

New Regulators of Wnt/β-Catenin Signaling Revealed by Integrative Molecular Screening

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The identification and characterization of previously unidentified signal transduction molecules has expanded our understanding of biological systems and facilitated the development of mechanism-based therapeutics. We present a highly validated small interfering RNA (siRNA) screen that functionally annotates the human genome for modulation of the Wnt/β-catenin signal transduction pathway. Merging these functional data with an extensive Wnt/β-catenin protein interaction network produces an integrated physical and functional map of the pathway. The power of this approach is illustrated by the positioning of siRNA screen hits into discrete physical complexes of proteins. Similarly, this approach allows one to filter discoveries made through protein-protein interaction screens for functional contribution to the phenotype of interest. Using this methodology, we characterized AGGF1 as a nuclear chromatin-associated protein that participates in β-catenin-mediated transcription in human colon cancer cells.

INTRODUCTION

Because of its fundamental roles in development and disease, the β-catenin-dependent Wnt signal transduction pathway (Wnt/β-catenin) has been extensively studied in normal and transformed human cells, as well as in model organisms representing diverse classes of metazoans. One specific cell model, the columnar epithelial cells of the gastrointestinal tract, is of considerable interest, as mutations of the adenomatous polyposis coli (APC) gene occur in ~80% of sporadic colon cancers (1, 2). These mutations activate β-catenin signaling, inhibit cellular differentiation, increase cellular proliferation, and ultimately result in the formation of precancerous intestinal polyps (3, 4). The comprehensive identification and mechanistic characterization of proteins that regulate Wnt/β-catenin signaling will contribute to the development of new therapeutics (5).

With the development of genome-scale RNA interference (RNAi) screening technologies, it is now possible to define the functional contribution of nearly every gene in the mammalian genome to specific signaling pathways or cellular processes (6). Although a powerful tool, RNAi screens alone provide little insight into the mechanisms by which specific proteins act. Conversely, by providing protein connectivity relationships, proteomic screening approaches can generate mechanistic insights but do not reveal the functional impact of specific proteins on the phenotype of interest (7). In the present study, we de-

scribe how the integration of protein-protein interaction networks (PINs) and human genome-wide RNAi screens of Wnt/β-catenin signaling identifies previously unknown modulators of the pathway, establishes physical relationships among those modulators, and ultimately facilitates the generation of mechanistic hypotheses.

RESULTS

Genome-scale small interfering RNA screen

We performed a near-saturation genome-wide small interfering RNA (siRNA) screen on the Wnt/β-catenin pathway in human DLD1 colon adenocarcinoma cells (Fig. 1A). These cells harbor inactivating mutations in APC and consequently display constitutively active Wnt/β-catenin signal transduction (8). DLD1 cells were engineered to express a β-catenin-responsive firefly luciferase reporter (BAR) to enable high-throughput measurement of Wnt signal transduction, as well as an EFlα-driven *Renilla* luciferase reporter for normalization purposes (9). Using these cells, we screened 28,124 siRNA pools in triplicate, each consisting of 3 unique siRNAs, targeting 20,042 messenger RNAs (mRNAs) (table S1, Fig. 1B, and Materials and Methods). siRNAs targeting 3% of mRNAs fulfilled the hit-calling criteria of a normalized fold change of greater than 3 or less than 0.33 with a Student's *t*-test *P* value less than 0.01. With this stringent data restriction, the primary screen yielded 740 genes that regulate Wnt/β-catenin signal transduction.

Secondary validation screens

Given that off-target silencing effects are inherent to siRNA screens and can thereby produce high false-positive discovery rates (10, 11), we implemented three validation screens, the first to increase the number of siRNAs tested, the second to eliminate cell type-specific hits, and a third to ensure that the hits were indeed regulators of endogenous β-catenin transcriptional target genes (Fig. 1A). In the first validation screen, we individually tested between three and nine nonoverlapping

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gene-specific siRNAs (Fig. 1B). Of the 740 genes that passed the hit criteria in the primary screen, 268 were confirmed by a minimum of two independent siRNAs, suggesting that these genes were not a result of off-target effects (tables S2 and S3 and Materials and Methods).

In the second validation screen, we broadened the general applicability of our screening hits by eliminating cell line–specific effects. Specifically, we repeated the secondary screen by individually testing three to nine independent siRNAs in SW480 cells, another APC mutant colorectal adenocarcinoma cell line (tables S4 and S5) (12). One hundred and nineteen genes were identified at the intersection of secondary screen data sets for DLD1 cells and SW480 cells (Fig. 1C and table S6). Therefore, of the 28,124 siRNAs tested against the Wnt/β-catenin signal transduction pathway, the secondary screen identified 119 genes that were confirmed with multiple siRNAs in multiple cell lines.

Definition of the colonic Wnt/β-catenin gene signature

In the third validation screen, we used endogenous β-catenin–regulated genes to monitor pathway activity (Fig. 1A). The rationale for this added layer of validation is that the primary and secondary screens used an artificial β-catenin reporter system, which was necessary for the high-throughput nature of the primary and secondary screens but is susceptible to luciferase-based and promoter-based artifacts. Thus, to test whether the hits were true modulators of endogenous β-catenin target genes, it was necessary to define the β-catenin gene signature in colon cancer cells. Using genome-wide complementary DNA (cDNA) microarray expression analyses, we separately profiled five nonoverlapping β-catenin–specific siRNAs in DLD1 cells. Of the 43,675 transcripts measured, 329 were regulated by all five β-catenin siRNAs (Fig. 2B). We next profiled SW480 cells after siRNA-mediated silencing of β-catenin, and

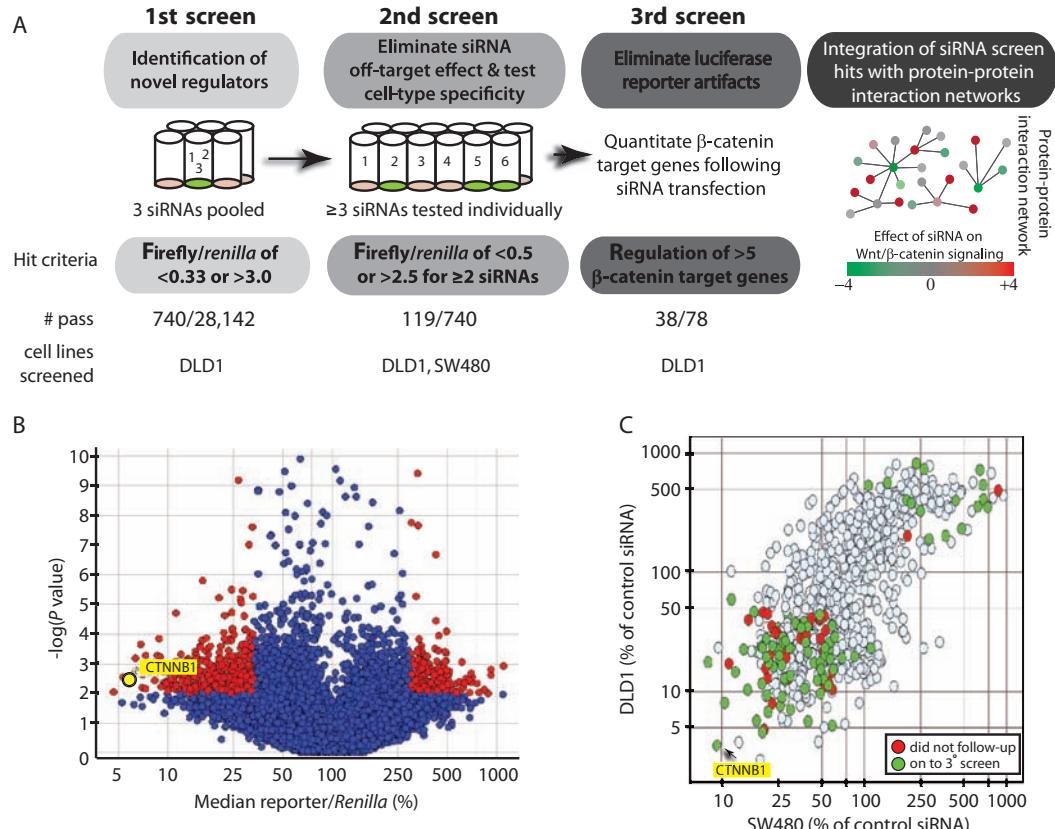
when compared to DLD1 cells, 40 genes were identified in common that define the “colonic β-catenin” gene signature (Fig. 2, C and D).

