Genetic Amplification of the NOTCH Modulator LNX2 Upregulates the WNT/β-Catenin Pathway in Colorectal Cancer

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Abstract

Chromosomal copy number alterations (aneuploidy) define the genomic landscape of most cancer cells, but identification of the oncogenic drivers behind these imbalances remains an unfinished task. In this study, we conducted a systematic analysis of colorectal carcinomas that integrated genomic copy number changes and gene expression profiles. This analysis revealed 44 highly overexpressed genes mapping to localized amplicons on chromosome 13, gains of which occur often in colorectal cancers (CRC). RNA interference (RNAi)–mediated silencing identified eight candidates whose loss-of-function reduced cell viability 20% or more in CRC cell lines. The functional space of the genes NUPL1, LNX2, POLR1D, POMP, SLC7A1, DIS3, KLF5, and GPR180 was established by global expression profiling after RNAi exposure. One candidate, LNX2, not previously known as an oncogene, was involved in regulating NOTCH signaling. Silencing LNX2 reduced NOTCH levels but also downregulated the transcription factor TCF7L2 and markedly reduced WNT signaling. LNX2 overexpression and chromosome 13 amplification therefore constitutively activates the WNT pathway, offering evidence of an aberrant NOTCH–WNT axis in CRC. Cancer Res; 73(6); 1–11. ©2012 AACR.

Introduction

Colorectal cancer (CRC) has an annual incidence of some 150,000 new cases and a mortality of more than 50,000 in the United States in 2010 (1). Despite the availability of screening programs, CRCs are often detected at advanced disease stages, at which surgery alone is no longer a curative therapeutic intervention. The development of CRC from premalignant precursors to invasive and metastatic disease involves accumulation of mutations in key regulator genes (2), as well as the acquisition of a recurrent pattern of genomic imbalances (3), a hallmark of epithelial cancers (4, 5). Like in many other tumors, these imbalances can be focal (i.e., limited to a chromosomal band or less) or they affect entire chromosome arms or chromosomes, most commonly resulting in low level gains or losses. Once acquired, which can occur as early as in dysplastic polyps, the distribution of chromosome-level imbalances is remarkably stable and consequently results in a pattern of genomic imbalances that is tumor type specific (6, 7). In CRC, such imbalances affect chromosomes 7, 8q, 13q, and 20 as gains and chromosomes 4, 5q, 8p, 17p, and 18 as losses (8). Chromosome-level genomic imbalances directly influence the expression levels of resident genes, therefore resulting in a massive transcriptional deregulation (9–14). It is therefore conceivable that the systematic integration of genomic imbalances with global gene expression profiles has the potential to reveal novel drivers of tumorigenesis (15, 16).

The pattern of genomic imbalances in CRC is peculiar because chromosome 13 is frequently gained or amplified. In many other carcinomas, this chromosome is lost (8), most likely because it contains the tumor suppressor gene RBP1. The consistent gain of chromosome 13 in CRC strongly suggests a tissue-derived selection. The identification of driver genes of this selection therefore requires the functional interrogation of potential candidates, which are found among those genes that are consistently overexpressed. To identify potential novel oncogenes on chromosome 13, we systematically mapped copy number changes by array Comparative Genomic Hybridization and overlaid the gene expression profiles obtained from the same tumors and cell lines. The involvement of candidate genes in growth and survival of CRC was then tested using RNA interference (RNAi), followed by gene expression profiling to
define a signature of loss-of-function (LOF). This led to the identification of affected cellular signaling pathways and ultimately to the identification of their role in the cancer cell.

Materials and Methods

Patient samples, cell lines, and nucleic acid extraction

Fifty-six patients diagnosed with colorectal adenocarcinomas (Unio Internationale Contra Cancrum II/III) recruited as part of the Clinical Research Unit KFO 179 at the Department of General Surgery, University Medicine Göttingen (Göttingen, Germany), were included in this study. This study has been approved by the ethical review committee from the University Medical Center (Göttingen, Germany), and informed consent was obtained from all patients. Supplementary Fig. S1 summarizes the experimental setup and the prioritization of targets.

