RESEARCH ARTICLE

Autocrine Activation of the Wnt/β-Catenin Pathway by CUX1 and GLIS1 in Breast Cancers

Charles Vadnais1,2, Peiman Shooshtarizadeh1, Charles V. Rajadurai1,2, Robert Lesurf1,2,5, Laura Hulea1,2, Sayeh Davoudi1, Chantal Cadieux2, Michael Hallett1,5, Morag Park1,2,3,4 and Alain Nepveu1,2,3,4,*

ABSTRACT

Autocrine activation of the Wnt/β-catenin pathway occurs in several cancers, notably in breast tumors, and is associated with higher expression of various Wnt ligands. Using various inhibitors of the FZD/LRP receptor complex, we demonstrate that some adenosquamous carcinomas that develop in MMTV-CUX1 transgenic mice model a form of autocrine activation of the Wnt/β-catenin pathway. By comparing expression profiles of laser-capture microdissected mammary tumors, we identify Glis1 as a transcription factor that is highly expressed in the subset of tumors with elevated Wnt gene expression. Analysis of human cancer datasets confirms that elevated WNT gene expression is associated with high levels of CUX1 and GLIS1 and correlates with genes of the epithelial-to-mesenchymal transition (EMT) signature: VIM, SNAI1 and TWIST1 are elevated whereas CDH1 and OCLN are decreased. Co-expression experiments demonstrate that CUX1 and GLIS1 cooperate to stimulate TCF/β-catenin transcriptional activity and to enhance cell migration and invasion. Altogether, these results provide additional evidence for the role of GLIS1 in reprogramming gene expression and suggest a hierarchical model for transcriptional regulation of the Wnt/β-catenin pathway and the epithelial-to-mesenchymal transition.

KEY WORDS: Breast cancer, Expression profiling, Mouse tumor model, Transcriptional regulation, Wnt/β-Catenin pathway

INTRODUCTION

The Wnt/β-catenin signaling pathway plays an important role in the development and regeneration of several tissues by stimulating the growth of stem cells and multipotential progenitors (MacDonald et al., 2009; Nusse, 2008). Wnts are secreted extracellular proteins that trigger a wide range of cellular responses upon receptor binding and activation. Most organisms contain multiple Wnt genes which initiate distinct cellular pathways, namely the canonical Wnt/β-catenin pathway, the planar cell polarity pathway, or the Wnt/calcium pathway. The β-catenin protein is maintained in a cytoplasmic complex that includes APC, Axin, and GSK3β. Phosphorylation by GSK3β causes the degradation of β-catenin unless the Wnt/β-catenin pathway is activated by Wnt ligands. Wnt proteins initiate the canonical pathway by binding and activating the Frizzled (Fzd) and LRP5/6 receptors. Activation of these receptors induces the dissociation of β-catenin from the degradation complex. As its concentration increases, β-catenin moves to nucleus where it displaces the Groucho corepressor from the TCF/Lef family of transcription factors with which it functions as a co-activator. As a consequence, the TCF/β-catenin complex now activates genes that were previously repressed by the TCF/Groucho complex. Many of the TCF/β-catenin target genes code for Wnt signaling components that are capable of enhancing or antagonizing Wnt pathway activity (MacDonald et al., 2009). Therefore, its own targets can regulate the Wnt pathway through feedback loop mechanisms (He et al., 1998). Activation of the FZD and LRP5/6 receptors can be inhibited by two types of secreted proteins. Secreted Frizzled-related proteins (sFRPs) and the Wnt inhibitory factor (WIF) bind Wnt proteins and prevent their interaction with LRP5/6 receptors, whereas Dickkopf (DKK) and sclerostin (SOST) compete with Wnt for binding to LRP5 and LRP6 (Dann et al., 2001; Hoang et al., 1996; Hsieh et al., 1999; Semenov and He, 2006).