To validate this signature set, we conducted a time-course analysis and found that 31 of the 40 target genes were regulated within 12 hours of β-catenin silencing, suggesting that most of the transcripts that compose the colonic β-catenin gene signature are directly regulated by β-catenin (Fig. 2D). Given the high frequency of mutational activation of the pathway in colon cancer, we hypothesized that this β-catenin gene signature would discriminate normal tissue from colon tumors (13, 14). Consistent with this hypothesis, unsupervised hierarchical clustering of 69 matched colon tumors and adjacent uninvolved tissues with this gene signature accurately segregated the tumors from 66 of the 69 matched normal samples (Fig. 2E). To illustrate polarity between the colonic β-catenin gene signature and the patient samples, we directly compared the β-catenin gene signature in DLD1 cells after siRNA-mediated silencing of β-catenin to the gene signatures of the patient samples. As expected, the β-catenin activated genes were overexpressed in colon tumors versus normal colon (Fig. 2E, bottom row).

Tertiary validation screen

Using this signature of β-catenin–responsive genes as readout for pathway activity, we next tested whether siRNAs identified in the secondary screen modulated the expression of endogenous β-catenin target genes. To this end, we used a microfluidic real-time polymerase chain reaction (PCR) platform and genome-wide cDNA microarray analysis to quantitate the expression of endogenous β-catenin target genes after siRNA transfection. Of the 119 genes that passed the secondary screen, 77 were tested by quantitative PCR and 13 by cDNA microarray profiling, with one overlap.

Fig. 1. An extensively validated mammalian genome-wide siRNA screen of the Wnt/β-catenin signal transduction pathway. (A) Schematic of the primary, secondary, and tertiary phases of the siRNA screen and integration of the data into a global protein-interaction network. (B) Volcano plot of the siRNA primary screen. siRNA pools were colored red if they met both quantitative and statistical thresholds. The siRNA pool targeting β-catenin (CTNNB1) is highlighted in yellow. (C) Scatter plot of secondary screen data showing normalized luciferase values in DLD1 and SW480 colorectal cancer cell lines. The data represent pooled siRNAs. Colored circles depict siRNA pools that confirmed when deconvoluted to multiple individual siRNAs. siRNA pools colored in gray did not confirm after deconvolution. β-Catenin is represented by its designated “official” name CTNNB1 in the figures and tables.



The Fluidigm (South San Francisco, CA) microfluidic real-time PCR platform permitted the simultaneous quantitation of 18 β -catenin target genes in 77 different samples. These 18 β -catenin-dependent target genes were selected from the 40 genes that compose the β -catenin gene signature. siRNAs targeting 29 of the 77 genes tested regulate at least 6 of the 18 endogenous Wnt/ β -catenin target genes ($P < 0.01$; table S7). By cDNA microarray expression analysis of 13 siRNA secondary screen hits, we found that siRNAs targeting 10 of the hits yielded expression profiles statistically similar to that of siRNAs targeting β -catenin (Fig. 3 and table S8). When we applied the β -catenin gene signature set as a third filter for hits passing the first two validation screens, our siRNA screens reduced 740 hits from the primary screen of more than 20,000 mRNAs to 38 triply validated regulators of Wnt/ β -catenin signal transduction (Table 1 and table S9)—29 genes from the microfluidic real-time PCR platform, 10 genes from cDNA expression profiling, with AGGF1 present in both analyses.

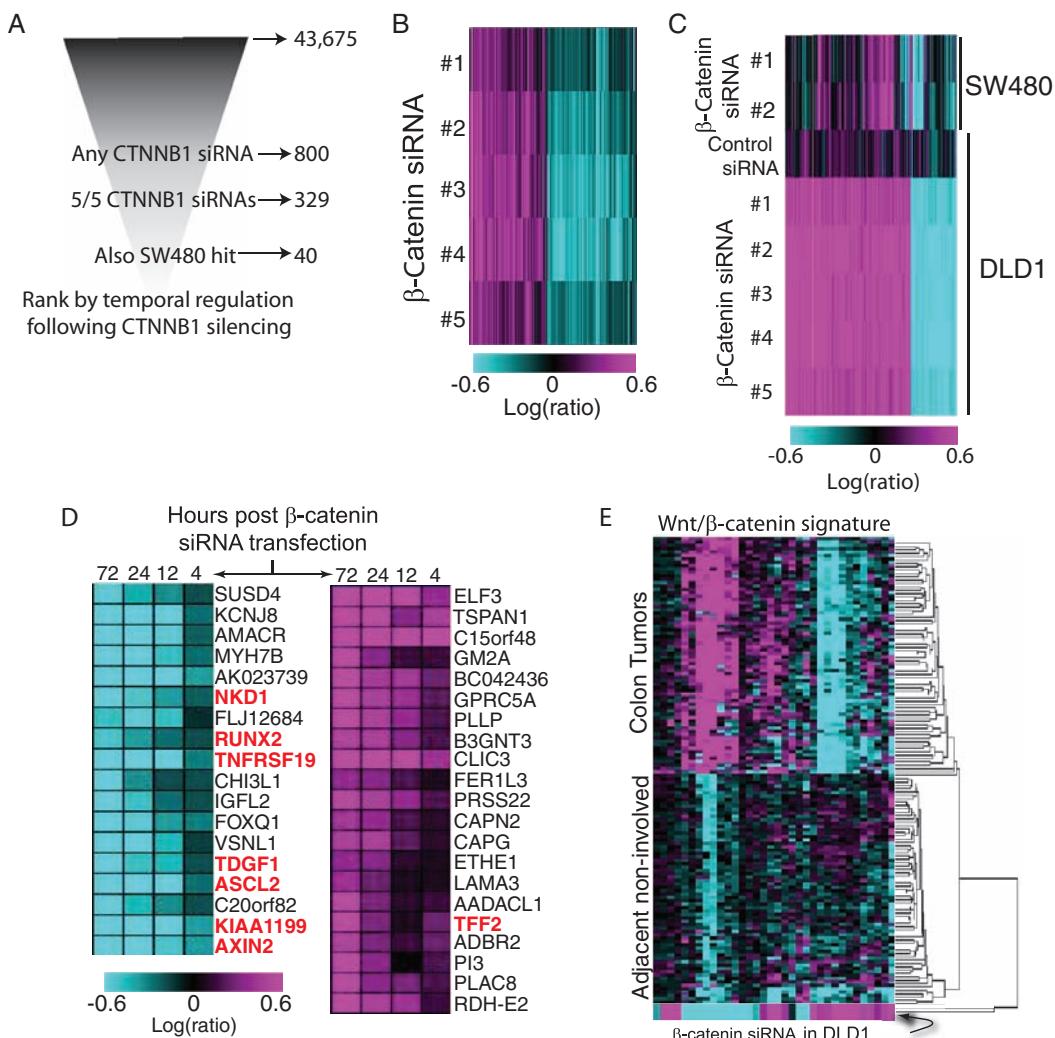
Integration of functional genomic data with proteomic interaction networks

RNAi-based screens, when combined with validation assays, are useful for the identification of genes that functionally contribute to a phenotype of

interest. However, such screens provide little mechanistic insight and do not establish physical relationships among screen hits. To address these limitations, we integrated our functional screen data with protein interactomes that compose much of the known Wnt/ β -catenin signal transduction pathway. The resulting network maps were used to test three assumptions: First, that siRNA screen hits encode established components of the Wnt pathway and their first-order interactors; second, because proteins do not act in isolation, physical interactions between siRNA screen hits will be detected; and third, mechanistic insight will be gained through association of hits with proteins of known biochemical function.