All of the cell lines were obtained from the American Type Culture Collection. DNA and RNA were extracted from the cell lines, primary tumors, and normal mucosa samples following standard procedures (17). Nucleic acid quantification was determined using a Nanodrop ND-1000 UV-VIS spectrophotometer (Nanodrop), and RNA quality was assessed using a Bioanalyzer 2100 (Agilent Technologies). Normal colon RNA from 5 different donors without a history of CRC was purchased from Ambion (Applied Biosystems).

Array CGH

Oligonucleotide-based array CGH was conducted according to the protocol provided by the manufacturer (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis, protocol version 4.0, June 2006, Agilent Technologies) with minor modifications.

Gene expression microarrays

Cell line and normal human colon RNA were labeled with Cy3, mixed, and hybridized to an oligonucleotide-based Whole Human Genome Microarray using the manufacturer’s recommendations (Agilent). Data were extracted using Agilent Technologies Feature Extraction software (v. 9.1).

Quantification of gene expression

Gene expression levels were validated by quantitative reverse transcription PCR (qRT-PCR) using Power SYBR Green technology (Applied Biosystems, Inc.) in the Applied Biosystems Prism 7000 sequence detector. Gene-specific PCR primers (Supplementary Table S1) were obtained from Operon Technologies, Inc. The genes RAB35, FBXL12, and OTUB1 were used for normalization. LNX2 and NOTCH1 expression were also measured by branch-DNA assay (Panomics).

RNAi-based analysis

The target sequences for the synthetic siRNAs (Qiagen Inc.) are listed in Supplementary Table S2. Lipid-based reverse transfections were conducted using Oligofectamine (Life Technologies) (5 ). Briefly, for analysis of the cell viability, each siRNA (2 pmol) was added to individual wells in a 96-well plate in 25 μL of serum-free RPMI and complexed with transfection reagent in 25 μL of serum-free RPMI to a final siRNA concentration of 20 nmol/L. Cells were then added in 50 μL RPMI supplemented with 20% FBS. Cellular viability was determined after 72 and 96 hours posttransfection using the CellTiter-Blue reagent (Promega).

Whole genome expression profiles were generated for 8 genes following RNAi to enable the determination of gene-specific LOF RNAi signatures. Reduction in target mRNA levels was confirmed by RT-PCR, and effects on cell viability were verified by functional assays. The gene expression data used to generate the gene-specific RNAi signatures have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus3 (GEO ID: GSE33824).

Immunoblotting

Immunoblotting was carried out according to standard procedures. Whole-cell lysates were obtained using SDS lysis buffer, sonicated, and denatured at 95°C for 10 minutes. Equal amounts of protein from whole-cell lysates were loaded in a NuPAGE Bis–Tris electrophoresis gel. The following antibodies were used: anti-KLF5 (Abcam), anti-SOX2 (L73B4; Cell Signaling Technology), anti-LNX2 (Abcam), anti-NOTCH1 (Epitomics), anti-NUMB (C29G11; Cell Signaling Technology), anti-HEY2/HRT2 (Millipore), anti-Hes1 (Epitomics), anti-TCF7L2 (EP2033Y; Abcam), anti-CTNNB1 (Abcam), anti-PARP (Abcam), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich).

Reporter assay

TOP-Flash reporter vector contains a luciferase open reading frame and 2 sets of 3 copies of T-cell factor (TCF)-binding sites, upstream of a minimal thymidine kinase promoter. FOP-Flash is a control vector, which is identical to TOP-Flash, but the TCF-binding sites are inactive due to mutations. Cells were first transfected with siRNA (day 0) using the standard protocol described earlier. The same cells were then cotransfected with the reporter DNA 48 hours post-siRNA transfection using Lipofectamine 2000 (Life Technologies). Cells were double-transfected with 100 ng of the reporter and 100 ng of Renilla construct for normalization. Lysates were harvested using the Dual-Glo Luciferase Assay System (Promega) and the luminescence analyzed with a Tecan i-control microplate reader (Tecan Group Ltd.).