Much of our knowledge of the Wnt pathway was originally derived from studies in drosophila. Failure to express either wingless or cut in the wing margin causes similar phenotypes from which their names were derived: wingless and cut wing (Couso et al., 1994; de Celis and Bray, 1997; Michelli et al., 1997; Neumann and Cohen, 1996). In the wing margin, cut is required in a cell-autonomous manner for wingless expression, whereas wingless is required for cut expression in neighboring cells (Michelli et al., 1997). Orthologs of cut in mammals are Cux1 homeobox 1 and 2, CUX1 and CUX2. In Drosophila imaginal discs, wingless is directly regulated by Cubitus interruptus, Ci, a transcription factor whose regulatory activity is under the control of the hedgehog ligand, Hh (Chen et al., 2000; Von Ohlen and Hooper, 1997). In mammals, there are several orthologs of Ci: Gli1, Gli2, Gli3, Gli5, Gli2 and Gli3 (Lichti-Kaiser et al., 2012; Riobo and Manning, 2007). Both Gli1 and Gli2 have been implicated in human cancers (Dahmane et al., 1997; Sheng et al., 2002). There is evidence to show that the Wnt/β-catenin pathway can be stimulated by Gli transcription factors, whether through activation of Wnt gene expression (Mullor et al., 2001), or indirectly via induction of Snail and downregulation of E-cadherin (Li et al., 2007). CUX1 was also shown to activate Snail gene expression and to cooperate with Snail in the repression of the E-cadherin gene (Kedinger et al., 2009).
CUX1 encodes several isoforms that exhibit strikingly different DNA binding properties (reviewed in (Nepveu, 2001; Sansregret and Nepveu, 2008)). The full-length protein, often called p200 CUX1, is very abundant, binds rapidly but only transiently to DNA and functions as an accessory factor in base excision repair (Randzan et al., 2014). The shorter isoforms p75 and p110 CUX1 bind stably to DNA and function as transcriptional repressors or activators depending on the promoter (Goulet et al., 2002; Harada et al., 1995; Harada et al., 2008; Moon et al., 2000; Moon et al., 2001). The p75 and p110 CUX1 isoforms exhibit similar DNA binding transcriptional activities (Cadieux et al., 2009; Goulet et al., 2002; Kedinger et al., 2009).

Cell-based assays demonstrated a role for CUX1 in cell cycle progression and cell proliferation (Sansregret et al., 2006; Truscott et al., 2007), strengthening of the spindle assembly checkpoint (Sansregret et al., 2011), cell migration and invasion (Kedinger et al., 2009; Michał et al., 2005), the DNA damage response (Vadnais et al., 2012), resistance to apoptotic signals (Ripka et al., 2010), as well as dendrite branching and spine development in cortical neurons (Cubelos et al., 2010) (reviewed by Hulea and Nepveu, 2012).

In colon cancer, the Wnt/β-catenin pathway is frequently activated following inactivation of the tumor suppressor APC or mutations in the β-catenin or Axin genes (Segditsas and Tomlinson, 2006). Recently, another mechanism of Wnt/β-catenin pathway activation was demonstrated where transcriptional activation of one or several of the Wnt genes leads to autocrine activation of Frizzled and LRP receptors and subsequent increase in nuclear β-catenin (Bafico et al., 2004). This autocrine activation has been shown in a sizeable proportion (20–25%) of breast cancers (Bafico et al., 2004; Benhaj et al., 2006; Schlang et al., 2007), lung cancers (Akiyama et al., 2009), neuroblastomas (Liu et al., 2008), acute myeloid leukemias and myelodysplastic syndromes (Xu et al., 2008). Only a few transcription factors so far have been implicated in transcriptional regulation of the Wnt/β-catenin pathway, including factors of the Gli family, CUX1 and ATF3 (Cadieux et al., 2009; Chen et al., 2000; Fiaschi et al., 2009; Grove et al., 1998; Micchelli et al., 1997; Von Ohlen and Hooper, 1997; Yan et al., 2011).

