IncRNA MIR100HG-derived miR-100 and miR-125b mediate cetuximab resistance via Wnt/β-catenin signaling

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De novo and acquired resistance, which are largely attributed to genetic alterations, are barriers to effective anti-epidermal-growth-factor-receptor (EGFR) therapy. To generate cetuximab-resistant cells, we exposed cetuximab-sensitive colorectal cancer cells to cetuximab in three-dimensional culture. Using whole-exome sequencing and transcriptional profiling, we found that the long non-coding RNA MIR100HG and two embedded microRNAs, miR-100 and miR-125b, were overexpressed in the absence of known genetic events linked to cetuximab resistance. MIR100HG, miR-100 and miR-125b overexpression was also observed in cetuximab-resistant colorectal cancer and head and neck squamous cell cancer cell lines and in tumors from colorectal cancer patients that progressed on cetuximab. miR-100 and miR-125b coordinately repressed five Wnt/β-catenin negative regulators, resulting in increased Wnt signaling, and Wnt inhibition in cetuximab-resistant cells restored cetuximab responsiveness. Our results describe a double-negative feedback loop between MIR100HG and the transcription factor GATA6, whereby GATA6 represses MIR100HG, but this repression is relieved by miR-125b targeting of GATA6. These findings identify a clinically actionable, epigenetic cause of cetuximab resistance.

Colorectal cancer (CRC) remains a leading cause of cancer-related death worldwide. Cetuximab and panitumumab are EGFR monoclonal antibodies (mAbs) that bind the extracellular domain of EGFR and enhance receptor internalization and degradation. These EGFR mAbs are common targeted agents for patients with wild-type KRAS metastatic CRC. When given as a monotherapy, EGFR mAbs elicit durable responses in 12–17% of patients, and up to 72% response rates are reported when the mAbs are combined with chemotherapy. However, drug resistance frequently arises. Intense efforts have led to the identification of many de novo and acquired genetic mechanisms of resistance to EGFR mAb therapy, including KRAS, NRAS, BRAF, PIK3CA and EGF mutations. However, little is known about non-genetic resistance mechanisms.

Non-coding RNAs (ncRNAs), particularly long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), are crucial for epigenetic regulation. Recently, a complex interaction between these two classes of regulatory ncRNAs has been discovered in which some lncRNAs are processed to produce miRNAs that repress target mRNAs. For example, lncRNA-H19-derived miR-675 suppresses the translation of insulin growth factor receptor, inhibiting cell proliferation in response to cellular stress or oncogenic signals. miR-17–92, generated from the lncRNA MIR17HG locus, attenuates TGF-β signaling to stimulate angiogenesis and tumor growth. The IncRNA MIR100HG-derived miR-100, let-7a-2 and miR-125b-1 cluster and the MIR99AHG-derived miRNA miR-99a, let-7c and miR-125b-2 cluster participate in the pathogenesis of acute megakaryoblastic leukemia. However, whether these lncRNAs or derived miRNAs contribute to drug resistance is largely unknown.

We identified a role for the lncRNA MIR100HG and two embedded miRNAs, miR-100 and miR-125b, in conferring cetuximab resistance. We found that MIR100HG, miR-100 and miR-125b were overexpressed in the setting of de novo and acquired cetuximab resistance in CRC and head and neck squamous cell cancer (HNSCC) cell lines. miR-100 and miR-125b coordinately downregulated five negative regulators (DKK1, DKK3, ZNRF3, RNF43 and APC2) of canonical Wnt/β-catenin signaling (hereafter referred to as Wnt signaling), leading to increased Wnt signaling. Wnt inhibition restored responsiveness to cetuximab in vitro and in vivo. We found that these events
occurred in CRC patients whose tumors progressed on cetuximab. We also discovered that MIR100HG overexpression was reinforced by miR-125b suppression of GATA6, which at steady state represses MIR100HG. Our findings identify an epigenetic cause of cetuximab resistance with diagnostic and therapeutic implications.

RESULTS
Establishment of cetuximab-resistant cells in three-dimensional culture
We placed single cells from a human KRAS/NRAS/BRCA wild-type, microsatellite-unstable CRC cell line, HCA-7, into three-dimensional (3D) culture in type-1 collagen to derive a cell line from colonies with cystic morphology, which we designated cystic colonies (CC)

Proliferation of CC was inhibited by cetuximab in 3D culture, but not in two-dimensional (2D) plastic culture

Figure 1 Characterization of CC-CR in 3D culture. (a) Schematic of experimental approach to establish cetuximab (CTX)-resistant cells in 3D culture. In the presence of CTX (3 µg/ml) in 3D type-1 collagen culture, greater than 95% of CC colonies died. Residual colonies were isolated and passaged into 3D culture for 150 rounds. (b) Top, differential interference contrast (DIC) and confocal images of representative CC and CC-CR in 2D and 3D culture. F-actin was stained with phalloidin (red). Scale bars represent 400, 1,000, 200 and 50 µm, respectively (from left to right). Bottom left, number of nuclei in the midplane of each colony. Bottom right, the morphology of colonies was divided into those with luminal, multi-layered, or solid morphology (n = 3 independent experiments performed in triplicate). **P < 0.01 by Student’s t test. (c) CC and CC-CR were cultured in 3D culture in the presence or absence of CTX (3 µg/ml) and colonies were counted after 18 d (n = 3 independent experiments performed in triplicate). **P < 0.01 by Student’s t test. (d) CC and CC-CR cells were cultured in 3D culture for 12 d and treated with CTX (10 µg/ml) for 24 h. Ki-67 (red) and cleaved Caspase-3 (Cleaved Casp-3, green) staining were imaged by confocal microscopy. Representative of four independent experiments. Scale bar represents 50 µm. Quantification is shown on the right (n = 4). **P < 0.01 by Student’s t test. (e) Immunoblots of 3D cell lysates from CC and CC-CR treated with CTX (10 µg/ml) for indicated time. β-actin served as the loading control. A representative blot from three independent experiments is shown. (f) Nude mice (n = 8) bearing subcutaneous tumors were treated with control saline or CTX at a dose of 1 mg per mouse, intraperitoneal (i.p.) injection, every 3 d. Tumor volumes were measured every 3 d using calipers. **P < 0.01 by repeated-measures analysis of variance (ANOVA) test followed by least significant difference (LSD) post hoc test. (g) Representative immunohistochemical images of Ki-67 and cleaved Casp-3 from CC and CC-CR xenografts before and after CTX treatment. Scale bar represents 50 µm. Data represent mean ± s.d. in b–d and f, n.s., not significant.
In cetuximab-treated CC, we observed reduced levels of p-EGFR, p-ERK1/2, p-AKT and Cyclin D1, as well as increased cleaved Caspase-3 and the pro-apoptotic marker BIM; these markers were largely unaffected in cetuximab-treated CC-CR (Fig. 1e). Next, we stably transduced CC and CC-CR with a GFP-expressing lentiviral vector, and then subcutaneously injected the cell lines into athymic nude mice. CC tumors were well differentiated and regressed following administration of cetuximab. CC-CR tumors were poorly differentiated and continued to grow in the presence of cetuximab, although not to the extent of untreated tumors (Fig. 1f,g and Supplementary Fig. 1c–e).

Upregulation of MIR100HG and embedded miR-100 and miR-125b in cetuximab-resistant cells

We first considered known mechanisms of cetuximab resistance in this 3D model. Using whole-exome sequencing and RNA sequencing (RNA-Seq), we found no known genetic events that were linked to cetuximab resistance, including all reported gene mutations, copy number changes and gene fusion events (Supplementary Table 1). Using RNA-Seq, we found that 141 transcripts were upregulated and 220 transcripts were downregulated in CC-CR compared with CC (fold change > 2 and false-discovery rate (FDR) < 0.01). Expression levels of ERBB1–4, the seven EGFR ligands and MET were comparable between CC and CC-CR (Supplementary Table 2). Immunofluorescence also revealed equivalent cell-surface EGFR staining in CC and CC-CR (Supplementary Fig. 1f). Small RNA-Seq detected seven miRNAs that were upregulated and 24 miRNAs that were downregulated in CC-CR compared with CC (fold change > 2 and FDR < 0.01). Notably, the most upregulated transcript in CC-CR was the lncRNA MIR100HG, and the two most upregulated miRNAs were miR-125b and miR-100 (Fig. 2a).