Flow cytometry cytotoxicity assay

Cells wereTrypsinized, harvested, and washed twice in 1× PBS. The cells were then washed with Annexin V–binding buffer provided with the Annexin V–PE Apoptosis Detection Kit (BD Biosciences). Cells were resuspended in 100 μL Annexin V–binding buffer. Samples were stained with 2.5 μL Annexin V–phycoerythrin (PE) and 5 μL 7-Aminoactinomycin D, and incubated for 15 minutes at room temperature. Next, 300 μL Annexin V–binding buffer was added postincubation. Samples were analyzed by flow cytometry on a fluorescence-activated cell sort ing (FACS) Calibur instrument (BD Biosciences) and FlowJo software.

Statistical and bioinformatic analysis

Agilent expression data were quantile normalized using the statistical computing language R (18). Differential
expression between siNeg- and siRNA-treated cells was calculated using empirically modified Bayes T-statistics with the R package Limma. Raw data for GSE14333, GSE17536, and GSE17537 were downloaded from the NCBI GEO. Each dataset was normalized separately using the robust multi-chip averaging (RMA) method from Affymetrix (19). One-way hierarchical clustering (average linkage) was conducted using JMP (version 8, SAS).

Transcription factor target gene lists were derived from peer-reviewed literature (Supplementary Table S3). All gene lists were cross-referenced to the Ingenuity Knowledge Base as well as NCBI Entrez Gene. Gene signatures were split into up- and downregulated lists and analyzed separately. Enrichment was calculated using a Benjamini–Hochberg corrected one-tailed Fisher exact test using Ingenuity Pathway Analysis (IPA, v.8.8).

Gene signatures were loaded into IPA, and each gene was mapped to a unique IPA object. As with the transcription factor analysis, gene signatures were parsed into up- and downregulated lists and analyzed separately. A Benjamini–Hochberg corrected one-tailed Fisher exact test was used to calculate enrichment.

Results

We report here a systematic functional genomic strategy for the identification of driver genes in CRC. This strategy was based on the hypothesis that genes that reside in regions of recurrent genomic amplification, and are consistently overexpressed, present bona fide oncogenes. Our approach was targeted to chromosome 13 as one of the most frequently and highly amplified chromosome in this disease.

Integration of aCGH and gene expression

High-resolution aCGH analysis confirmed the consistent gain of chromosome 13 in CRCs and derived cell lines. While in most instances the entire chromosome was gained, we observed 2 common regions of recurrent focal amplifications. The genome coordinates of these amplicons map to chr13:20,856,880-29,466,246 and chr13:103,927,403-114,125,347, for the proximal and distal amplicon, respectively (Fig. 1A). All colon cancer samples and cell lines were also profiled on 44K gene expression arrays using RNA from a pool of 5 normal colon mucosa samples as reference. We identified 29 (37%) of 78 genes annotated in the microarray as located in the 2 amplified regions that were significantly overexpressed in the samples compared with normal colon mucosa (P < 0.05), supporting a positive correlation between copy number changes and gene expression (Fig. 1B). While our analysis was focused on genes that mapped to the 2 amplicons, we included several other genes located on chromosome 13 because they were consistently overexpressed. Overall, the integration of the array-based genomic copy number changes and gene expression datasets from CRC tumors and cell lines generated a list of 67 candidate genes. Functional validation of these candidate CRC genes requires in vitro models. To aid in the identification of suitable model systems for assessment of gene function, we further quantified the expression of these 67 candidate genes by qRT-PCR in 25 CRC cell lines (Supplementary Fig. S2). Overall, out of the 67 genes, overexpression of 44 genes (65%) was validated in 25 cell lines. We therefore conclude, consistent with previous analyses that the cell lines are adequate in vitro models (20). The list of 44 genes is presented in Supplementary Table S4. On the basis of this analysis, we chose the cell line SW480 for our functional analysis because it exhibits a gain of chromosome 13. We also used the cell line DLD-1, which does not show a gain of chromosome 13, but the candidate genes were overexpressed in an amplification-independent manner.