We have previously used tandem affinity purification and mass spectrometric analyses (TAP-MS) to define the PINs for seven proteins in the Wnt/ β -catenin signal transduction pathway (9, 15). Augmentation of these data with TAP-MS analysis of 16 proteins identified in our siRNA screen yielded a functionally biased Wnt/ β -catenin interactome (table S10). We integrated our siRNA secondary screen data with this physical map and discovered 41 proteins that both interact with a “bait” protein and regulate Wnt/ β -catenin signal transduction (Fig. 4 and fig. S1). Of these 41 proteins, 31 are of immediate interest, as they were not previously known to physically or functionally associate with the Wnt/ β -catenin signal transduction pathway.

Fig. 2. Definition of the β -catenin transcriptional signature in colonocytes. (A) Schematized synopsis of the experimental approach and data reduction. (B) Heat map illustration of microarray expression profiles for DLD1 cells transfected with five nonoverlapping siRNAs targeting β -catenin (CTNNB1). (C) Heat map depiction of genes regulated by β -catenin in DLD1 cells and SW480 cells. (D) Heat map representation of cDNA microarray data for the 40 genes that define the β -catenin gene signature after time-course analysis of siRNA-mediated silencing of β -catenin in DLD1 cells. Genes listed in red have been previously reported to be regulated by β -catenin. Of the 40, 31 were regulated within 12 hours of knockdown of β -catenin. (E) Correct assignment of normal and tumor colon tissue by unsupervised hierarchical clustering with the β -catenin gene signature. To illustrate polarity, the expression profile of DLD1 cells transfected with β -catenin siRNAs is shown as the last row.



In a complementary approach designed to extend our interactome, we curated the literature and the STRING database for protein-protein interactions specific to 20 core Wnt/β-catenin pathway components (table S11) (16). From these 20 proteins, we identified 114 first-order interactors, which are associated through 559 interactions. Overlay of the functional siRNA data revealed that 7 of the 20 bait proteins and 14 of the 114 first-order interactors regulate Wnt/β-catenin signaling. These data show the specificity of our siRNA screen to the Wnt/β-catenin PIN ($P < 0.0001$, Fisher's exact test; table S12). We next created a STRING-curated PIN for 297 proteins that met threshold criteria from the DLD1 secondary screen (table S13). Of these 297 proteins, 84 had at least one interaction with another siRNA screen hit, an observation that establishes physical interconnectivity among siRNA screen hits ($P < 0.0001$, Fisher's exact test; table S12). Using common nodes, we combined these two integrated networks to yield an “extended” functionally annotated Wnt/β-catenin PIN

(Fig. 5A and fig. S1). These integrative PINs, which were defined by TAP-MS and interactions culled from publicly available databases, validate our functional siRNA screen data, as well as identify complexes of proteins important for Wnt/β-catenin signaling. The third assumption we tested, and perhaps the most apparent, was that integration of functional siRNA screen data on a proteomic interaction network would reveal mechanistic insight.

AGGF1, a chromatin-associated protein required for β-catenin-mediated transcription

We chose AGGF1 for further exploration because (i) it passed the tertiary siRNA screen, (ii) TAP-MS analysis revealed that it associates with a protein complex of known function, and (iii) it is implicated in human disease. Specifically, *AGGF1* is mutated in Klippel-Trenaunay syndrome (KTS), a human vascular overgrowth disease characterized by capillary malformations and soft tissue hypertrophy (17–19). By TAP-MS analysis, we

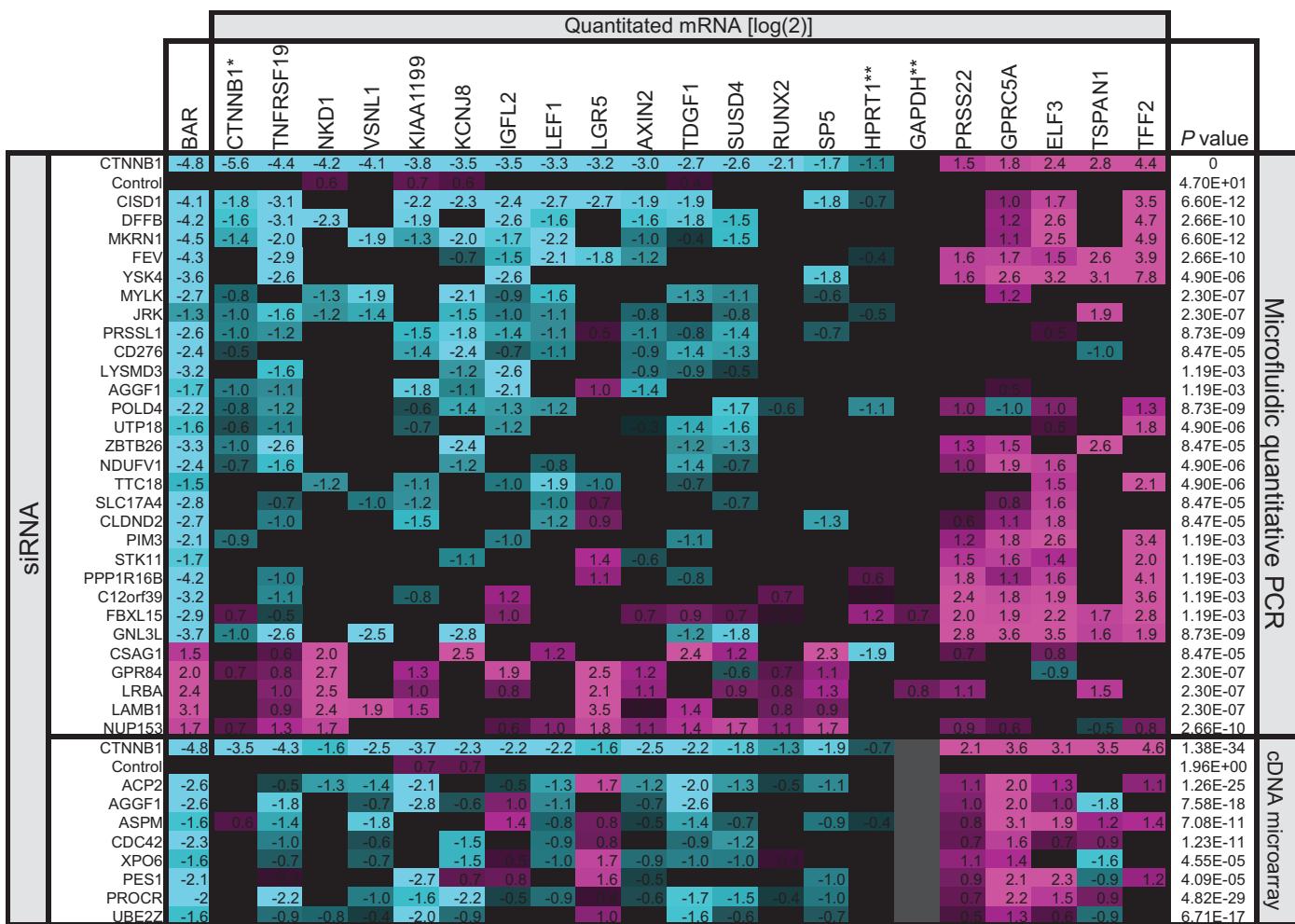


Fig. 3. The tertiary validation screen uses endogenous β-catenin target genes as an indicator of pathway activation. Heat map representation of gene expression quantification after siRNA transfection in DLD1 cells is shown (37 of 77 siRNA targets are shown; see table S7 for complete data set). For most of the siRNAs, quantitative PCR was used to determine the expression of the β-catenin-regulated genes after siRNA transfection of DLD1 cells. Additionally, several of the siRNAs were screened by genome-

wide microarray analysis of transfected DLD1 cells. Data are $\log(2)$ transformed and the average of three independent experiments was taken. The P value of a siRNA's signature overlap with the WNT/β-catenin signature was calculated with the use of the hypergeometric distribution and Bonferroni corrected. *We quantitated the β-catenin (CTNNB1) mRNA to identify siRNAs that regulated Wnt/β-catenin signal transduction through control of β-catenin mRNA levels. **These mRNAs encode housekeeping proteins.