MIR100HG is the host gene of the miR-100, let-7a-2 and miR-125b-1 cluster on chromosome 11 (Fig. 2b). Quantitative reverse transcription PCR (qRT-PCR) analysis confirmed the upregulation of endogenous MIR100HG expression in CC-CR in the presence or absence of cetuximab (Fig. 2c). pri-miR-100, pri-miR-125b-1 and their corresponding mature miRNA, miR-100 and miR-125b, were also enriched in CC-CR (Fig. 2c and Supplementary Fig. 2a). Although pri-let-7a-2 was upregulated in CC-CR, mature let-7a expression was unchanged compared with CC (Supplementary Fig. 2b). The transcriptional start site (TSS) of MIR100HG was confirmed by 5’ RACE-PCR (Supplementary Fig. 2c). Analysis of The Cancer Genome Atlas (TCGA) CRC data repository revealed that miR-100 and miR-125b expression was tightly correlated with MIR100HG expression (Fig. 2d). RNA fluorescence in situ hybridization (FISH) revealed highly enriched MIR100HG, miR-100 and miR-125b expression in CC-CR tumor xenografts (Fig. 2e). In contrast, let-7a expression did not correlate with that of MIR100HG (Supplementary Fig. 2d).

To assess whether MIR100HG, miR-100 and miR-125b overexpression extended beyond this one cell line, we examined their expression in a panel of 30 CRC cell lines placed on a continuum of cetuximab sensitivity and resistance based on published reports (Supplementary Table 3). Expression of MIR100HG, miR-100 and miR-125b were enriched in more cetuximab-resistant lines than the more sensitive lines (Fig. 2f). Their expression inversely correlated with cetuximab resistance, regardless of KRAS/BRCA mutation status (Supplementary Fig. 2e,f). For example, two of the cetuximab-sensitive lines (GEO and SW403) expressed low levels of MIR100HG, miR-100 and miR-125b despite harboring mutant KRAS. In addition, we also observed upregulation of MIR100HG, miR-100 and miR-125b in the setting of cetuximab resistance in HNSCC cell lines (Supplementary Fig. 3a). Thus, MIR100HG, miR-100 and miR-125b were upregulated in the setting of cetuximab resistance in CRC and HNSCC cell lines, and this phenomenon occurred in both acquired and de novo resistance. These findings led us to further explore the function of MIR100HG, miR-100 and miR-125b in cetuximab resistance.

miR-100 and miR-125b cooperativity drives cetuximab resistance

Given that a major role of certain lncRNAs is the production of embedded miRNAs, we asked whether cetuximab resistance is mediated by miR-100 and miR-125b overexpression. To this end, we delivered lentiviral-overexpression and sponge constructs into CC and CC-CR, respectively, to generate stable cell lines expressing each miRNA, the miR-100/miR-125b bicistron or their corresponding sponges (Supplementary Fig. 3b,c). Although the miR-100 sponge had no significant effect on colony number in CC-CR in 3D culture, both the miR-125b and bicistron sponges significantly reduced colony number (Fig. 3a). In the presence of cetuximab, the miR-100 sponge modestly reduced colony number, whereas the reduction in colony number was more pronounced with the miR-125b sponge and the bicistron sponge (Fig. 3a). Opposite effects were observed in CC following overexpression of miR-100 and miR-125b, both individually and together. The miR-100/miR-125b bicistron, but not individual miRNAs, increased colony number in CC (Fig. 3b). Following cetuximab treatment, the miR-100/miR-125b bicistron conferred the strongest pro-survival effect; when introduced individually, miR-125b had a greater effect than miR-100 (Fig. 3b). Similar opposing effects were observed in morphological changes, as well as Ki-67 and cleaved Caspase-3 staining following the expression of the different sponges in CC-CR and the different miRNAs in CC (Fig. 3c,d and Supplementary Fig. 3d,e). In addition, overexpression of the miR-100/miR-125b bicistron in Caco-2 cells (low endogenous miR-100/miR-125b expression) rendered cells less responsive to cetuximab, whereas inhibition of the miR-100/miR-125b bicistron in DLD-1 cells and HNSCC SCC25-derived CTX-R7 cells (both with high endogenous miR-100/miR-125b expression) restored cetuximab responsiveness (Supplementary Fig. 4). Similar results were observed when CC-CR and CC cells with the differing manipulations were established as subcutaneous xenografts in nude mice and treated with cetuximab (Fig. 3e,f and Supplementary Fig. 5). Together, these results are consistent with a model in which miR-100 and miR-125b cooperate to confer cetuximab resistance.

miR-100 and miR-125b repress multiple Wnt negative regulators and increase Wnt signaling