Transgenic mice expressing the p75 or p110 CUX1 isoform under the control of the mouse mammary tumor virus (MMTV) regulatory sequences were previously shown to develop mammary tumors of diverse histologic types (Cadieux et al., 2009). Interestingly, approximately one third of the mammary tumors resemble tumors previously characterized in MMTV-Wnt1 transgenic mice and were classified as adenosquamous carcinomas (Li et al., 2000). Analysis of 6 of these adenosquamous carcinomas revealed the presence of β-catenin in the nucleus and increased expression of either WNT1, WNT6, WNT8B and WNT10A or combination thereof. Evidence from multiple experimental approaches indicated that CUX1 is directly involved in the regulation of several Wnt genes. RNAi-mediated CUX1 knockdown caused a decrease in the expression of WNT1, WNT6, WNT8B and WNT10A, with concomitant downregulation of reporter assay and reporter assays demonstrated that p75 and p110 CUX1 bind to and activate the promoter of each of these genes (Cadieux et al., 2009). These findings led us to formulate two hypotheses. First, that MMTV-CUX1 mammary tumors with high WNT expression represent a model for autocrine activation of the Wnt/β-catenin pathway. Secondly, since only one third of the mammary tumors in MMTV-CUX1 transgenic mice exhibit high WNT expression, we hypothesized that other factors are needed, in addition to CUX1, for transcriptional activation of this pathway. In the present study, we employed various types of inhibitors to establish that activation of the FZD/LRP receptor complex is required for the augmentation of TCF/β-catenin transcriptional activity observed in MMTV-CUX1 mammary tumor cells. Furthermore, by comparing the expression profiles of laser-capture microdissected tumor cells from a panel of randomly selected MMTV-CUX1 mammary tumors, we identified GLIS1 as a transcription factor that is significantly overexpressed in tumors with high WNT gene expression. Co-expression experiments in MCF10A untransformed mammary epithelial cells demonstrated that CUX1 and GLIS1 cooperate in the autocrine activation of the Wnt/β-catenin pathway and in stimulating cell migration and invasion.

MATERIALS AND METHODS

Cell Culture

All lines were maintained in Dulbecco’s modified minimum essential medium (DMEM) supplemented with penicillin/streptomycin, glutamine, and 10% fetal bovine serum (all from Invitrogen). Cells were cultured in a humidified incubator at 37 °C and 5% CO2.

Protein Extracts

Nuclear extracts were prepared using a procedure adapted from Lee et al. (Lee et al., 1988). Briefly, cells were submitted to three freeze/thaw cycles in Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT). Nuclei were then resuspended in Buffer C (20 mM Hepes, pH 7.9, 25% glycerol 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA) and incubated at 4°C for 30 min. After 15 min of centrifugation, the supernatant was collected. Buffers A and C were supplemented with protease inhibitor mix tablet (Roche Applied Science).

Immunoblotting

Proteins extracts were resuspended in Laemmli buffer, boiled for 5 min, resolved by SDS-PAGE, and electropheretically transferred to a PVDF membrane. Membranes were blocked in TBS-T (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween X-100) containing 5% milk and 2% bovine serum albumin. Membranes were then incubated with primary antibodies diluted in TBS-T, washed in TBS-T, and incubated with species-specific secondary antibodies conjugated to horseradish peroxidase for 45 min at room temperature. Proteins were then visualized using the ECL system of Amersham Biosciences according to the manufacturer’s instructions. The following antibodies and dilutions were used: Cux1 (Moon et al., 2001) (1:1142), γ-tubulin (1:15,000, Sigma), α-actin (1:10,000, Santa Cruz) β-catenin (1:2000, BD Biosciences).

siRNA knockdown

CUX1 knockdown was performed by transfecting cells with a pair of siRNA constructs specific for CUX1 mRNA (5’ GAAUCUU-CUCGGUUGAACCUUUGAA and 3’ GCUUCAGAGCGAUAAUA-CACUAUA) using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions.

Lentiviral constructions

The lentivirus vectors were constructed by inserting the p110 CUX1 sequence into a pRev vector containing Hygromycin resistance and the Glis1 sequence into a pLent6 vector containing a Puromycin resistance gene. Lentiviruses were produced by transient transfection of 293FT cells by using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen).

RNA extraction and Real Time PCR

RNA was extracted using TRIzol reagent (Invitrogen), and cDNA was prepared using Superscript II RNase H-reverse transcriptase kit (Invitrogen) following the manufacturer’s instructions. Real time PCR
was performed on a LightCycler instrument using the FastStart DNA Master SYBR Green kit (Roche Applied Science) and specific primer pairs for each gene.

**Luciferase assays**

Luciferase assays were performed as previously described (Cadieux et al., 2009) with minor modifications in HS578T and HEK293T cells. For TOP/FOP reporter assay, Cells plated at 8x10^4 per well in 12-well plates were cotransfected with 0.25 µg of either the TOP 8x or FOP 8x reporter plasmids (Addgene) and 0.5 µg of either CUX1 p110 or empty vector as effector. As a control for transfection efficiency, the β-galactosidase protein (Sigma) was included in the transfection mix and the luciferase activity was normalized based on β-galactosidase activity.