Table 1. Tertiary siRNA screen hits

Gene symbol	Functional notes	P value*	BAR†	Gene symbol	Functional notes	P value*	BAR†
CISD1	Mitochondrial respiration	6.6×10^{-12}	-4.13	CSAG1	Chondrosarcoma-associated gene 1	8.5×10^{-5}	1.51
MKRN1	E3 ubiquitin ligase; transcriptional regulator	6.6×10^{-12}	-4.50	LYSMD3	Putative peptidoglycan-binding	1.2×10^{-3}	-3.20
DFFB	Apoptosis; caspase activated DNase	2.7×10^{-10}	-4.17	PIM3	Oncogene; serine/threonine protein kinase	1.2×10^{-3}	-2.13
FEV	ETS transcription factor	2.7×10^{-10}	-4.32	STK11	LKB1; Peutz–Jeghers syndrome	1.2×10^{-3}	-1.73
NUP153	Nuclear pore complex member	2.7×10^{-10}	1.73	PPP1R16B	Protein phosphatase 1 regulatory subunit	1.2×10^{-3}	-4.21
PRSSL1	Protease	8.7×10^{-9}	-2.55	C12orf39	Nothing known	1.2×10^{-3}	-3.24
POLD4	DNA replication and repair	8.7×10^{-9}	-2.23	FBXL15	Circadian rhythm	1.2×10^{-3}	-2.93
GNL3L	Nucleolar pre-RNA processing	8.7×10^{-9}	-3.65	AGGF1	Angiogenesis	2.3×10^{-15}	-2.63
MYLK	Myosin light chain kinase	2.3×10^{-7}	-2.68	SLC25A39	Mitochondrial carrier protein	3.0×10^{-14}	-0.43
JRK	Homolog of the mouse jerky gene	2.3×10^{-7}	-1.27	AKT1	Phosphorylates GSK3B	4.6×10^{-12}	-1.25
GPR84	G protein–coupled receptor 84	2.3×10^{-7}	2.04	UBE2Z	Ubiquitin-conjugating enzyme	5.0×10^{-12}	-1.57
LRBA	Probable tie to Chediak–Higashi syndrome	2.3×10^{-7}	2.42	PROCR	Receptor for activated protein C	1.3×10^{-6}	-2.01
LAMB1	Laminin, beta 1	2.3×10^{-7}	3.07	ASPM	Microcephaly associated	3.8×10^{-4}	-1.60
YSK4	Yeast Sps1/Ste20-related kinase 4	4.9×10^{-6}	-3.63	PES1	Ribosome biogenesis	1.8×10^{-3}	-2.09
UTP18	Small subunit processome component	4.9×10^{-6}	-1.62	XPO6	Nucleocytoplasmic shuttle	1.1×10^{-2}	-1.56
NDUFV1	Mitochondrial respiration	4.9×10^{-6}	-2.40	ACP2	Lysosomal acid phosphatase	2.2×10^{-2}	-2.64
TTC18	Tetratricopeptide repeat domain 18	4.9×10^{-6}	-1.51	CDC42	Small GTPase of the Rho subfamily	4.5×10^{-2}	-2.25
CD276	Probable costimulatory B7 molecule	8.5×10^{-5}	-2.42				
ZBTB26	Zinc finger and BTB domain containing 26	8.5×10^{-5}	-3.26				
SLC17A4	Sodium/phosphate cotransporter	8.5×10^{-5}	-2.76				
CLDND2	Claudin domain containing 2	8.5×10^{-5}	-2.70				

*P values were calculated as described in Materials and Methods.
†BAR(log2) values from DLD1 secondary screen.

discovered that AGGF1 associates with the SWI/SNF chromatin remodeling complex (Fig. 5B, table S10, and fig. S1). By physically moving nucleosomes along DNA, the SWI/SNF complex creates a chromatin structure that can either facilitate or impede gene transcription (20, 21). Together, these data suggest a model whereby AGGF1 regulates Wnt/β-catenin signaling by controlling the chromatin structure of β-catenin target genes.

In support of this model, the BRG1 (“official name,” SMARCA4) subunit of human SWI/SNF both directly binds β-catenin and is required for β-catenin–mediated transcription (22, 23). We find that AGGF1, BAF57 (SMARCE1), and BRG1 are required for β-catenin–dependent transcriptional activation of BAR (Fig. 6, A and B). Second, using a Gal4-β-catenin reporter system, we show a requisite role for hSWI/SNF in TCF/LEF-

independent β -catenin-mediated transcriptional activation (Fig. 6C). Third, AGGF1 and BAF57 are required for the expression of the endogenous Wnt/ β -catenin target genes *LEF1* and *AXIN2* (Fig. 6D and fig. S2).

Because Wnt/ β -catenin signaling is highly conserved in evolution, we next asked if AGGF1 contributed to Wnt/ β -catenin signaling *in vivo*. Ectopic stimulation of Wnt/ β -catenin signaling during early zebrafish development results in posteriorization of the embryonic axis, as evidenced visually by the elimination of anterior structures (24). Two different antisense-morpholino oligonucleotides directed against *aggf1* inhibited Wnt/ β -catenin-induced posteriorization of the embryonic axis (Fig. 6E). Together with our cell-based findings, these data show an evolutionarily conserved and requisite role for AGGF1 in Wnt/ β -catenin signaling.

AGGF1 was first described as a secreted angiogenic growth factor, whereas our model predicts a nuclear role for AGGF1 in regulating Wnt signaling (18). Because of these disparities, we rigorously tested the possibility that AGGF1 functions in the nucleus. We found that like the SWI/SNF member BAF57 and histone H3, most of AGGF1 localized in the nuclei DLD1 and HEK293T cells (Fig. 7A). Additionally, in a direct examination of whether extracellular AGGF1 can regulate Wnt/ β -catenin signal transduction, we supplemented the DLD1 growth media with increasing amounts of recombinant human AGGF1 protein and found no change in β -catenin-mediated transcription (fig. S3).

To directly test our model of transcriptional regulation by AGGF1, chromatin immunoprecipitation (ChIP) was used to define the promoter occupancy of *LEF1* and *AXIN2*, both of which are β -catenin-regulated

genes that require AGGF1 and BAF57 for expression (Fig. 5D and fig. S2). We found trimethylated lysine 4 on histone 3 (H3K4; a mark of transcriptional activity) and β -catenin associated with the *LEF1* and *AXIN2* promoters (Fig. 7B and fig. S2). Similarly, two different antibodies specific for AGGF1 revealed its presence on both promoters, but not downstream coding sequences. Quantitatively, antibodies specific for AGGF1 and β -catenin provide a significant enrichment of the *LEF1* promoter compared to downstream genomic sequence (Fig. 7C). In addition to *LEF1* and *AXIN2*, comparison of cDNA microarray profiles of DLD1 cells after AGGF1 or β -catenin siRNA transfection identified 134 genes that are regulated by both proteins ($P = 1.4E-93$; Fig. 7D and table S14). Taken together, these data suggest that AGGF1 associates with and regulates 40% of Wnt/ β -catenin target genes and further raises the possibility that AGGF1 functionally contributes to Wnt/ β -catenin signaling in colon cancer. Consistent with a role in contributing to Wnt/ β -catenin signaling in colon tumors, analysis of cDNA microarrays of 65 matched colon tumors and adjacent uninvolved tissues revealed overexpression of *AGGF1* in colon tumors (Fig. 7E and table S15).