To understand how miR-100 and miR-125b influence cetuximab responsiveness, we considered the most-downregulated genes in CC-CR and CC and gene fusion events (Supplementary Table 4). Using RNA-Seq, we found that 141 transcripts were upregulated and 220 transcripts were downregulated in CC-CR compared with CC (fold change > 2 and false-discovery rate (FDR) < 0.01). Each miRNA, the miR-100/miR-125b bicistron or their corresponding sponge significantly reduced Wnt signaling (Fig. 4a). To identify Wnt signaling targets, we performed a de novo stream index of RAS signaling (Supplementary Fig. 6a) and data analysis. Analysis of a large human CRC data set (n = 458 cases) also revealed that MIR100HG expression levels positively correlated with the Wnt score (Supplementary Fig. 6a), whereas no correlation was observed between MIR100HG and the Ras-Az score (Supplementary Fig. 6a), which measures MEK activation as a downstream index of RAS signaling. To identify miRNAs with potential to be functional enrichment analysis further identified Wnt pathway enrichment in miR-100 and miR-125b putative targets (Supplementary Table 4). Thus, we considered whether miR-100 and miR-125b might target components of Wnt signaling. Using
Figure 2 Transcriptome profiling of CC and CC-CR in 3D culture. (a) Left, heatmap of the top 50 differentially expressed transcripts in CC-CR versus CC from three independent 3D culture experiments. Gene expression values are gene-wise z-transformed and are colored red for high abundance and blue for low abundance, as indicated in the scale bar. Right, miRNA heatmap showing miRNAs altered (greater than twofold and FDR < 0.01) in CC-CR compared with CC xenografts. Concomitantly, high miR-100 (green) and miR-125b (red) signals were observed in CC-CR tumors; the yellow fluorescent signal indicates coexpression of miR-100 and miR-125b. Scale bars represent 50 μm. (b) Genomic organization of lncRNA MIR100HG, host gene of the miR-100/let-7a-2/miR-125b-1 cluster, on human chromosome 11 (hsa chr11). (c) qRT-PCR showing upregulation of lncRNA MIR100HG, miR-100 and miR-125b in CC-CR compared with CC in 3D culture. In CC-CR, cells were treated with CTX (CTX+, 3 μg/ml) or normal culture medium (CTX−) for 14 consecutive days in 3D culture. ACTB or U6 snRNA served as the internal control, respectively. Fold changes were calculated by CT method. **P < 0.01 by one-way ANOVA followed by Dunnett's test. (d) Scatter plots of MIR100HG versus miR-100 or miR-125b expression in the TCGA CRC data repository. Pearson correlation coefficients (r) and P values are shown. (e) RNA FISH showing high MIR100HG (red) expression in CC-CR mouse tumor xenografts compared with CC xenografts. Concomitantly, high miR-100 (green) and miR-125b (red) signals were observed in CC-CR tumors; the yellow fluorescent signal indicates coexpression of miR-100 and miR-125b. Scale bars represent 50 μm. (f) qRT-PCR analysis of MIR100HG, miR-100 and miR-125b expression levels among a panel of 30 CRC cell lines ranked by their responsiveness to cetuximab (Supplementary Table 3). ACTB or U6 snRNA served as the internal control. Fold changes were normalized to CC. n.d., not detected. n = 3 independent experiments performed in triplicate. Data represent mean ± s.d. in c and f.
Figure 3 Cooperativity of miR-100 and miR-125b in cetuximab (CTX) resistance. (a,b) Indicated cells were grown in 3D culture in normal medium (CTL) or treated with CTX (3 µg/ml) in 3D culture. The resultant colonies were counted after 18 d (sponge, Sp). n = 3 independent experiments performed in triplicate. *P < 0.05, **P < 0.01 by one-way ANOVA followed by Dunnett’s test compared with CTL-Sp or miR-CTL. (c,d) Left, indicated cells were cultured in 3D culture for 12 d and CTX (10 µg/ml) was added for 24 h before cells were fixed, stained for cleaved Casp-3 (cyan) and Ki-67 (magenta). Scale bars represent 50 µm. Right, quantification of the morphological changes among indicated cell lines. n = 4 independent experiments. (e,f) Left, indicated cells were injected subcutaneously into nude mice (n = 8). After tumor size reached approximately 100 mm³, the mice received CTX treatment (1 mg per mouse, i.p. injection every 3 d). Representative fluorescent images of GFP signals captured from subcutaneous tumors are shown. Middle, growth curve of tumors in nude mice (n = 8) injected with cells as indicated. **P < 0.01 by repeated-measures ANOVA test followed by Dunnett’s test. Right, tumors (n = 8) were isolated on day 28 after treatment and tumor weight was calculated. **P < 0.01 by one-way ANOVA followed by Dunnett’s test compared with CTL-Sp or miR-CTL. Data represent mean ± s.d. n.s., not significant.
computational target prediction, we found that the 3' untranslated regions (UTRs) of DKK1 and DKK3 contain binding sites for miR-100 and miR-125b, respectively (Fig. 4a). Given that clustered miRNAs are coexpressed and often coordinately regulate molecular pathways by targeting different components of the same pathway, we searched for other negative regulators of Wnt signaling that contain putative binding sites for miR-100 or miR-125b, and identified zinc and ring finger 3 (ZNRF3), ring finger protein 43 (RNF43) and APC2 as potential targets of miR-100 or miR-125b alone or in combination (Fig. 4a and Supplementary Table 5). Decreased protein levels of these five Wnt negative regulators in CC-CR compared with CC were confirmed by both immunoblots in cell lines and immunostaining in xenografts (Supplementary Fig. 6b,c). Using 3' UTR luciferase reporter assays, we confirmed these five candidates are direct targets of miR-100 and/or miR-125b in both CC and Caco-2 cells; repression of these genes was rescued by mutations in the corresponding binding sites (Fig. 4b,c and Supplementary Fig. 6d). Immunoblots confirmed the regulation of the predicted targets by miR-100 and miR-125b alone or in combination in CC and CC-CR (Fig. 4d). Consistently, this regulation was also observed in Caco-2 cells (low endogenous miR-100/miR-125b expression) and HuTu80 cells (high endogenous miR-100/miR-125b expression) (Supplementary Fig. 6e).

We next examined whether miR-100- and miR-125b-induced downregulation of these Wnt negative regulators results in increased Wnt signaling. Although total β-catenin levels were not significantly altered, CC-CR exhibited increased active tyrosine phosphorylated β-catenin levels (Fig. 4f). Consistent with previous reports, we also observed a decrease in nuclear β-catenin (Fig. 4f). We next examined whether miR-100- and miR-125b-induced downregulation of these Wnt negative regulators results in increased Wnt signaling. Although total β-catenin levels were not significantly altered, CC-CR exhibited increased active tyrosine phosphorylated β-catenin levels (Fig. 4f). Consistent with previous reports, we also observed a decrease in nuclear β-catenin (Fig. 4f).
p-Y489 β-catenin and increased nuclear β-catenin compared with CC in 3D culture (Fig. 4c and Supplementary Fig. 6f). Consistently, β-catenin was largely confined to the plasma membrane in CC xenografts, whereas it was largely nuclear in CC-CR xenografts (Fig. 4f). Moreover, mRNA expression of a panel of Wnt target genes was significantly enriched in CC-CR versus CC (Fig. 4g). Cetuximab blocked Wnt3a-induced Wnt activation in CC, but had no obvious effect on Wnt3a-induced Wnt signaling in CC-CR (Supplementary Fig. 6g). Cetuximab treatment also led to a marked and persistent decrease in Wnt target genes in CC over 48 h, whereas expression of those genes in CC-CR was only modestly decreased at early time points after treatment, and rebounded at later time points (Supplementary Fig. 6h). Furthermore, nuclear β-catenin levels increased in CC and Caco-2 cells stably overexpressing either miR-100 or miR-125b, and the increase was greater in cells expressing the miR-100/miR-125b bicistron (Supplementary Fig. 7a). In contrast, nuclear β-catenin levels were reduced following overexpression of the miR-100/miR-125b bicistron sponge in CC-CR, DLD-1 and CTX-R7 cells (Supplementary Fig. 7a). Corresponding changes in Wnt target genes were also observed (Fig. 4h and Supplementary Fig. 4e). Consistent with these findings, nuclear β-catenin immunoreactivity increased in CC nude mouse xenografts expressing the miR-100/miR-125b bicistron and decreased in their CC-CR counterparts expressing the bicistronic sponge (Supplementary Fig. 7b).

On the basis of our findings that Wnt signaling is increased in CC-CR, we hypothesized that cetuximab responsiveness may be restored by suppression of Wnt signaling. Given that DKK1 and DKK3 are secreted Wnt antagonists and are among the most downregulated genes in CC-CR, we tested whether their overexpression could overcome cetuximab resistance using a doxycycline-inducible lentiviral system22. Although induction of DKK1 or DKK3 resulted in a slight reduction in colony number, this effect was augmented by the addition of cetuximab (Supplementary Fig. 8a,b). Moreover, administration of recombinant DKK1 and DKK3 enhanced the ability of cetuximab to decrease proliferation and increase apoptosis (Supplementary Fig. 8c).

Furthermore, nuclear β-catenin expression decreased when DKK1 or DKK3 was inductively expressed in CC-CR in the presence of cetuximab (Supplementary Fig. 8d). We next tested whether pharmacological inhibition of Wnt activity sensitizes CC-CR to cetuximab using a tankyrase inhibitor, XAV-939 (ref. 23), and a β-catenin degradation complex, ICG-001 (ref. 24). Both compounds caused a concentration-dependent reduction in colony number, and cetuximab growth inhibition was enhanced by their addition (Supplementary Fig. 8e). ICG-001 also enhanced the growth inhibitory effects of cetuximab in other CRC and HNSCC cell lines with high expression of MIR100HG (Supplementary Figs. 4e and 8f). In CC-CR nude mouse xenografts, administration of cetuximab and ICG-001 individually only slowed tumor growth; however, combined treatment resulted in tumor regression (Fig. 4i–k and Supplementary Fig. 8g,h). Thus, blockade of Wnt signaling, either upstream or downstream of the APC/β-catenin degradation complex, restores cetuximab responsiveness to cetuximab-resistant cells.

Reciprocal negative regulation between GATA6 and MIR100HG/miR-125b

To explore the mechanism(s) by which miR-100 and miR-125b are upregulated in CC-CR, we investigated transcriptional regulation of the host gene MIR100HG. Possible transcription factors containing binding sites in the 2.5-kb promoter of MIR100HG were mapped in silico using the Match program (version 1.0)25 and cross-referenced with the RNA-Seq data set (Supplementary Table 6). Among these transcription factors, we focused on the zinc-finger transcription factor GATA6, which was downregulated at both the mRNA and protein level in CC-CR in 3D culture and in nude mouse xenografts (Figs. 2a and 5a–c).