Functional analysis: loss-of-function compromises cell viability

To test the hypothesis that one or more genes located on chromosome 13, which are frequently amplified and overexpressed in CRC, are required for tumor survival and/or
growth, we used RNAi to identify genes whose LOF reduces the viability of either SW480 and/or DLD-1 CRC cells. We initially assessed viability by using 2 siRNAs for each of the 44 overexpressed genes (Supplementary Fig. S3A). On the basis of the results of the viability assays, 17 genes were selected for analysis with 2 additional siRNAs per gene. For 9 of those 17 genes, the additional siRNAs failed to recapitulate a loss of viability following gene silencing (Supplementary Fig. S3B). We therefore selected 8 genes that significantly induced a 15% or greater decrease in viability ($P < 0.05$) in one or more cell lines for further experimentation (Fig. 2).

The LOF of NUP1L1, DIS3, POMP, and LNX2 resulted in a substantial reduction of viability of both SW480 and DLD-1 cells with at least 2 siRNAs. SW480 cells were more sensitive to the LOF of KLF5, GPR180, SLC7A1, and POLR1D than the DLD-1 cells; this may be attributable to slight differences of the expression levels of these genes (Supplementary Table S5). However, in all instances the observed decrease of viability was paralleled by an efficient reduction of the target mRNA compared with control transfections with siNeg, as determined by qRT-PCR (Supplementary Fig. S4). Because only KLF5 has been functionally associated with CRC (21), we thus decided to use an unbiased approach to query the potential functional role of these 8 genes in the context of CRC.

Transcriptional signatures associated with loss-of-function by RNAi

Whole genome expression profiles of cells in which RNAi has been applied to silence a single gene can be used to identify, in an unbiased manner, genes and pathways perturbed as a consequence of this silencing, and thus the potential function of a specific gene. Gene expression profiles were generated from SW480 cells transfected with siRNAs corresponding to NUP1L1, LNX2, POLR1D, POMP, SLC7A1, DIS3, KLF5, and GPR180 (2 different siRNAs corresponding to each gene, triplicate-independent transfections for each siRNA), and with a negative control siRNA (siNeg). To define gene-specific expression profiles following RNAi, i.e., an RNAi signature, we first considered a significant change following silencing of each target gene as a probe that showed a ±0.6 or more log$_2$ ratio fold change in expression (corresponding to ~1.5-fold linear change in expression) with a $q$ value 0.05 or less when the siNeg expression profile was compared with the expression profiles generated for each gene-specific siRNA. This resulted in a remarkable correlation by the 2 siRNAs corresponding to each gene (Supplementary Fig. S5). All expression profiles showed a significant decrease in the expression levels of the target gene and the number of genes within each signature varied considerably from fewer than 50 genes to nearly 4,000 genes (Supplementary Fig. S6 and Supplementary Table S6).

The RNAi signatures for KLF5, NUP1L1, POMP, and LNX2 show altered expression of specific CRC-associated genes

To understand the biologic significance of these more extensive changes to the transcriptome, we began by investigating whether the LOF signatures were enriched for targets of transcription factors associated with CRC, including the vitamin D receptor (VDR), NF-κB, SMAD4, TCF7L2, β-catenin, and MYC (Fig. 3). The enrichment for the targets of CRC-associated transcription factors was very different for each of the 5 genes; silencing of POMP showed enrichment for the upregulation of targets of SMAD4 and VDR, as did NUP1L1, whereas the LNX2 RNAi signature showed highly significant enrichment for the downregulation of targets of TCF7L2. The silencing of DIS3 showed slight enrichment for gene targets of MYC within the downregulated portion of its RNAi signature, whereas silencing of KLF5 was associated with enrichment for the upregulation of TCF7L2 and NF-κB targets.