DISCUSSION

The conserved Wnt/ β -catenin signaling pathway acts to regulate cell fate and proliferation in embryonic development and to regulate regeneration and tissue homeostasis in adults (4, 25). Dysregulation of this pathway is linked to a myriad of diseases, including cancer, Alzheimer's disease, and bone den-

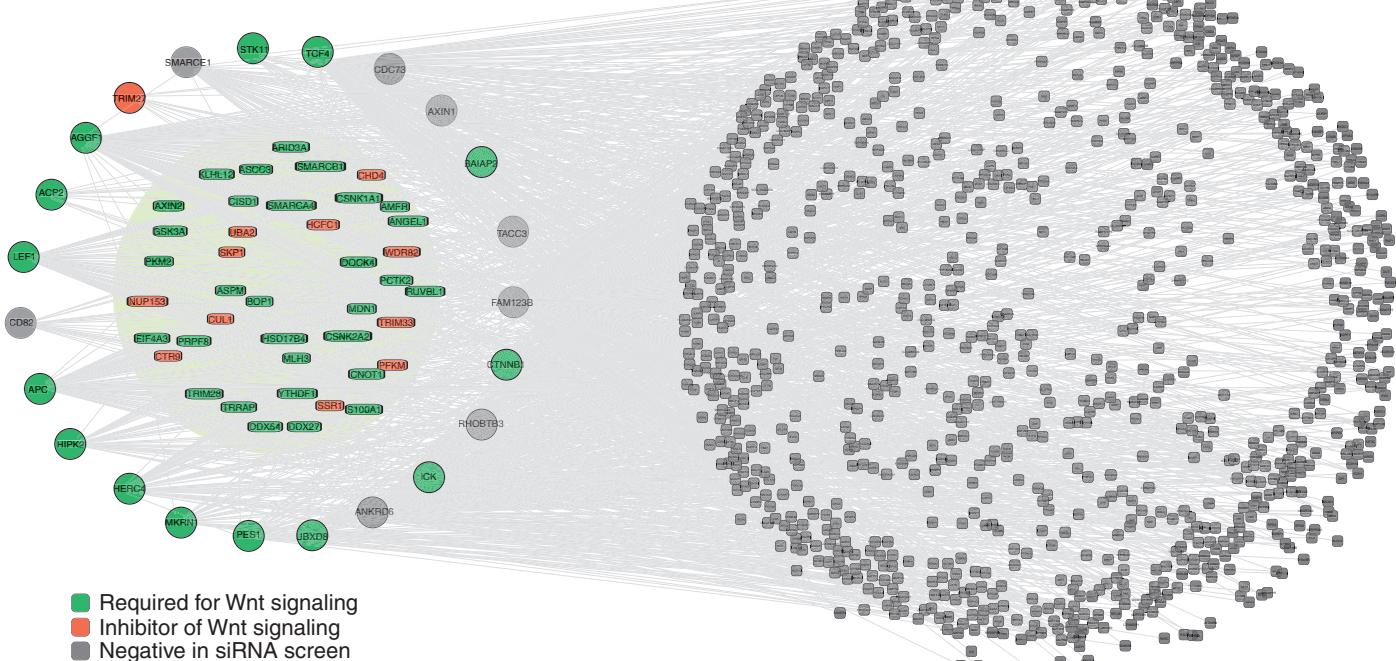


Fig. 4. Integration of siRNA screen hits on a mass spectrometry-based PIN. Circles and rectangles (nodes) represent proteins and lines (edges) describe the relationship between nodes. Red and green nodes denote protein inhibitors and activators of the pathway, respectively. Because the tertiary screen was not comprehensive and highly restrictive, we used the DLD1 siRNA secondary screen hits for proteomic integration (table S13). (A) Affinity purifica-

tion and mass spectrometry of 23 bait proteins (outer circle nodes, left), including 7 established Wnt pathway genes and 16 proteins identified at various levels in the siRNA screen. Forty-one proteins were identified as first-order interactors and hits in the siRNA screen (inner circle, square nodes, left). Most proteins identified by our TAP-MS approach did not meet siRNA secondary screen criteria (right cloud, gray) (see also fig. S1).

sity syndromes (5). Through rigorous siRNA screening with three levels of validation, we report 38 triply validated and 119 doubly validated regulators of the Wnt/β-catenin pathway (Table 1 and table S6, respectively). When we integrated the siRNA screen hits with proteomic analyses, we identified 112 proteins that were jointly detected in both screens (table S16). This integrative approach offers a powerful means of rapidly placing siRNA hits into the context of known protein complexes while simultaneously leveraging each screen to validate hits in the other screen in an unbiased manner.

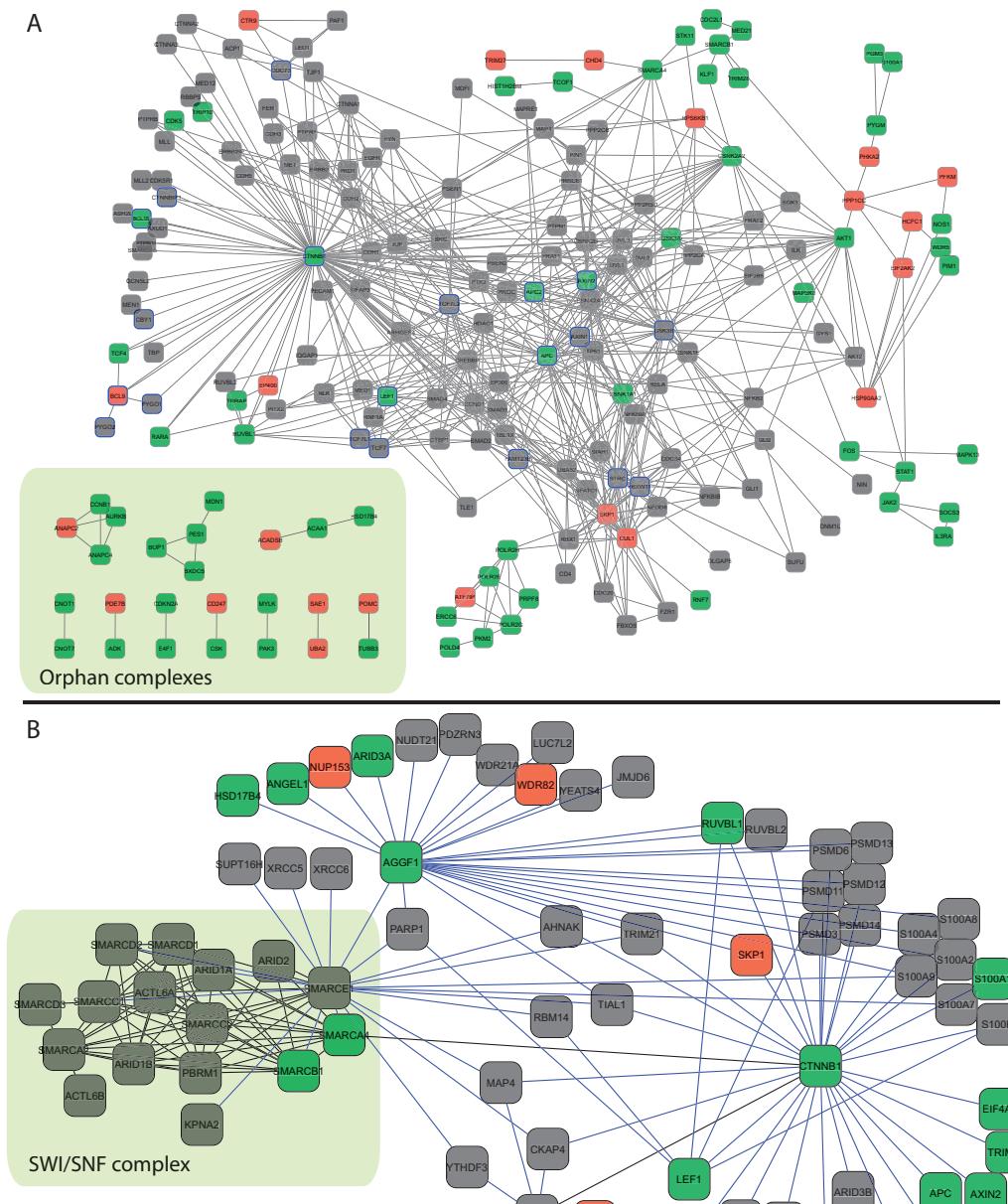
Comparative analysis of Wnt/β-catenin siRNA screens

Comparison of our data with previous genome-wide RNAi screens to identify modulators of β-catenin signaling in *Drosophila* (23) and human cervical cancer cells (26) reveals that only established core com-

ponents of the Wnt pathway were identified in all three screens, which include β-catenin, TCF7, BCL9, and AXIN (table S17). This raises the question of whether there are substantial differences in the proteins that regulate Wnt/β-catenin signaling in different cell types. In comparing hits from our own screens we found surprisingly few similarities between genes identified by functional siRNA screens in DLD1 and SW480 colorectal cells (44% of 268 genes tested).