GATA6 is critical for gut endoderm development, and it both promotes and suppresses gastrointestinal and pancreatic neoplasia26–29. We found that MIR100HG expression decreased in cetuximab-treated CC, whereas GATA6 mRNA progressively increased over 48 h (Fig. 5d); however, this phenomenon did not occur in CC-CR (data not shown). GATA6 knockdown in CC (Fig. 5e, top, and Supplementary Fig. 9a) resulted in MIR100HG upregulation, and its expression no longer decreased following cetuximab treatment (Fig. 5e, bottom), suggesting a repressive effect of GATA6 on MIR100HG. Luciferase reporter assays revealed that overexpression of GATA6 (Supplementary Fig. 9b) resulted in a concentration-dependent inhibition of MIR100HG promoter activity (Fig. 5f).

Four putative GATA binding sites (G/A)GATA(A/T) were identified in the MIR100HG promoter region (Fig. 5g). Sequential deletions and mutations of these binding sites revealed that GATA-binding site 2 (−1,198 upstream of the TSS) is the major site for GATA6 repression of MIR100HG transcriptional activity (Fig. 5h). GATA6 repression of MIR100HG was also validated in HuTu80 cells, which have low expression of GATA6 and high expression of MIR100HG (Supplementary Fig. 9c,d). Chromatin occupancy of GATA6 at GATA-binding site 2 was confirmed by chromatin immunoprecipitation and electromobility shift assay using nuclear extracts from CC cells (Supplementary Fig. 9e,f).

Of interest, we found that the 3′ UTR of GATA6 harbors a putative binding site for miR-125b (Supplementary Table 5). In both CC and Caco-2 cells, introduction of miR-125b reduced luciferase activity of the wild-type 3′ UTR reporter construct, but not when the miR-125b site was mutated (Fig. 5i and Supplementary Fig. 9g). As predicted, GATA6 levels were reduced in CC and Caco-2 cells stably expressing miR-125b, and conversely increased in CC-CR and HuTu80 cells expressing the miR-125b sponge (Fig. 5j and Supplementary Fig. 9h).

Further analysis of the TCGA data repository revealed that GATA6 was significantly downregulated, whereas MIR100HG was significantly upregulated, in stage IV CRC patients (Fig. 5k). In addition, CRCs with lower quartile expression of GATA6 tended to have higher expression of MIR100HG in the TCGA data repository (Fig. 5k), as well as in two additional CRC data sets (Supplementary Fig. 9i). Taken together, these findings suggest that a double-negative regulatory circuit between GATA6, MIR100HG and miR-125b underlies cetuximab resistance.

Increased MIR100HG, miR-100 and miR-125b expression in CRC specimens at time of progression on cetuximab

To examine whether this mode of cetuximab resistance occurs in human CRC, we obtained paired tumor specimens from ten individuals before the start of cetuximab treatment and at the time of tumor progression (Supplementary Table 7). KRAS/NRAS/BRAF mutations were excluded in tumor specimens obtained before treatment with cetuximab. qRT-PCR revealed that miR-100 and miR-125b were coordinately overexpressed ($r_s = 0.842, P < 0.01$) in tumors that progressed on treatment compared with pre-treatment levels ($P < 0.05$; Fig. 6a). In addition, nuclear β-catenin immunoreactivity was significantly higher in tumors that progressed on cetuximab (Fig. 6b). miR-125b expression directly correlated with nuclear β-catenin staining ($r_s = 0.636, P < 0.05$); the correlation between miR-100 expression and nuclear β-catenin staining did not reach statistical significance.
Figure 5 GATA6 transcriptionally represses MIR100HG and is targeted by miR-125b in a double-negative feedback loop. (a) Immunoblot of GATA6 in CC and CC-CR cells cultured in 3D culture. In CC-CR, cells were treated with CTX (CTX+, 3 µg/ml) or normal culture medium (CTX−) for 14 consecutive days in 3D culture before protein extraction. Representative of three independent experiments. (b) Immunofluorescence of GATA6 (green) and nuclei (blue). Scale bar represents 50 µm. (c) Representative IHC of GATA6 in CC and CC-CR xenografts (n = 8). Scale bar represents 50 µm. (d) qRT-PCR analysis of MIR100HG and GATA6 expression at indicated time points following CTX treatment (10 µg/ml) of CC in 3D culture. n = 3 independent experiments. (e) CC cells were transfected with two independent siRNAs against GATA6 or control (siCTL), treated with CTX (10 µg/ml) and subjected to qRT-PCR analysis. n = 2 independent experiments performed in triplicate. **P < 0.01 by Student’s t test. (f) Luciferase reporter assays were performed by co-transfection of pGL3-MIR100HG promoter luciferase reporter with increasing concentrations of pcDNA3.1-GATA6 plasmid or empty vector control (CTL), along with a Renilla luciferase reporter. Luciferase activity was measured 36 h post-transfection and normalized to Renilla values. n = 3 independent experiments performed in triplicate. **P < 0.01 by one-way ANOVA followed by Dunnett’s test. (g) A schematic representation of consecutive deletion and mutation constructs spanning the -2,000 to +500 region of the MIR100HG promoter. The putative GATA6-binding sites in the MIR100HG promoter are shown in black boxes. (h) The luciferase vector pGL3 driven by either wild-type, deletion or mutant (MUT) promoter was transfected in CC-CR, and luciferase activity was measured. n = 3 independent experiments. *P < 0.05, **P < 0.01 by Student’s t test. (i) Luciferase reporter analysis of a wild-type (WT) or mutant (MUT) GATA6 3’ UTR activity following addition of either synthetic miR-125b or a negative control miR-CTL. **P < 0.01 by Student’s t test. (j) Immunoblot of GATA6 in stable miR-125b-transduced CC and 125b-Sp-transduced CC-CR. Representative of three independent experiments. (k) Box plots showing expression of GATA6 (left) and MIR100HG (middle) by stage from the TCGA CRC data repository. Right, MIR100HG expression in the low (<25%) and high (>75%) quartile of GATA6 expression. *P < 0.05, **P < 0.01 by Mann–Whitney U test. n.s., not significant. Data represent mean ± s.d. in d–f, h and i.
Conversely and consistent with our pre-clinical findings, there was reduced nuclear GATA6 expression in tumors that advanced on cetuximab (Fig. 6c). However, we did not find a significant inverse correlation between miR-100 and GATA6 (r = −0.455, P = 0.187) or between miR-125b and GATA6 (r = −0.515, P = 0.128). Using FISH analysis, we found that the MIR100HG, miR-100 and miR-125b signals were increased in tumors that progressed on treatment. In these same samples, there was increased β-catenin staining and reduced GATA6 staining (Fig. 6d). We excluded MET amplification by FISH in all ten paired specimens and sequenced the post-treatment tumors for mutations in KRAS/NRAS/BRAF (Supplementary Fig. 9j and Supplementary Table 8). NRAS and KRAS mutations were detected in two cases, respectively; we confirmed that these were likely acquired events by re-sequencing the pre-treatment DNA. In both cases, MIR100HG, miR-100 and miR-125b signals were increased in tumors that progressed on treatment. In the remaining eight cases that lacked genetic resistance events, five cases exhibited upregulated MIR100HG, miR-100 and miR-125b in the tumors post-treatment. These clinical data support our pre-clinical findings and demonstrate that upregulation of MIR100HG, miR-100 and miR-125b occurs in the setting of acquired cetuximab resistance in CRC patients, and this upregulation may both coincide with and be independent of genetic mutations associated with cetuximab resistance.