**Laser-Capture-Microdissection**

Tumors from the previously generated p75-CUX1 and p110-CUX1 transgenic mice (Cadieux et al., 2009) were embedded in Tissue Freezing Medium (Triangle Biomedical Sciences #TFM-5) and flash frozen in liquid nitrogen. 10 µm slices of tissue were prepared in a Microm HM505E Cryostat at −30°C on positively charged slides (Fisherbrand Superfrost/Plus #12-550-55). Slides were then stained using a shortened H&E protocol (Ponzo et al., 2009). Epithelial cells were then isolated by IR (InfraRed) pulse on an Arcturus XT Laser Capture Microdissection instrument within 30–45 minutes of staining to obtain 100–150 pulses of material of 20–25 µm in diameter. RNA was isolated from the microdissection caps using the Picopure RNA isolation kit from Arcturus according to the manufacturer’s instructions, including the optional DNase treatment using the Qiagen RNase-Free DNase kit (#79254).

**Expression Profiling**

RNA isolated from microdissected tissue was amplified using the Arcturus RiboAmp HS PLUS RNA amplification kit according to the manufacturer’s instructions for 2 rounds of amplification. Amplified mRNA (aRNA) was labeled using the Arcturus Turbo Labeling Cy5 and Cy3 kits using slightly modified instructions: The labeling reaction was carried out using 5 µg of aRNA in a 20 µl volume instead of 50 µl to increase the dye incorporation rate. Labeled aRNA was hybridized to Agilent’s Whole Mouse Genome Microarray (G4112F), washed and scanned on an Agilent 5 µm scanner model G2505B according to the manufacturer’s instructions.

**Expression profiling data analysis**

Expression profiling array images were processed using the Feature Extraction software from Agilent. The raw data was then analyzed using the LIMMA (Smyth, 2005) package on the R platform (http://www.r-project.org). The “ggplot” R package was also used.

**Public dataset recovery and Meta-analysis**

Human breast cancer expression profiling datasets were obtained from the Oncomine database (https://www.oncomine.org). We retrieved all datasets comprising 100 or more samples and for which expression of at least one of CUX1 and GLIS1 as well as at least 15 out of the 19 Wnt ligand genes were available (As of November 2012). Note that datasets using Affymetrix platforms contain probes for the CASP transcript at least one of CUX1 and GLIS1 as well as at least 15 out of the 19 Wnt ligand genes were available (As of November 2012). Note that datasets using Affymetrix platforms contain probes for the CASP transcript at least one of CUX1 and GLIS1 as well as at least 15 out of the 19 Wnt ligand genes were available (As of November 2012). Note that datasets using Affymetrix platforms contain probes for the CASP transcript at least one of CUX1 and GLIS1 as well as at least 15 out of the 19 Wnt ligand genes were available (As of November 2012).

**Live-cell Imaging**

1x10^5 cells were plated on 10 µg/ml fibronectin coated plates for three hours and then subjected to time lapse-video microscopy with a frame taken every 5 minutes for a total of 16 hrs. The last forty frames from each condition were subjected to analysis using the Metamorph software.

**Invasion assays**

Modified Boyden chambers with 8 mm pore size were coated with a mixture of Matrigel (BD biosciences) with final concentration of 1.25 mg/ml and acid purified Rat tail Collagen I (Gibco) with final concentration of 2.5 mg/ml for 2 hrs at 37°C. Cells (1x10^6) were seeded on the bottom of the filters and were incubated at 37°C with 5% CO2 for 5 hrs to adhere. After 5 hrs, filters were inverted and top compartment was filled with 10% FBS and the bottom compartment was filled with plain DMEM and cells were allowed to invade for 48 hrs towards serum. Cells were stained with Calcein-AM (Invitrogen) as per the manufacturer’s instructions and confocal Z-stacks were acquired every 10 µm. Cells invading 20 µm into the matrix and beyond were quantified as percentage of total cells.