Despite the utility of comparing siRNA screen hits across disparate platforms, caution should be taken when either comparing negative data between independent screens or attempting to predict hit rates in future siRNA screens. Differences between siRNA screen hits are expected if there are differences in the thresholds for hit selection, the length of time between siRNA introduction and phenotypic scoring, the efficacy

Fig. 5. Integration of siRNA screen hits on a literature-based PIN. Circles and squares (nodes) represent proteins and lines (edges) describe the relationship between nodes. Red and green nodes denote protein inhibitors and activators of the pathway, respectively. (A) A PIN of the Wnt pathway and siRNA secondary screen hits culled from the literature and public databases. Seventeen known Wnt pathway proteins were used as seeds in the STRING database, returning 100 first-order interactors with a total of 534 edges. Additional 3 core Wnt nodes and 14 first-order interactors were manually curated; connected through 26 edges. Seven of the 20 Wnt pathway seed proteins (blue border) and 14 of the 114 first-order interactors passed threshold in the secondary screen. Next, we used all 320 proteins that passed threshold in the secondary screen as input for STRING; 297 were present in the database and 84 had at least one interaction with another secondary screen hit; a total of 109 edges. (B) Combining nodes and edges derived from mass spectrometry experiments (blue edges) and from bioinformatic resources (black edges), a PIN for AGGF1 that includes β-catenin (CTNNB1) and the SWI/SNF complex is revealed (see also fig. S1).



of siRNA-mediated silencing, or the presence of background mutations with epistatic consequences (such as the *APC* mutation in the DLD1 cells). Because of these intricacies, future studies with suitable controls will be necessary to understand the differences between the siRNA hits in the various cell lines. The results of such studies will provide insight into the hypothesis that “the” human Wnt/β-catenin pathway consists of a conserved core pathway decorated with context-dependent modulators.

Linking multiple protein complexes to Wnt/β-catenin signaling

It is generally time-consuming and laborious to develop criteria for determining which siRNA hits to pursue, and one may readily omit genes based on subjective standards. We have confronted this limitation by integrating hits from our siRNA screen with an extended PIN for the Wnt/β-catenin pathway. Specifically, we created PINs for the Wnt/β-catenin signal transduction pathway with the use of all known protein-protein interactions and TAP-MS-derived interaction data for 25 additional proteins. Combinatorial analysis of siRNA and proteomic data has several advantages compared to the analysis of either screen in isolation. First, mapping siRNA data to PINs illuminates physical relationships that are invisible to siRNA screens. Second, the dual-screening approach facilitates the rescue of false negatives from the siRNA screen by physically linking relatively weak siRNA hits to the Wnt/β-catenin pathway. This point is illustrated

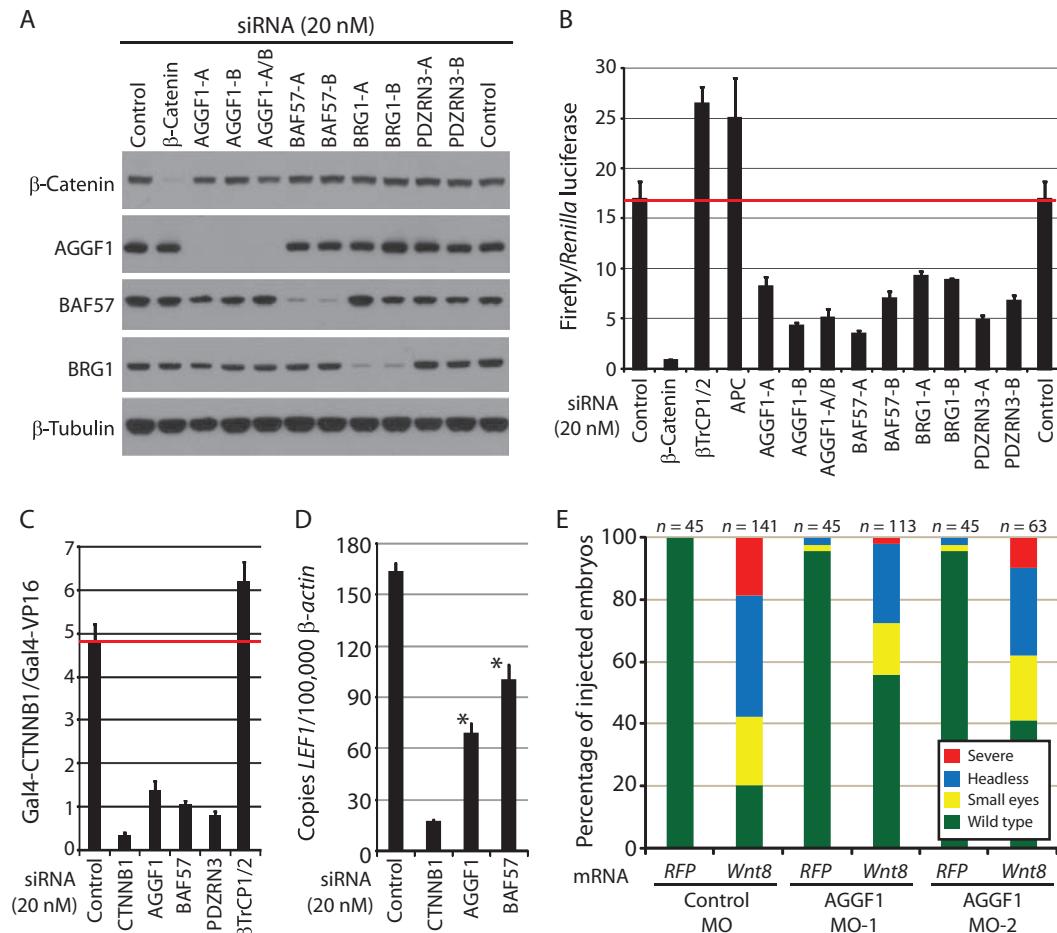
by PDZRN3, which failed to meet siRNA secondary screen thresholds, but was functionally explored because of its association with AGGF1.

The integrated map of Wnt/β-catenin signal transduction reveals the functional importance and physical associations for multiple proteins. For example, the CHD4 (also known as Mi-2) protein negatively regulates Wnt/β-catenin signaling and associates with three proteins that similarly affect the pathway—WTX, Brg1 (SMARCA4), and TRIM27. CHD4 is a component of NuRD chromatin remodeling complex, which, when taken together with AGGF1 and the hSWI/SNF complex, highlights the importance of chromatin remodeling on Wnt/β-catenin signaling (27, 28). As another example, we found four proteins required for Wnt signaling—PES1, BOP1, MDN1, and BXDC5—which are all members of a complex involved in ribosomal biogenesis (29, 30). Interestingly, the PES1-BOP1 complex controls chromosomal stability in colorectal tumors, where constitutive activation of Wnt/β-catenin signaling is common (31, 32). From our screens we selected AGGF1 for more detailed investigation, described below.