Consistent with the enrichment for NF-κB targets within those genes showing altered expression following LOF of the
Figure 3. Comparison of RNA signatures derived from candidate gene silencing with gene expression modules of transcription factors involved in CRC. Determination of the overlap of signature genes after RNAi-mediated silencing of POMP, NUPL1, KLF5, DIS3, and LNX2 with genes known to be either downregulated (A) or upregulated (B) by CRC-associated transcription factors TCF7L2, β-catenin, SMAD4, VDR, MYC, and NFκB (color code at the bottom of the figure). The red-dashed line indicates a significance threshold of P < 0.01.

KLF5 transcription factor, pathway analysis of the KLF5 RNAi signature generated a network centered upon NF-κB (Supplementary Fig. S7A). This network included the most downregulated gene in the KLF5 RNAi signature, FGF18, a gene involved in CRC (Fig. 4A; Supplementary Table S6; refs. 22–24). We have consistently observed increased expression of FGF18 in primary colon tumors and derived cell lines. Upon silencing of KLF5, the expression of FGF18 showed a 4-fold linear reduction in expression (Fig. 4B), suggesting that potentially the amplification and overexpression of KLF5 is contributing to the growth stimulation mediated by FGF18. The most upregulated gene within the KLF5 RNAi signature was the transcription factor SOX2 (Fig. 4A and Supplementary Table S6). Both in cell lines and in primary tumors, SOX2 expression is depleted when compared with normal colon mucosa, however, RNAi against KLF5 induced expression of SOX2 both at the mRNA and protein levels (Fig. 4B and C). SOX2 and KLF5 have both been linked to embryonic stem cell self-renewal with SOX2 the better characterized of these transcription factors (25, 26). These data though suggest a direct transcriptional link between suppression of KLF5 expression and upregulation of SOX2 that may have implications for both stem cell and cancer biology.

Silencing of NUPL1 decreased POU2AF1 expression (4-fold linear change), as well as another CRC-associated gene KIAA1199, a downstream target of VDR. Others and we have noted upregulation of KIAA1199 in our primary tumor dataset and in early colonic lesions (27). The expression of the VDR and SMAD4 transcription factor were also significantly enriched in the POMP RNAi upregulated gene signature (P < 1E–7), which overall was the largest RNAi signature including nearly 4,000 genes. Although the expression of TCF7L2 was decreased in the POMP transcriptomic signature, only slightly significant enrichment for this specific transcription factor was observed in the downregulated gene set (P = 0.036; Fig. 3A). Interestingly, significant enrichment for MYC was identified among the downregulated genes following LOF of POMP (P = 0.001).