DISCUSSION

MIR100HG is a polycistronic miRNA host gene that encodes miR-100, let-7a-2 and miR-125b-1 in its third intron. MIR100HG was first reported to participate in fate determination of human mesenchymal stem cells and was later found to be highly expressed in acute megakaryoblastic leukemia. Increased MIR100HG expression is associated with a poor prognosis in cervical cancer, whereas its expression is reduced in breast cancer as a result of hypermethylation. Increased expression of miR-100 and miR-125b are also correlated with gastric cancer progression in clinical samples. In our study, concomitant upregulation of MIR100HG, miR-100 and miR-125b occurred in the setting of acquired and de novo cetuximab resistance in CRC and HNSCC cell lines. Moreover, we found that these events could co-occur with KRAS/NRAS/BRAF mutation and in the tumors of CRC patients that progressed on cetuximab. Analysis of the TCGA CRC data repository revealed a stage-dependent increase in MIR100HG, miR-100 and miR-125b expression in tumors that advanced on cetuximab.
of MIR100HG expression. These data support the hypothesis that MIR100HG, miR-100 and miR-125b are potential predictive biomarkers for cetuximab resistance.

We identified that miR-100 and miR-125b coordinately contribute to cetuximab resistance by targeting five negative regulators of Wnt signaling, miR-100 targeted DKK1 and ZNRF3, and miR-125b targeted ZNRF3, RNF43, DKK3 and APC2. Wnt signaling is tightly regulated and negative regulators act on many different levels. DKK1 and DKK3 are secreted Wnt signaling antagonists of the Dickkopf family. DKK1 acts by binding and internalizing the Wnt co-receptor LRPS/6 (ref. 36), whereas it is unclear how DKK3 attenuates Wnt signaling. ZNRF3 and RNF43 are two closely related transmembrane E3 ubiquitin ligases that antagonize Wnt signaling through ubiquitination and degradation of the Wnt receptor Frizzled and its co-receptor LRPS/6 (refs. 38, 39). Although inactivating mutations have been reported for ZNRF3/RNF43 (ref. 40), our data suggest that downregulation by miR-100 and miR-125b may represent an alternative mechanism of attenuating ZNRF3/RNF43 function. APC2 targets β-catenin for destruction and is functionally complementary to APC; it was recently reported that APC2 recruits TNKS into the β-catenin destruction complex to regulate β-catenin proteolysis. We previously identified APC as a target of miR-125b in leukemia cells, but did not examine APC in CC or CC-CR, as it is mutated in these cells. However, it was recently reported that miR-125b targets APC in mutant β-catenin HCT116 cells that have wild-type APC. Our results show that miR-100 and miR-125b work together to target these five Wnt negative regulators, providing a previously unknown regulatory mode for clustered miRNAs to cooperatively regulate this pathway.

We cannot exclude the possibility that miR-100 and miR-125b contribute to cetuximab resistance through means other than Wnt signaling. For example, miR-125b can enhance tumor formation in the skin by targeting vacuolar protein-sorting 4 homolog B (Vps4b) and indirectly prolonging EGFR activity. However, we observed no difference in VPS4b expression between CC and CC-CR. We also have not excluded an effect of the full-length 3-kb MIR100HG transcript on Wnt signaling.

Our findings add to the literature describing crosstalk between EGFR and Wnt signaling. For example, in APC-mutant CRC, increased EGFR signaling enhances Wnt activity, supporting the notion that Wnt signaling is further modulated in the setting of an impaired β-catenin degradation complex. In a reciprocal manner, binding of Wnt ligands to their GPCR Frizzled receptors results in EGFR transactivation via metallloprotease-dependent, cell-surface ectodomain cleavage of EGFR ligands. Moreover, increased Wnt signaling confers resistance to EGFR tyrosine kinase inhibitors in lung cancer. The precise mechanism by which increased Wnt signaling confers cetuximab resistance is uncertain. It has been reported that Wnt signaling increases EGFR expression in liver. We observed that cetuximab did not reduce p-EGFR, pERK1/2 or p-AKT in CC-CR, whereas it does in CC. Although there is equivalent cell-surface EGFR staining in CC and CC-CR, this does not exclude differences in the rates of EGFR internalization, recycling and degradation. Going forward, a system-wide approach should prove useful to help unravel mechanisms underlying the EGFR/Wnt crosstalk in this system.

The role of GATA6 in cancer is complex and context dependent; even in the same tumor type, conflicting evidence exists. For example, GATA6 promotes pancreatic carcinogenesis by activating Wnt signaling. Separate studies have found that it serves a tumor-suppressive role by maintaining a pancreatic differentiation program. Likewise, in colonic neoplasia, opposing actions have been reported. In colonic adenomas, GATA6 represses BMP expression, thereby enabling stem-cell self-renewal, and in CRC cell lines it enhances expression of Lgr5 and REG4 to promote clonogenicity and growth, respectively. In contrast, our data support a tumor-suppressive role for GATA6 in CRC. Analysis of the TCGA CRC repository revealed a reduction in MIR100HG expression in stage IV CRC along with increased MIR100HG expression. Reduced expression of GATA6 would permit increased expression of MIR100HG, and the corresponding increased expression of miR-125b would reinforce repression of GATA6. In this context, GATA6 serves a permissive tumor-suppressive role by preventing Wnt-signaling-enhanced cetuximab resistance.

Our findings have important therapeutic implications for CRC and HNSCC. It is increasingly appreciated that there are gradients of Wnt signaling in CRC and that Wnt signaling can be modulated in the setting of APC loss of function. In our model, MIR100HG-, miR-100- and miR-125b-mediated Wnt activation represents cells adapting to survive under EGFR inhibition by activating compensatory pathways. We found that induction of DKK1 or DKK3 individually, or the combined addition of recombinant DKK1 and DKK3, overcomes cetuximab resistance in CC-CR. Both XAV-939, a tankyrase inhibitor, and ICG-001, a β-catenin-CBP inhibitor, augmented the growth inhibitory effects of cetuximab. We propose that future trials in individuals with wild-type KRAS/NRAS/BRAF CRC should consider the levels of MIR100HG expression.

In summary, we have identified a complex circuitry underlying cetuximab resistance by upregulation of MIR100HG and its embedded miRNAs (see Supplementary Fig. 10). miR-100 and miR-125b coordinately activate Wnt signaling by reducing expression of five negative regulators of Wnt signaling. miR-125b reinforces upregulation of MIR100HG by inhibiting GATA6 expression, which normally suppresses MIR100HG. We found that inhibition of Wnt signaling can overcome this mode of cetuximab resistance, underscoring the potential clinical relevance of the interactions between EGFR and Wnt signaling.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.L., X.Z., C.L., D.F. and R.J.C. designed the research. Y.L., X.Z., Q.L., C.L., R.G.-D., Z.C., B.S., I.W., H.H., T.W., M.Y. and S.H. performed experiments, analyzed data, and prepared figures and tables. T.Y., E.L., K.S.-D., C.H.C., S.E., J.-H.K. and D.F. contributed new reagents and/or analytical tools. Y.L., X.Z., Q.L., C.L., I.L.F., T.J.Y., E.L., J.G.P., C.H.C., D.F. and R.J.C. analyzed the data and provided critical input.
Y.L., X.Z. and R.J.C. wrote the paper. R.J.C. and D.F. conceived the project, and Y.L., X.Z. and R.J.C. wrote the paper. R.J.C. and D.F. conceived the project, and

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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2D and 3D cell culture. NCI-H508, Caco-2, SW403, SW498, HT29, SK-CO-1, DLD-1, SW480, SW837, SW48, SW620, LoVo, COLO205, T84, LS174T, NCI-H716, HCT8, HCT15, SW1116, RKO, COLO320DM, HtTu80, LS123 and HCT116 cell lines were from the American Type Culture Collection (ATCC). HCA-7, its derivatives CC and CC-CR; DiFi, GEo, LIM1215, and LIM2405 were maintained in the Coffey lab. The SNUC4 cell line was from the Korean Cell Line Bank and the V99 cell line was provided by J. Mariadason (Olivia Newton-John Cancer Research Institute). HNSCC cell lines SCC25, its derived cetuximab-resistant sublines (CTX-R1, R3, R4, R5, R7 and R8), and UNC10 were maintained in C.H.C.'s laboratory. All cell lines were confirmed to be free of mycoplasma contamination. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Corning) supplemented with 10% bovine growth serum, glutamine, nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone) in 5% CO2 at 37°C. 3D collagen cultures were set up using 3 layers of type-I collagen PureCol (Advanced BioMatrix) in triplicate as previously described. Human recombinant DKK1 (rDKK1) and DKK3 (rDKKK3) are from R&D Systems. Drugs are used as follows: cetuximab (Merck KGaA), Wnt pathway inhibitor ICG-001 and XAV-939 (Selleck Chemicals). Colonies were counted by GelCount colony counter (Oxford Optronix).