AGGF1 nuclear activity in Wnt signaling

We found that AGGF1, which is linked to KTS (18, 19), is (i) required for Wnt/β-catenin signaling in our luciferase-based siRNA screen, (ii) required for zebrafish embryos to respond to Wnt/β-catenin signaling, and (iii) regulates 40% of Wnt/β-catenin target genes in DLD1 cells, as determined by cDNA microarray expression profiling. Through pro-

Fig. 6. Requisite role of AGGF1 for β-catenin-mediated transcription. (A) siRNAs complementary to the indicated mRNAs were transfected into DLD1 cells 72 hours before protein isolation and Western blot analysis. (B) DLD1 cells expressing BAR and *Renilla* were transfected with the indicated siRNAs 72 hours before cell lysis and luciferase quantitation. Error bars represent standard deviation across biological quadruplicates. (C) DLD1 cells stably expressing a UAS-luciferase reporter and either Gal4-β-catenin fusion protein (Gal4-CTNNB1) or Gal4-VP16 fusion protein were transfected with the indicated siRNAs for 72 hours before luciferase quantitation. Error bars represent standard deviation across biological quadruples. (D) Quantitative PCR of *LEF1* and β-actin mRNA after siRNA-mediated silencing of β-catenin, AGGF1, or BAF57 in DLD1 cells (* $P < 0.05$; Students *t* test). (E) One-cell-stage zebrafish embryos were injected with *Wnt8* mRNA or red fluorescent protein (RFP) mRNA with either control morpholino or one of two different *aggf1* morpholinos. Embryos were scored for degree of anterior truncation 48 hours after fertilization.



teomic analyses, we found that AGGF1 associates with at least nine different nuclear proteins, among which is BAF57, an established component in the SWI/SNF chromatin remodeling complex (33). Through ChIP studies of the β -catenin target genes *AXIN2* and *LEF1*, we established with two independent AGGF1 antibodies that AGGF1 is present in a protein-DNA complex that contains β -catenin and TCF/LEF DNA binding sites. Moreover, we confirmed a report that AGGF1 localizes to the nucleus, although this group also observed the secretion of AGGF1 after the induction of angiogenesis (18). Given the investment of effort needed to execute a detailed analysis of a candidate component of a signaling pathway, such as that applied to AGGF1, the integration of proteomic and siRNA data affords an efficient tool for selecting hits for subsequent investigation.

CONCLUSIONS

Despite intensive investigation, the Wnt field has yet to come to a consensus on several fundamental questions. What are the components of the Wnt/ β -catenin pathway that are required for signaling throughout the animal kingdom and that constitute the minimal core pathway? What is the “complete” functional pathway in a specific cell type? Does this extended functional pathway differ between different cell types and, if so, what are the identities and roles of the context-dependent modulators? What are the nodes in the signaling pathway that are engaged in cross talk with other signaling pathways, and how do these define larger signaling networks? As discussed above, the pres-

ent study begins to address these questions. In the process, we applied stringent criteria for conducting siRNA screens and we propose that integrating proteomic screens with siRNA screens will prove to be a powerful approach.

MATERIALS AND METHODS

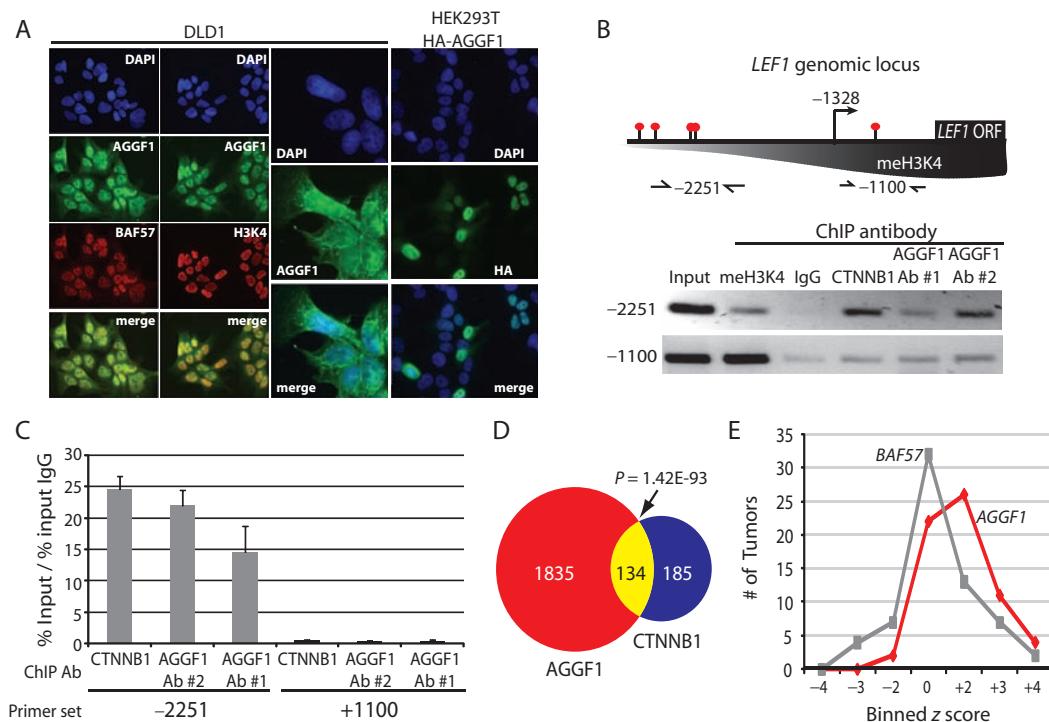
Reagents

DLD1 and SW480 cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The siRNA sequences, morpholino sequences, and commercial antibodies used to examine the AGGF1-SWI/SNF complex are listed in table S18. All siRNA that passed the secondary screen are listed in table S19. All siRNA transfections, irrespective of cell type, used RNAiMAX (Invitrogen, Carlsbad, CA).

High-throughput siRNA screens

The genome-wide siRNA screen was performed as previously described, with minor modifications (34). Briefly, cells were reverse-transfected in 1536-well plates, with a final concentration of pooled siRNA at 25 nM. Seventy-two hours after transfection, firefly luciferase and *Renilla* luciferase were quantitated. The pilot-scale screens were completed essentially as described above, except that cells were reverse-transfected in 384-well plates and cell viability was controlled by AlamarBlue staining. All siRNAs were designed with a proprietary algorithm. We provide sequences for all siRNAs that met the hit-calling criteria in the secondary screen (table S19).

Fig. 7. AGGF1 is a chromatin-associated protein involved in regulating 40% of β -catenin target genes. (A) Subcellular localization of endogenous AGGF1, BAF57, and H3K4 as determined by immunofluorescence in DLD1 cells. HEK293T cells stably expressing HA-tagged AGGF1 were stained with a hemagglutinin antibody (HA). For both DLD1 and HEK293T cells, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). (B) Schematic of the *LEF1* genomic locus; putative TCF/LEF DNA binding sites are indicated in red. The indicated antibodies were used for ChIP analysis of two different regions of the *LEF1* genomic locus. Two different antibodies specific for AGGF1 were analyzed and data were confirmed in three biological replicates. (C) qPCR for two regions of the *LEF1* genomic locus following ChIP analysis with the indicated antibodies. Data were normalized to the IgG ChIP signal and represented as percent of input and representative of three biological replicates. Error bars depict standard deviation from the mean. (D) Venn diagram schematic of microarray expression profiles for DLD1 cells transfected with siRNAs against AGGF1 or β -catenin. The gene signatures were defined by the overlap of two independent AGGF1



siRNAs or five independent β -catenin siRNAs. (E) AGGF1 and BAF57 mRNAs were quantitated by cDNA microarray analyses of 65 colon tumors and matched adjacent normal tissue. Data are represented by number of tumors showing indicated z scores relative to standard deviation of matched normal.