Loss of LNX2 function results in changes in NOTCH and WNT/β-catenin pathway

Silencing of LNX2, ligand of numb-protein X 2, had a profound effect on viability and resulted in the deregulation of some 680 genes (Fig. 5A). LNX2 is thought to serves as a scaffold for the membrane protein NUMB. NUMB was originally identified as an important cell fate determinant that is asymmetrically inherited during mitosis and controls the fate of sibling cells by inhibiting the NOTCH signaling pathway in neural tissue. The LNX2 RNAi signature showed downregulation of NOTCH1. To confirm that silencing of LNX2 reduces the expression of NOTCH1, we conducted an independent set of transfections and used a different mRNA assay (Fig. 5B) and also examined NOTCH1 protein levels (Fig. 5C). We consistently observed a downregulation of NOTCH1 expression following LNX2 silencing. While we observed no effect on NUMB mRNA levels, NUMB protein levels were decreased following LNX2 LOF, as too was the expression of JAGGED1, the ligand for the receptor NOTCH1 (Fig. 5C). In addition, pathway analysis of the LNX2 RNAi signature identified NOTCH1 as part of a large molecular network (Supplementary Fig. S7B). To examine whether the effects on the levels of NOTCH1 were associated with a decrease in the NOTCH signaling pathway, we inquired known downstream targets of NOTCH1 within the LNX2 RNAi signature. Interestingly, several NOTCH1 signaling targets, including HES5, HES6, HEY2, and LFNG were downregulated (P < 0.05; Fig. 5D). Furthermore, Western blot analysis confirmed the reduction of HEY2 and also identified a decrease of HES1 (Fig. 5E), showing that the expression of downstream targets of the NOTCH signaling pathways is reduced. These data suggest a direct link between LNX2 expression and NOTCH1 signaling in SW480 cells.
The other notable feature of the LNX2 RNAi signature was the highly significant enrichment for targets of the transcription factor TCF7L2 (Fig. 3A). TCF7L2 is the main effector of canonical WNT signaling. Of the 272 genes downregulated following silencing of LNX2, 78 are putative TCF7L2 target genes ($P = 1.96 \times 10^{-8}$, Benjamini–Hochberg corrected Fisher exact test). These 78 genes included the proliferative and antiapoptotic genes MYB, CCND1, BCL2, POU2AF1, and ERBB4. Similarly, target genes for the TCF7L2-binding partner and transcriptional coactivator β-catenin (CTNNB1) were also enriched within this downregulated set (28 of 272; $P = 0.049$). Eleven downregulated targets of β-catenin were also targets of TCF7L2. After combining the downregulated TCF7L2 and β-catenin gene targets into a single list (95 total), we found it to be enriched for functional ontologies such as gastrointestinal disease ($P = 0.001$), cancer ($P = 0.002$), and cell cycle ($P = 0.008$).

The transcription factor TCF7L2 is the dominant effector of the WNT pathway and responsible for transcriptional regulation of genes involved in WNT signaling. The enrichment for TCF7L2 gene targets in the LNX2 RNAi signature suggests a potential link between the WNT pathway and LNX2 function, and as an extension of this, between chromosome 13 amplification and activation of WNT signaling. To test this hypothesis, we first examined the expression levels of TCF7L2 in the expression profiles obtained following LNX2 silencing and did observe a decrease in TCF7L2 expression (Fig. 6A). This was confirmed at the protein level 96 hours post-LNX2 siRNA transfection (Fig. 6B). Furthermore, TCF7L2 is present in one of the top networks generated by pathway analysis of the LNX2 RNAi signature (Supplementary Fig. S7C). Importantly, we next investigated the role of LNX2 in regulating WNT pathway activity using a reporter assay. Using the TOP-FOP-Flash reporter system, we showed a significantly decreased WNT/β-catenin activity in SW480 cells after gene silencing of LNX2 when compared with negative control ($P < 0.0001$; Fig. 6C). In addition, by Western blot analysis, we observed a decrease of the protein levels of CTNNB1 upon treatment with siRNAs against LNX2 (Supplementary Fig. S8).

Finally, toward a complete characterization of the mechanism underlying the reduction in viability following LNX2 silencing, we assessed 2 cellular markers of apoptosis: Annexin V levels by flow cytometry analysis and cleavage of PARP using Western blot analysis. A time-course at 48, 72, and 96 hours posttransfection showed a sequential activation of apoptosis, which on average for the 2 siRNAs reached some 45% of Annexin V–positive cells at 96 hours (Fig. 6D). This activation perfectly correlates with increased levels of cleaved PARP and
the absence of LNX2 protein (Fig. 6E). Our analysis therefore suggests that silencing of LNX2 results in altered NOTCH1 and WNT signaling and activation of apoptosis.