RNA-Seq analysis. Total RNA from cells embedded in collagen was isolated by TRIzol Reagent (Invitrogen) and then purified using RNeasy Mini Kit (Qiagen). The concentration and integrity of total RNA were estimated using the Qubit 2.0 Fluorometer (Invitrogen) and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. Polyadenylated RNAs were isolated using NEBNext Magnetic Oligo Fluorometer (Invitrogen) and Agilent 2100 Bioanalyzer (Agilent Technologies), the concentration and integrity of total RNA were estimated using the Qubit 2.0 Fluorometer (Invitrogen). The miRNA preparation was performed using TRIzol Reagent (Invitrogen) and chloroform extraction followed by ethanol precipitation. Approximately 1 μg of total RNA from each sample was used for small RNA library preparation using NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs) following the manufacturer's protocol. Small RNA-Seq analysis. Approximately 1 μg of total RNA from each sample was used for small RNA library preparation using NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs) following the manufacturer's protocol. Post PCR material was purified using QIAquick PCR Purification Kit (Qiagen). Post PCR yield and concentration of the prepared libraries were assessed using Qubit 2.0 Fluorometer and DNA 1000 chip on Agilent 2100 Bioanalyzer. Selection of small RNA was done on the Pippin Prep instrument (Sage Science). Accurate quantification for sequencing applications was performed using the qPCR-based KAPA Biosystems Library Quantification Kit (Kapa Biosystems). Paired-end (PE) sequencing (75 bp) was performed on the NextSeq 500 sequencer (Illumina). RNA-Seq reads were aligned to the human genome hg19 using TopHat2 (ref. 59), and the number of reads mapped to each gene was calculated by HTseq (http://htseq.readthedocs.io/). Differentially expressed genes between CC and CC-CR were detected by edgeR based on negative binomial distribution. The P values were adjusted by Benjamini and Hochberg's multiple correction procedures. Differential expression was determined based on fold-change (FC) and FDR with [log(FC)] > 1 and FDR < 0.01.

Small RNA-Seq analysis. Human DKK1 expression vector pcDNA3-DKK1-FLAG was kindly provided by C.A. Iacobuzio-Donahue (Memorial Sloan Kettering Cancer Center). GATA6 Silencer Select siRNAs (ID s5605, s5606) and the Silencer Select negative control siRNA were used for transfection (Life Technologies). GATA6 expression plasmids or siRNAs were transfection-confirmed by sequencing. Small RNA-Seq analysis. Approximately 1 μg of total RNA from each sample was used for small RNA library preparation using NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs) following the manufacturer's protocol. Post PCR material was purified using QIAquick PCR Purification Kit (Qiagen). Post PCR yield and concentration of the prepared libraries were assessed using Qubit 2.0 Fluorometer and DNA 1000 chip on Agilent 2100 Bioanalyzer. Selection of small RNA was done on the Pippin Prep instrument (Sage Science). Accurate quantification for sequencing applications was performed using the qPCR-based KAPA Biosystems Library Quantification Kit (Kapa Biosystems). Paired-end (PE) sequencing (75 bp) was performed on the NextSeq 500 sequencer (Illumina). RNA-Seq reads were aligned to the human genome hg19 using TopHat2 (ref. 59), and the number of reads mapped to each gene was calculated by HTseq (http://htseq.readthedocs.io/). Differentially expressed genes between CC and CC-CR were detected by edgeR based on negative binomial distribution. The P values were adjusted by Benjamini and Hochberg's multiple correction procedures. Differential expression was determined based on fold-change (FC) and FDR with [log(FC)] > 1 and FDR < 0.01.

Whole-exome sequencing. DNA extraction was performed by the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's instructions. The DNA was quantified by Nanodrop spectrophotometer (Thermo Fisher Scientific). Genomic DNA was sequenced using Illumina HiSeq 2500. Reads were aligned to the human genome hg19 with BWA, sorted, and indexed with SAMtools. Duplicate reads were marked by Picard (http://broadinstitute.github.io/picard), SNPs and Indels were called simultaneously on CC and CC-CR samples by SAMtools with base quality 20, reads with mapping quality 20, and mapping quality downgrading coefficient of 50. SNPs and Indels with strand bias P < 0.01, base quality bias P < 0.01, or end distance bias P < 0.01 were filtered out. Furthermore, SNPs within 3 bp around a gap were removed. SNPs and Indels were annotated, and their effects were predicted by snpEff and snpSift. Steekle was used to detect SNVs and indels that were present at a significantly different frequency between CC and CC-CR samples with default parameters except turning off the depth filter for exome sequencing data.

Constructs, oligonucleotides, infection and transfection of human cell lines. miRNA expression lentiviral vectors LeGO-cO:miR-100, LeGO-cO:miR-125b, LeGO-cO:miR-100/125b bicistron, and control empty vector were used as described previously. Lentivirus produced in HEK293 cells was generated and collected using standard protocols. GFP-positive infected cells were selected in Blasticidin S (10 μg/ml) followed by flow sorting. Stable miRNA knockdown was achieved by introducing the lentiviral miRNA sponge constructs that target either miR-100, miR-125b alone or both. Briefly, eight repeats of anti-sense miR-100 (5′-CAACAAGCTCGATCTAGGGGT-3′) and/or anti-sense miR-125b (5′-TCACAAAGTTTGGTCTAGAGGA-3′) were synthesized and followed using standard protocols, and then cloned into the pGLV3/H1/GFP vector (GenePharma). A control sponge was used, which includes eight repeats of an artificial miRNA (5′-AAGTTTCAGAAGAAAGTACACA-3′) that is not complementary to any known miRNA. GFP-positive infected cells were selected in puromycin (1 μg/ml) followed by flow sorting.

Human DKK1 expression vector pcDNA3-DKK1-FLAG was kindly provided by S. Aaronson (Mount Sinai School of Medicine). Human DKK3 expression vector pcCS2-DKK3-flag was from Addgene (plasmid #15496). Lentiviral-inducible expression constructs containing DKK1 or DKK3 under the control of a doxycycline-inducible promoter were constructed by transferring each ORF into the pLm2-ducer lentivector vector. Infected cells were selected in G418 (200 μg/ml) to generate stable cell lines. Tetracycline-reduced FBS (Clontech) was substituted for all media for cells transduced with the pLm2-ducer 20 vectors. To induce expression of DKK1 or DKK3, 1 μg/ml doxycycline (Sigma-Aldrich) was added to the culture medium.

pcDNA3.1-GATA6 and pcDNA3.1-mutant (mut) GATA6 were used for site-directed mutagenesis were kindly provided by C.A. Iacobuzio-Donahue (Memorial Sloan Kettering Cancer Center). GATA6 Silencer Select siRNAs (ID s5605, s5606) and the Silencer Select negative control siRNA were used for transfection (Life Technologies). GATA6 expression plasmids or siRNAs were transfected into indicated reporter cells using Lipofectamine 2000 or RNAiMAX Reagent (Thermo Fisher Scientific), respectively. Experiments were performed 48 h after transfection.