**RESULTS**

**Elevated Wnt Gene Expression in Mammary Tumor Cells Leads to Autocrine Activation of the Wnt/β-catenin Pathway**

To be able to investigate whether elevated Wnt gene expression in MMTV-CUX1 mammary tumors leads to autocrine activation of the Wnt/β-catenin pathway, we first established cell lines from the p75-80 adenosquamous carcinoma and the p75-534 solid carcinoma. The numbers p75-80 and p75-534 refer to the particular tumor bearing mice in the transgenic mouse cohort expressing the 75 CUX1 isoform under the control of MMTV regulatory sequences, as detailed in the introduction. Based on the histological features of these tumors, we predicted that cell lines derived from these tumors would respectively express high and low levels of Wnt genes (Cadieux et al., 2009). We found out later that the correlation between histological types and Wnt gene expression is not absolute (see below, Fig. 4). Nevertheless, RT-qPCR analysis indicated that expression of Wnt1, Wnt2, Wnt6, Wnt7a, Wnt7b, Wnt8a and especially Wnt10a and Wnt10b was much higher in the p75-80 than in the p75-534 cell line (Fig. 1A). In addition, immunofluorescence using an antibody specific for the active form of the β-catenin protein showed higher nuclear levels in the p75-80 line (Fig. 1B). As a functional assay to monitor the transcriptional activity of TCF/β-catenin complexes in these two cell lines, we measured the expression ratio of the TOP and FOP luciferase reporter plasmids. The two plasmids are identical except for the presence of 8 TCF binding sites in the TOP reporter, whereas these sites are inactivated in the FOP reporter. The TOP reporter, was activated in the p75-80 but not in the p75-534 cell line, confirming that the β-catenin pathway is activated in the former (Fig. 1C). Importantly, activation of the TOP reporter in p75-80 cells was prevented in the presence of niclosamide, or upon cotransfection of plasmids expressing sFRP1, sFRP2 or SOST (Fig. 1C). As niclosamide promotes Fzr(1) internalization and down-regulates Dvl-2 expression (Chen et al., 2009b), whereas sFRP1/2 and SOST/DKK inhibit activation of the FZP-LRP5/6 receptor by respectively binding
to Wnt ligands or the LRP5/6 receptor (Dann et al., 2001; Hoang et al., 1996; Hsieh et al., 1999; Semenov and He, 2006), these results indicate that elevated TCF/β-catenin transcriptional activity in p75-80 cells results from autocrine activation of the Wnt pathway.

CUX1 Is Required for Maximal Expression of the Wnt Genes in Human Tumor Cell Lines

A number of human cancer cell lines have been reported to display autocrine activation of the Wnt/β-catenin pathway (Akiri et al., 2009; Bafico et al., 2004; Vijayakumar et al., 2011). To verify whether CUX1 is required for expression of Wnt genes in these human cancer cells, we performed siRNA-mediated knockdown of CUX1 in six cell lines, two each from breast (MDA157 and MDA231), ovarian (PA-1 and SKOV3) and lung (H2347 and A427), and we performed RT-PCR analysis to measure the expression of Wnt genes previously identified as transcriptional targets of CUX1 in chromatin immunoprecipitation (ChIP) assays: Wnt 1, 2, 4, 6, 8b and 10a (Cadiex et al., 2009; Harada et al., 2008; and unpublished observations). In every cell line, CUX1 knockdown caused a significant decrease in the expression of three or more WNT genes (Fig. 2). We conclude that CUX1 is required for maximal expression of WNT genes in human tumor cell lines that display autocrine activation of the Wnt/β-catenin pathway.

Ectopic Expression of p110 CUX1 Leads to Autocrine Activation of the Wnt/β-Catenin Pathway in Human Tumor Cell Lines

We next verified whether ectopic CUX1 expression would activate the Wnt/β-catenin pathway in cell lines that do not display autocrine activation of this pathway. Nuclear β-catenin expression was significantly higher in HEK293 cells stably expressing p110 CUX1 (Fig. 3A). In agreement with these results, co-expression of p110 CUX1 with the TOP and FOP reporter plasmids led to a striking elevation in the TOP/FOP ratio (Fig. 3B). Similar increases in nuclear β-catenin expression and TCF/β-catenin transcriptional activity were observed in Hs578T and MCF-7 human breast cancer cells upon ectopic expression of p110 CUX1 (Fig. 3C,D). Importantly, co-expression of sFRP1, sFRP2, SOST or DKK1 or transfection in the presence of the niclosamide or IWP-2 inhibitors in HEK293 cells in every case annihilated the stimulatory effect of p110 CUX1 (Fig. 3B). As mentioned earlier, sFRP1/2, SOST, DKK and niclosamide inhibit the Wnt/β-catenin pathway by preventing activation of the FRP-LRP5/6 receptor. In addition, IWP-2 blocks signaling by Wnt ligands by inhibiting Porcupine (Porcn), which is required for palmitoylation and subsequent secretion of Wnt ligands (Chen et al., 2009a). Together, these results demonstrate that p110 CUX1 can activate the Wnt/β-catenin pathway through an autocrine loop.
Activation of the Wnt/β-Catenin Pathway in MMTV-CUX1 Mammary Tumors Is Associated with High Expression of Glis1