**The LNX2-TCF7L2 module is recapitulated in clinical samples and correlates with expression of LNX2**

To detect biologically relevant modularity using predefined ontologies and gene expression data (28), we constructed a LNX2-TCF7L2 module from the 78 TCF7L2 target genes, listed in Supplementary Table S7, which were significantly down-regulated after silencing LNX2. To further investigate LNX2 association with this gene module, we analyzed the correlation between LNX2 expression and the average expression of all module genes using 3 independent CRC gene expression datasets containing more than 500 patient samples. This meta-analysis resulted in highly significant correlations in all 3 datasets (GSE14333: N = 290, R = 0.52, P = 2.2E−16; GSE17536: N = 177, R = 0.44, P = 5.6E−10; GSE17537: N = 49, R = 0.39, P = 0.006; shown for dataset GSE14333 in Fig. 6F). Next, using an additional unpublished dataset, we proved that this correlation was also present in normal samples (R = 0.53; P = 7.01E−06) and indeed found that the gene module could form distinct clusters separating normal and cancer specimens (Supplementary Fig. S9). Taken together, these data strongly
support the assertion that LNX2 serves as a regulator of TCF7L2 transcriptional activity and of subsequent downstream WNT signaling. This is entirely consistent with a central involvement of this gene in CRC and with the frequent gain and amplification of chromosome 13.

Discussion

One major mechanism to constitutively overexpress cancer-promoting genes is through genomic amplification of their respective locus. Genes that reside in regions of recurrent genomic amplification and are overexpressed are prime candidates for bona fide oncogenes (29). The gain of chromosome 13 is one of the most common aberrations in CRC and is found in some 60% to 80% of cases, often in the form of a high-level gain of the entire chromosome (3, 8, 30). It is now abundantly clear that gene expression levels are positively correlated with genomic copy number (13). Gains or losses of entire chromosomes or chromosome arms therefore result in aberrant transcript levels of many, if not most, of the resident genes (11, 12, 14, 31). This, of course, hampers the identification of candidates that drive the acquisition of such recurrent genomic imbalances. It has also been shown that the low level upregulation of genes on commonly gained chromosomes often involves genes that favor metabolic activity, and therefore may act in concert with specific oncogenes to promote growth and tumorigenesis (10). While we explicitly do not exclude the possibility that additional driver genes on chromosome 13 play a role in CRC [for instance, CDK8 (32) and CDX2 (33)], we considered it a reasonable strategy to initially concentrate on the expression of a module of genes regulated by the transcription factor TCF7L2 and downregulated signature genes after silencing LNX2 in a set of 290 primary CRC samples. Y-axis, expression levels of LNX2 in primary CRCs; x-axis, expression levels of the TCF7L2 module. The correlation indicates a direct influence of LNX2 on the expression of TCF7L2-regulated genes and consequently the genomic amplification of chromosome 13 and activation of WNT-signaling.
unbiased manner, explored the function of these candidates in CRC cells using genome-wide expression profiling after gene silencing.

Only 2 of the candidate genes studied, KLF5 and POLR1D, had been previously implicated in CRC tumorigenesis (21, 34, 35); however, limited functional association with molecular pathways involved in colorectal carcinogenesis has been reported. High-resolution DNA copy number profiles of stage II colon cancers identified KLF5, which encodes a Zn-finger transcription factor of the Kruppel-like family, as residing in a distinct focal amplification (34). The presence of this amplification is associated with poor overall survival (36). In our study, silencing of KLF5 strongly induced the expression of SOX2, a transcription factor involved in the maintenance of the undifferentiated state in embryonic stem cells. Although SOX2 has been proposed as an amplified lineage-survival oncogene in squamous cell lung cancer (37) and esophageal squamous cell carcinomas (38), its role in CRC remains to be elucidated.