For 3` UTR luciferase reporter assay, the 3` UTR fragments of DKK1, DKK3, ZNRF3, RNF43, and APC2 containing miR-100 or miR-125b putative target sites were amplified and cloned downstream of the SV-40 promoter-driven Renilla luciferase cassette in psiCHECK-2 (Promega). For luciferase reporter assays to measure promoter activities, PCR products of sequential deletion fragments of human MIR100HG promoter were cloned into pGL3-Basic vector (Promega). A site-directed mutagenesis kit (Agilent Technologies) was used to mutate the miR-100, miR-125b, or GATA6 binding sites of these vectors. All sequences were confirmed by sequencing.

qRT-PCR. Analysis of mRNA and miRNA levels was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems). For mRNA detection, cDNA was generated with the QuantiTect Reverse Transcription Kit (Qiagen). Diluted cDNA samples were amplified to establish a standard curve for calculation of relative target concentrations using Express SYBR GreenER qPCR SuperMix with Premixed ROX (Life Technologies). The housekeeping gene ACTB was used as an internal control. The primers for the genes of interest were synthesized by RealTimePrimers.com or Sigma-Aldrich (Supplementary Table 9). Analysis of IncRNA and miRNA levels was performed with the use of the TaqMan fast advanced master mix (Applied Biosystems). TaqMan IncRNA, miRNA, and pri-miRNA expression assays (Life technologies) were used...
according to the manufacturer's instructions, with ACTB or U6 small nuclear RNA (U6 snRNA) as the internal control (Supplementary Table 10). The relative expression of RNAs was calculated using the comparative Ct method.

5' rapid amplification of cDNA ends (RACE). 5' RACE was used to determine transcriptional initiation sites of IncRNA MIR100HG using FirstChoice RLM-RACE Kit (Thermo Fisher Scientific), and Zero Blunt TOPO PCR Cloning Kit (Life Technologies) was used for sequencing according to the manufacturer's instructions. Two reverse primers for the TSS of MIR100HG were used in a nested PCR with the two 5' primers from the kit.Outer primer: 5'-AAACCGGGGGCTCCAGTTGACAT-C3'; inner primer: 5'-TCTTTGCC ATCCCGTTGTCACTGGT-3'.

Western blot analysis. Whole-cell lysates were prepared using RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (Roche). The nuclear extract was isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific). Protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Primary antibodies were against DKK1 (Santa Cruz sc-25516; 1:150), ZNRF3 (sc-86958; 1:150), RNF3 (sc-165398; 1:150), GATA6 (sc-7244; 1:150), Lamin A/C (sc-7922; 1:200), and GAPDH (sc-20575; 1:200); DKK3 (Abcam #2459; 1:1,000), APC2 (Abcam #80018; 1:500), ZNRF3 (Abcam #122353; 1:1,000), RNF3 (Abcam #81425; 1:1,000), and p-ERK1/2 (Abcam #4695; 1:1,000); β-catenin (BD Biosciences #610154; 1:2,000); EGFR (Millipore #06-847; 1:500); AKT (CST #9272; 1:1,000), and p-LRP6 (Millipore #07-2187; 1:500); ERK1/2 (Cell Signaling Technology, #4370; 1:1,000); p-ERK1/2 (Cell Signaling Technology, #4370; 1:1,000); p-β-catenin (S552; CST #9566; 1:1,000); p-ERK1/2 (CST #4370; 1:1,000), AKT (CST #9272; 1:1,000), p-AKT (CST #4060; 1:1,000), cleaved Caspase-3 (CST #9664; 1:1,000), CyclinD1 (CST #2978; 1:1,000), BIM (CST #2933; 1:1,000), p-β-catenin (S52; CST #9566; 1:1,000), LRP6 (CST #2560; 1:1,000), and GATA6 (CST #5851; 1:1,000); p-β-catenin (S17; CST #2292; 1:200), and GAPDH (sc-20357; 1:200). Then sections were incubated with Envision System HRP-labeled polyclonal and chemiluminescent detection was performed.

Immunohistochemistry (IHC). IHC for target molecules was performed on serial sections from tumor tissues of nude mice xenografts and CRC patients. Tissue sections were deparaffinized, subjected to antigen retrieval using tar- get antigen retrieval solution (Dako), and incubated with primary antibodies against Ki-67 (Dako M7240; 1:150), cleaved Caspase-3 (CST #9661; 1:200), β-catenin (BD Biosciences #610154; 1:800), GATA6 (Abcam #2260; 1:200), DKK1 (Abcam #61034; 1:100), DKK3 (Abcam #115869; 1:50), ZNRF3 (Abcam #122353; 1:150), RNF3 (Abcam #129401; 1:150), and APC2 (Abcam #113370; 1:200). Then sections were incubated with Envision System HRP-labeled polyclonal anti-rabbit (Dako K4003) or anti-mouse secondary antibodies (Dako K4001). The results of IHC were scored by two independent observers. Ki-67 and cleaved Caspase-3 staining was quantified by calculating positively stained cells in at least five randomly chosen HFPs of each sample. Quantification of other molecules was based on intensity and extent of staining according to the histological scoring method as previously described.

In vivo tumor growth in xenograft model. In vivo cetuximab treatment was performed using 6–8 week-old female athymic BALB/c nude mice. All experiments were conducted under protocols approved by the Fourth Medical University Institutional Animal Care and Use Committee. Suspensions of the corresponding cells were subcutaneously injected into the flanks (6 × 10^6 tumor cells/150 µl PBS per spot; 6–8 mice in each group). Animals were weighed, and the tumor size was measured using bilateral caliper measurements. Tumor volume was calculated using the formula: tumor maximum diameter (L) × the right angle diameter to that axis (W)^2/2. When the tumors reached the determined size (around 100 mm^3), mice were randomized into control and treatment groups. Cetuximab treatment was given at a dose of 1 mg/mouse, intraperitoneal (i.p.) injection, every 3 d. For Wnt pathway inhibitor ICG-001 in vivo treatment, the sodium phosphate form of ICG-001, synthesized by Vanderbilt Institute of Chemical Biology (VICB) Synthesis Core, was administered at a dose of 150 mg/kg body weight, i.p. injection every day. For in vivo imaging system (IVIS, PerkinElmer) was used to detect GFP fluorescence in tumor-bearing mice. After 4 weeks of treatment, mice were sacrificed according to institutional ethical guidelines. Postmortem examination included tumor size and weight measurements, and then tumors were paraffin-embedded to perform hematoxylin and eosin staining. The sample size for the experiments was based on the pilot studies and determined to ensure a power at 0.8 with type 1 error (α) at 0.05 of expected difference. Postmortem examination and data analysis were done by two investigators blinded to the group allocations.
Human CRC samples and subjects. All human CRC samples were obtained from the Xijing Hospital of Digestive Diseases. The study was approved by the Ethics Committee of Xijing Hospital with written informed consent obtained from all subjects. The pathological status of the specimens was provided by the Department of Pathology. In total, we analyzed ten pairs of tumor specimen pre- and post-cetuximab treatment. Pre-cetuximab treated specimens were retrospectively obtained during surgical or biopsy under colonoscopy on subjects with CRC. After computed tomography (CT) of tumor lesions demonstrated tumor re-growth (disease progression) following initial response to cetuximab-based therapy, post-cetuximab treated specimens were collected whenever possible at the time of progression. RNA was extracted with RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion). IHC and FISH analyses were performed on formalin-fixed, paraffin-embedded (FFPE) tumor tissue sections. Blind evaluation was done by two pathologists.

FISH assays. Locked nucleic acid-in situ hybridization (LNA-ISH) with tyramide signal amplification (TSA) was performed to detect lncRNA and miRNA as previously described. LNA probes were synthesized (Exiqon) including double biotin-labeled probe against MIR100HG, double digoxigenin (DIG)-labeled probe against miR-125b, double fluorescein-labeled probe against miR-100, DIG-labeled probe against U6 snRNA, and DIG-labeled scramble probe. Anti-Digoxigenin HRP Conjugate, anti-Fluorescein HRP Conjugate, Streptavidin-HRP Conjugate, and TSA Cy3 and Fluorescein Kit (all from PerkinElmer) were used for TSA methods. Confocal fluorescence microscopy was performed using a Zeiss LSM 710 confocal microscope.

