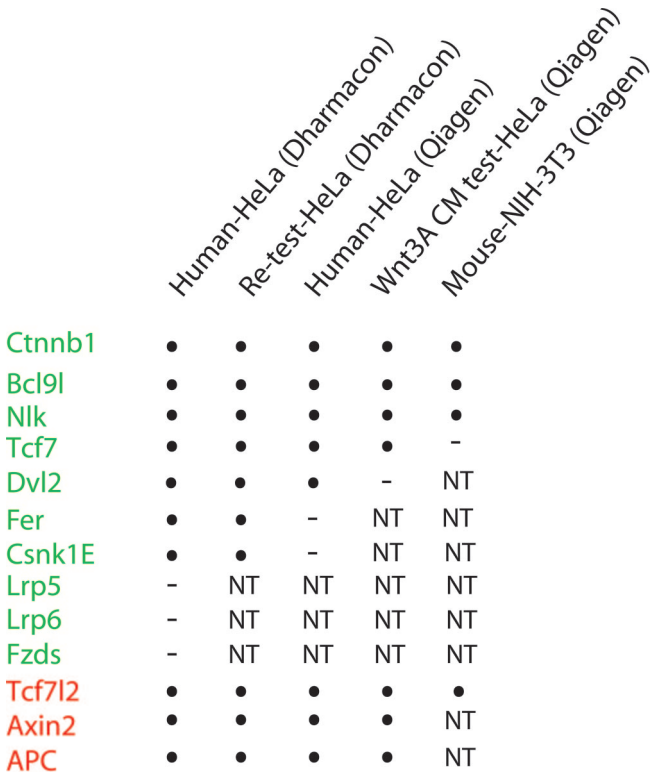


Fig. S4. Summary of screening data for several known Wnt/β-catenin pathway components. ●= pass; ''-'' = failed, NT = not tested.

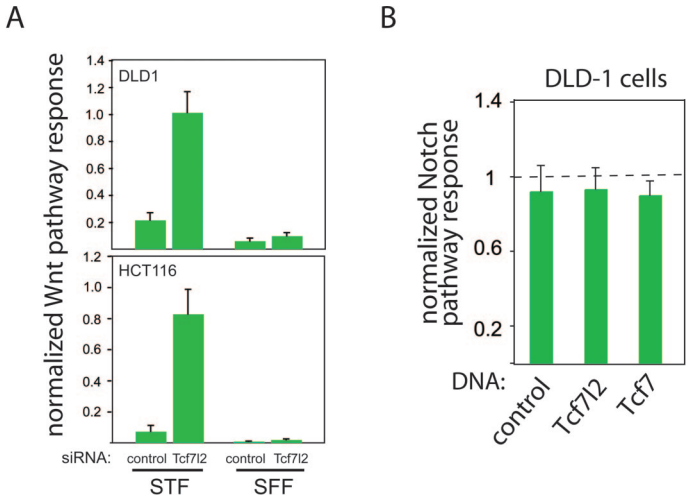


Fig. S5. Reporter controls in CRC cells. (*A*) SuperTopFlash (STF) or control SuperFopFlash (SFF) reporter with mutated TCF binding sites, were transfected with SV40-RL reporter into DLD-1 or HCT116 cells with or without *Tcf7l2* siRNAs. Wnt pathway response is represented as FL activity normalized to RL activity. In both cell lines, STF activity is several fold that of SFF activity. In the presence or absence of *Tcf7l2* siRNA, activity of STF is greatly augmented whereas that of SFF remains nearly unchanged. Data represented as mean ± SD ($n = 3$). (*B*) The cell growth effects of expressing either Tcf7l2 or Tcf7 proteins are likely specific to transcriptional modulation of TCF target genes. DLD-1 cells transfected with an activated Notch construct and Notch responsive reporter (see Experimental Procedures), and either Tcf7l2 or Tcf7 constructs do not exhibit altered Notch pathway activity.

Tumor Name	Nucleotide (genomic)	Mutant ID	
Cx014	g.chr10:114902178C>T:397R>R/W	mutant 4	Wood et al. Science 2007
Hx031	g.chr10:114902178C>T:397R>R/W	mutant 4	
Hx103	g.chr10:114890936_114890937het_insT		
Hx114	g.chr10:114700540het_delT	mutant 2	
Hx139	g.chr10:114700575G>T:24E>E/X	mutant 1	
Hx164	g.chr10:114901527A>T:329K>K/I		
Co82	g.chr10:114907768A>G: IVS10-2A>G; splice acceptor		Sjoblom et al. Science 2006
Hx172	g.chr10:114907769G>C (homozygous): IVS10-1G>C; splice acceptor		
Mx41	g.chr10:114915323C>T: 465R>R/C	mutant 3	

Fig. S6. Tcf7l2 mutations identified in CRC. Mutations are based on (1, 2). Note that the IVS10 mutations correlate with IVS11 sequence in the annotation by (2). IVS = intronic sequence.

1. Sjoblom T, *et al.* (2006) The consensus coding sequences of human breast and colorectal cancers. *Science* 314:268–274.
2. Wood LD, *et al.* (2007) The genomic landscapes of human breast and colorectal cancers. *Science* 318:1108–1113.
3. Duval A, *et al.* (2000) The human T-cell transcription factor-4 gene: Structure, extensive characterization of alternative splicings, and mutational analysis in colorectal cancer cell lines. *Cancer Res* 60:3872–3879.

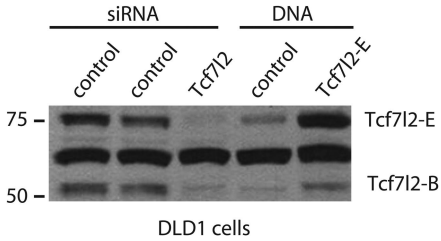


Fig. S7. The predominant form of Tcf7l2 expressed in DLD-1 cells co-migrates with the ''E'' form.

Other Supporting Information Files

Table S1

Table S2

Table S3

Table S4

Table S5

Table S6

Table S7