The LOF of LNX2 resulted in a striking reduction of viability of CRC cell lines, and LNX2 showed the highest enrichment of TCF7L2 target genes (P < 10E−12) and evidence of disruption of NOTCH signaling. LNX2 was a particularly interesting candidate because we and others have shown that it localizes in a minimal region of amplification at chromosome band 13q12.2 (chr13:26,694,966-27,905,729; Supplementary Fig. S10; ref. 30). The important role of LNX2 is also supported by the overexpression observed in tumors compared with normal mucosa (Supplementary Fig. S11), and by data from the Protein Atlas, where it is shown to be strongly and exclusively overexpressed in CRC (39). One role of LNX proteins is to serve as a molecular scaffold that guides NUMB to specific sites in the cell (40). The LNX2 family-related member, LNX1, is required for NUMB ubiquitylation causing proteasome-dependent degradation and enhanced NOTCH signaling (41). The tight regulation of WNT and NOTCH signaling is essential for the development and homeostasis of intestinal epithelial cells (42–44). Aberrant NOTCH signaling, on the other hand, is intricately involved in colorectal tumorigenesis (45). Our data show that silencing of LNX2 results in downregulation of NOTCH1 and several downstream targets, including HE51, HE55, HE56, HEY2, and LFNG. In fact, the effect of NOTCH1 on cell proliferation depends on active WNT (its effect on differentiation does not; refs. 43, 46). The cross-talk between the WNT/β-catenin pathway and NOTCH has been shown to be involved in the control of cell proliferation and colorectal tumorigenesis in mice (46). One of the candidates to mediate such a cross-talk is JAGGED1 (47, 48). Interestingly, expression of JAGGED1 was decreased after silencing of LNX2, suggesting that LNX2 may operate upstream of JAGGED1 in its contribution to the control of NOTCH and WNT/β-catenin pathway cooperation. However, we cannot exclude that deregulation of JAGGED1 was a direct effect of deregulation of the WNT/β-catenin pathway (47). Our observation that silencing of LNX2 not only resulted in reduced NOTCH activity, but also in a significant reduction of the expression of TCF7L2, is entirely consistent with the interplay of NOTCH and WNT signaling. Reduced expression of TCF7L2 was accompanied by the intuitive depression of WNT/β-catenin activity as shown using the TOP-FOP-Flash reporter assay subsequent to LNX2-silencing. β-Catenin and TCF7L2 are the downstream effectors of the WNT signaling cascade (49), and according to our data the expression of these 2 molecules is modulated by LNX2. This is the first data that suggest that the WNT and NOTCH signaling pathways are coregulated in CRC. As a result of LNX2 upregulation, mediated by the amplification of chromosome 13, there is an increase in WNT/β-catenin signaling, thus promoting proliferation through TCF7L2 transcriptional targets (Supplementary Fig. S12). We believe that the significant reduction of viability as a consequence of LOF of LNX2 is a reflection of the fact that it stimulates 2 critical cancer pathways simultaneously. The correlation of the expression of LNX2-TCF7L2 signature genes with LNX2 levels was validated in a large set of clinical samples, indicating that genes that depend on expression of the transcription factor TCF7L2 correlate with those genes that are affected when suppressing the expression of an upstream regulator of the WNT signaling pathway.

The finding that there is such an abundance of chromosome 13 genes involved in crucial CRC signaling pathways is entirely consistent with the frequent genomic amplification of chromosome 13 in CRC at the transition from dysplastic adenomas to invasive disease (3). This supports the hypothesis of tumor-specific pathway addiction. It seems indeed to be the case that not only one, but several, if not many, genes on that chromosome activate pathways required for colorectal carcinogenesis (32, 33), a notion that reinforces the idea that this chromosome needs to be present in extra copies in the majority of CRC in a tissue-specific manner. In essence, chromosome 13 is a CRC chromosome.

In summary, we have identified a set of genes that are highly and consistently amplified and overexpressed in CRC, and we have inferred the mechanism of action by which these genes might have a role in the cellular viability of CRC cells. By analyzing the gene expression signatures after RNAi-mediated gene silencing, followed by targeted functional analysis, we identified LNX2, a gene that was not associated with cancer before, as mediating the cross-talk between WNT and NOTCH signaling cascades and regulating WNT/β-catenin activity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
References


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