For detection of MET amplification, MET/CEP7 dual-color probes (Cyto test) were used for recognizing the MET gene status following the manufacturer’s protocol. Analysis was according to the University of Colorado Cancer Center (UCCC) criteria. A MET/CEP 7 ratio was established based on counting at least 200 cells.

Targeted Sanger sequencing of KRAS, NRAS and BRAF. KRAS/NRAS/BRAF mutations had been excluded in tumor specimens obtained before treatment with cetuximab. This study was carried out on 12 FFPE blocks of colorectal carcinomas (ten blocks obtained after disease progression upon cetuximab treatment and case 2 and four blocks before treatment). Genomic DNA was isolated using QIAamp DNA FFPE Tissue Kit (QIAGEN). Oncogenic alleles of KRAS (codon G12, G13, Q61, and K117) and NRAS (codon G12, G13, and Q61), and BRAF (codon G465, G468, Y472, D593, F594, L596, L597, T598, V600 and K601) were sequenced by targeted Sanger sequencing with PCR primers listed (Supplementary Table 1). The PCR products were then sequenced using BigDye Terminator 3.1 Cycle Sequencing Kit on a 96-capillary 3730XL DNA Sequencer (Applied Biosystems).

Statistical analysis. Statistical analysis was performed by the SPSS 18.0 (SPSS) and R (version 3.3.1). The statistical significance between data sets was expressed as P values, and P < 0.05 was considered statistically significant. Two-tailed unpaired or paired Student’s t test, ANOVA (Dunnnett’s or LSD post hoc test), non-parametric signed rank test, Mann–Whitney U test, and Pearson correlation coefficients were used according to the type of experiment.

For Pearson correlation of MIR100HG expression with 64-gene Wnt scores and RAS_AZ scores on 458 CRCs, the log2 expression values of MIR100HG were obtained on 458 CRC tumors (ten samples without suitable microarray data were excluded from 468 CRCs previously reported). A set of 64 consensus β-catenin (upregulated) targeted genes were adopted from a recent study, and a mean log; expression of the 64 genes was calculated as the Wnt/β-catenin pathway score on 458 CRC samples as previously described. The RAS_AZ signature score, which measures MEK activation as a downstream index of RAS activity, was previously developed and was pre-calculated on 458 CRC samples. For comparison, one of the Wnt scores between CC and CC-CR, the 64-gene Wnt scores (log; expression) were calculated for CC and CC-CR (each with three replicates) similarly as described above and then subjected to the two-tailed unpaired Student’s t test.

RNA-Seq and small RNA-Seq data of CRC was obtained from TCGA Firehose developed by the Broad GDAC (https://confluence.broadinstitute.org/display/GDAC/Dashboard-Stddata). mRNA expression array data of CRC/ colon cancer was acquired from published studies (GEO accession: GSE14333, GSE39582). The gene expression abundances were log2 transformed, and Pearson correlation coefficients were used to measure the correlation between MIR100HG and miR-100, miR-125b and let-7a. Mann–Whitney U test was used to determine the expression difference of MIR100HG in the lower (0–25%) and higher (>75%) quartiles of GATA6 expression.

Data availability. RNA-Seq and small RNA-Seq data are available at the NCBI Gene Expression Omnibus (GEO) repository with accession GSE82236. Whole-exome sequencing data are available at the GEO repository with accession GSE76352. A Life Sciences Reporting Summary is available.

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Experimental design

1. Sample size
   Describe how sample size was determined.
   Sample size was chosen based on similar studies performed in our lab and those reported in the literature. For nude mice studies, the sample size for the experiments was based on the pilot studies and determined to ensure a power at 0.8 with type 1 error ($\alpha$) at 0.05 of expected difference.

2. Data exclusions
   Describe any data exclusions.
   No data was excluded from the studies.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All attempts at replication were successful. For nude mice studies, they were performed with at least six mice for one group.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Nude mice were allocated into groups of equal average baseline body weight.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   For nude mice studies, postmortem examination and data analysis were done by two investigators blinded to the group allocations. For human CRC specimen study, blind evaluation was done by two pathologists.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a

   Confirmed

   - The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - The test results (e.g. $P$ values) given as exact values whenever possible and with confidence intervals noted
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Statistical analysis was performed by the SPSS 18.0 (SPSS Inc.) and R (version 3.3.1).

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Type-I collagen PureCol was purchased from Advanced BioMatrix. Human recombinant DKK1 (rDKK1) and DKK3 (rDKK3) were purchased from R&D Systems. Cetuximab was purchased from Merck KGaA, and Wnt pathway inhibitors ICG-001 and XAV-939 were purchased from Selleck Chemicals.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The following antibodies were used for immuno-blot: DKK1 (Santa Cruz sc-25516), ZNRF3 (sc-86958), RNF43 (sc-165398), GATA6 (sc-7244), Lamin A/C (sc-7292), and GAPDH (sc-20357); DKK3 (Abcam #2459), APC2 (Abcam #80018), ZNRF3 (Abcam #122353), RNF43 (Abcam #84125) and p-EGFR Y1068 (Abcam #5644); β-Catenin (BD Biosciences #610154); EGFR (Millipore #06-847) and p-LRP6 (Millipore #07-2187); ERK1/2 (Cell Signaling Technology, CST, #4695), p-ERK1/2 (CST #4370), AKT (CST #9272), p-AKT (CST #4060), cleaved Caspase-3 (CST #9664), Cyclin-D1 (CST #2978), BIM (CST #2933), p-β-catenin (SS52) (CST #9566), LRPS (CST #2560), and GATA6 (CST #5851); β-actin (Sigma-Aldrich A1978). For immunofluorescence, cleaved Caspase-3 antibody (Abcam #2302), Ki-67 antibody (Dako M7240), β-catenin (Y489) (Developmental Studies Hybridoma Bank, IA), and GATA6 (CST #5851) were used. For immunohistochemistry, Ki67 antibody (Dako M7240), cleaved Caspase-3 (CST #9661), β-catenin (BD Biosciences #610154), GATA6 (Abcam #122353), DKK1 (Abcam #61034), DKK3 (Abcam #115869), ZNRF3 (Abcam #122353), RNF43 (Abcam #129401), and APC2 (Abcam #113370) were used.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

NCI-H508, Caco-2, SW403, SW948, HT29, SK-CO-1, DLD-1, SW480, SW837, SW48, SW620, LoVo, COLO205, T84, LS174T, NCI-H716, HCT8, HCT15, SW1116, RKO, COLO320DM, HuTu80, LS123, and HCT116 cell lines were from the American Type Culture Collection (ATCC). HCA-7, its derivatives CC and CC-DR, DFi, GEO, LIM1215, and LIM2405 were maintained in the Coffey lab. The SNUC4 cell line was from the Korean Cell Line Bank and the V9P cell line was provided by John Mariadason (Olivia Newton-John Cancer Research Institute, Melbourne, Australia). HNSCC cell lines SCC25, its derived cetuximab-resistant sublines (CTX-R1, R3, R4, R5, R7, and R8), and UNC10 were maintained in Christine Chung's laboratory.

b. Describe the method of cell line authentication used.

Cell lines were authenticated using short tandem repeat (STR) analysis.

All cell lines were tested negative for mycoplasma contamination.

No commonly misidentified cell lines were used.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

6-8 week-old female athymic BALB/c nude mice were randomly divided into different experimental groups. The animal study was approved by the IACUC at the Fourth Military Medical University, China. All mice were housed in standard conditions.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

All relevant information on human CRC specimens, such as age, gender, diagnosis and treatment categories was shown in Extended Data Table. 7. Genotypic information was included in Extended Data Table. 8.