Gene expression studies

For determination of the β -catenin gene signature, the cell lines DLD1 and SW480 were transfected with the indicated siRNAs or water (mock) with RNAiMAX (Invitrogen). The sequences of the five β -catenin siRNAs used are listed in table S18. Following the 72-hour incubation, RNA was extracted from the cells with RNeasy kits (Qiagen, Valencia, CA), following the manufacturer's protocols, including the on-column DNase digestion step. Samples were then submitted for microarray gene expression profiling on the Affymetrix platform.

For the microfluidic quantitative real-time fluorescence polymerase chain reaction (qPCR) tertiary screen data, pools of three siRNAs to each “hit” target were transfected into DLD1 cells. Physical and financial limitations of the Fluidigm microfluidic qPCR platform limited our analysis to 77 of the 119 genes that met threshold criteria in the secondary screen. These 77 genes were selected based on magnitude of BAR modulation in the secondary screen, as well as by biological and disease interest. RNA was isolated after 72 hours with the RNeasy Mini 96-well plate kit (Qiagen). This RNA was run in a reverse transcriptase reaction with the ABI Archive Kit (Applied Biosystems, Foster City, CA). The resulting cDNA was run in a pre-amp reaction with the use of the ABI Pre-Amp Master Mix (Applied Biosystems). On-Demand TaqMan Assays (Applied Biosystems) for the WNT signature transcripts to be assayed were mixed with BioMark Assay Loading Buffer (Fluidigm) in preparation for loading. The BioMark 46.46 chip creates all possible combinations of 46 assay wells and 46 sample wells for a total of 2116 qPCR reactions. In this experiment, duplicate assays were loaded so that duplicate C_T values for each sample could be obtained. The chip was run in the Biomark System (Fluidigm) instrument for 40 cycles. The entire process from transfection to qPCR was run three times.

Tertiary screen data analysis

C_T values from the three Biomark runs were converted to fold changes by standard calculations, with glucuronidase β as the input control and duplicate mock-transfected samples as the negative control. Additionally, glyceraldehyde-3-phosphate dehydrogenase and hypoxanthine-guanine phosphoribosyltransferase 1 were run as input controls. Because no siRNA pools tested target these genes, the regulation of these genes by all siRNAs can be used as the negative control sample in a *t* test. The six replicate regulations of a given siRNA pool on a given signature gene are used as the positive control sample. In this way, *P* values were calculated for each siRNA pool-signature gene combination. The Bonferroni correction was applied. Only gene regulations whose *P* value was less than 0.05 were considered significant.

For a given siRNA pool, the overlap with the Wnt/ β -catenin 18-gene set tested in the Biomark qPCR platform was evaluated by counting the number of genes that were significantly regulated and that were regulated with the correct polarity with respect to the β -catenin (CTNNB1) siRNA. For example, for siRNA pools that negatively regulate BAR, the proper direction was regulating the gene signature in the same direction as that of CTNNB1 siRNAs. A *P* value was calculated from this count with the binomial distribution assuming the odds of a gene being regulated at random by a given siRNA to be 1 out of 20. These *P* values were Bonferroni corrected. Only those siRNA pools with a *P* value less than 0.01 were considered to significantly overlap with the tested 18-gene Wnt/ β -catenin signature set. We set thresholds on 6 of 18 β -catenin target genes to bias hit selection toward general regulators of Wnt/ β -catenin signal transduction.

For cDNA microarray expression analyses, *P* values were placed on the regulation of a gene with the use of a proprietary error model. A *P* value less than 0.01 was considered a significant signature overlap. The *P* value of a siRNA's signature overlap with the Wnt/ β -catenin signa-

ture was calculated with the use of the hypergeometric distribution and Bonferroni corrected.

Affinity purification and mass spectrometry

TAP-MS experiments were performed as previously described (9, 15). The PINs for BAF57, APC, and CDC73 were determined by immunoprecipitation of the endogenous protein from DLD1 cells. Briefly, cells were harvested from 15 \times 150-mm tissue culture plates, washed in PBS, lysed, and cleared by centrifugation. The supernatant was cleared two times with protein G agarose beads before immunoprecipitation with 10 μ g of antibody. Antibody complexes were washed four times in lysis buffer, four times in ammonium bicarbonate (pH 7.0), and eluted in sodium citrate (pH 2.0). We provide in table S10 unfiltered mass spectrometry data for all baits analyzed, as well as PIN files generated in cytoscape (<http://www.cytoscape.org/>) as Supplementary Materials (MSDATA.cys, WNT+DLDSEC.cys, AGGF1.cys) (35).

GAL4-luciferase reporter assays

A 5 \times UAS-luciferase reporter and an EF1 α -driven *Renilla* luciferase reporter were introduced into DLD1 cells by lentiviral transduction. To these stable cells, we then introduced β -catenin(SA)-GAL4DBD or VP16-GAL4DBD fusion constructs by lentiviral transduction (DBD, DNA binding domain). The resulting polyclonal cell lines were used to test AGGF1 and BAF57 siRNAs.

Chromatin immunoprecipitation

ChIP experiments were carried out as previously described with the following modifications (36). Briefly, cells were lysed in nuclei lysis buffer (1% SDS, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) with proteinase inhibitors added and sonicated to an average DNA size of 300 to 1500 bp. Sonicated DNA was then diluted fourfold in immunoprecipitation dilution buffer (16.7 mM Tris-HCl, pH 8.0, 11.2 mM EDTA, 0.01% SDS, 150 mM NaCl). Immunoprecipitations were performed overnight at 4°C with β -catenin, AGGF1, H3K4Me3, and immunoglobulin G (IgG) control antibodies. Antibody complexes were captured with protein G agarose slurry and eluted in 1% SDS + 0.1 M NaHCO₃. After proteinase K treatment for 2 hours at 55°C, DNA was purified and analyzed by quantitative PCR as described previously (9).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/1/45/ra12/DC1

Fig. S1. Scalable vector graphic representation of the integration of siRNA screen hits on protein-protein interaction networks.

Fig. S2. AGGF1 is required for the expression of both *LEF1* and *AXIN2* and associates with the *LEF1* and *AXIN2* promoters.

Fig. S3. Recombinant human AGGF1 (rhAGGF1) does not regulate β -catenin-mediated transcription when added to the media of DLD1 cells.

Table S1. Genome-wide primary siRNA screen data from DLD1 cells.

Table S2. Genome-wide secondary siRNA screen data from DLD1 cells.

Table S3. Pilot-scale secondary siRNA screen data from DLD1 cells.

Table S4. Genome-wide secondary siRNA screen data from SW480 cells.

Table S5. Pilot-scale secondary siRNA screen data from SW480 cells.

Table S6. Compiled siRNA screen hits that passed the secondary screen.

Table S7. Tertiary screen Fluidigm real-time PCR data from DLD1 cells.

Table S8. Tertiary screen cDNA microarray data from DLD1 cells.

Table S9. Thirty-eight hits that passed tertiary screen.

Table S10. Workbook containing filtered and compiled TAP-MS data.

Table S11. STRING-based protein-protein interaction data for core components of the Wnt pathway.

Table S12. Statistics on the specificity of the siRNA screen hits to the Wnt pathway and on the interconnectivity of proteins identified in the screen.

Table S13. STRING-based protein-protein interaction data for siRNA screen hits.

Table S14. Genes regulated by both β -catenin and AGGF1.

- Table S15. AGGF1 and BAF57 expression in colon cancer.
 Table S16. One hundred twelve genes identified by siRNA screens and proteomic interaction maps.
 Table S17. Evolutionarily conserved modulators of Wnt/β-catenin signaling.
 Table S18. Reagents used in this study.
 Table S19. siRNA sequences for all genes that passed the secondary screen.
 MSDATA.cys. Cytoscape file for Fig. 4.
 WNT+DLDSEC.cys Cytoscape file for Fig. 5A.
 AGGF1.cys Cytoscape file for Fig. 5B.

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