Since activation of the β-catenin pathway was observed in a fraction of mammary tumors from MMTV-CUX1 transgenic mice, we reasoned that it might require other transcription factors in addition to CUX1. As an approach to identify such transcription factors, we looked for factors whose expression was elevated in these mammary tumors. To ensure that changes in gene expression would not be masked by noise coming from stromal cells, mammary epithelial tumor cells were microdissected prior to performing expression profiling by microarray analysis. In total, 17 tumors of various histological types were analyzed and clustered using an unsupervised algorithm according to Wnt gene expression (Fig. 4). This procedure revealed three large clusters that displayed low, medium and high Wnt gene expression. Importantly, a distinct algorithm that linearly orders tumors over a gene signature (BreSAT) identified the same set of tumors as having the highest expression of Wnt genes (supplementary material Fig. S1). One gene coding for a transcription factor was clearly overexpressed in tumors with high Wnt gene expression: Glis1. In addition, we observed a correlation with genes whose expression is associated with epithelial-to-mesenchymal transition (EMT): Vim, Cdh2, Snai1, Snai2 and Twist1 were elevated, whereas Cdh1 and Ocln were down-regulated (Fig. 4). In contrast, elevated Wnt gene expression did not correlate with that of β-Catenin mRNA, Wnt receptors or markers of stem and progenitor cells (supplementary material Fig. S2).
Higher Expression of WNT Genes in Human Breast Cancers Correlates With CUX1, GLIS1 and EMT Markers

We verified whether high WNT gene expression correlates with that of CUX1 and GLIS1 in human breast cancers. As previously discussed, Affymetrix microarrays do not have a relevant probe for CUX1 (Sansregret et al., 2011), but the Oncomine database still contained three breast cancer datasets with data on CUX1 expression (Esserman et al., 2012; Glück et al., 2012; Cancer Genome Atlas Network, 2012). These datasets were sorted according to expression of WNT genes using the BreSAT algorithm. As mentioned above, this tool allows one to investigate a continuous trend across the data. The heatmap generated from one breast cancer dataset is presented in Fig. 5A as an example (Esserman et al., 2012). The top 25% of samples ranked according to WNT expression exhibited significantly higher expression of CUX1 and GLIS1 genes than the bottom 25% of samples (Fig. 5A,B). Overall, a correlation between CUX1 and WNT levels was observed in the 3 out of 3 breast cancer datasets that had a relevant CUX1 probe (Esserman, Glück, TCGA), and between Glis1 and WNT in 7 out of 8 datasets with Glis1 probes (supplementary material Table S1). In the Esserman dataset, we also observed a correlation with most, but not all, genes of the EMT signature: VIM, SNAI1 and TWIST1 were elevated, whereas CDH1 and OCLN were decreased (Fig. 5A,C). A significant correlation between WNT, CUX1, GLIS1 and EMT markers was also observed in the Glück and the TCGA datasets (supplementary material Fig. S3).

Ectopic Expression of GLIS1 in an MMTV-CUX1 Tumor Cell Line Leads to Wnt Genes Activation

Expression profiling analysis in human and mouse mammary tumors suggested that high Wnt gene expression requires both CUX1 and GLIS1. We verified this hypothesis using the “non-Wnt” cell line, p75-534, which displays low expression levels of Wnt and Glis1 mRNA. To test whether higher GLIS1 expression was able to convert p75-534 cells to a “Wnt” phenotype, we ectopically expressed GLIS1 using a lentiviral vector and then measured the expression of Wnt mRNAs and β-catenin protein. We observed a 10-fold increase in the expression of Wnt2, Wnt2b, Wnt7b and Wnt10a and a 2-fold increase in Wnt5a, Wnt6, Wnt7a, and Wnt9b (Fig. 6A). In agreement with these findings, the steady-state levels of β-catenin protein was significantly increased (Fig. 6B). We conclude that higher expression of GLIS1 in mammary tumor cells from MMTV-CUX1 transgenic mice leads to the transcriptional activation of several Wnt genes.

GLIS1 and p110 CUX1 Cooperate to Activate the Wnt/β-Catenin Pathway and to Stimulate Cell Motility and Invasiveness

To verify whether GLIS1 and p110 CUX1 cooperate to activate the Wnt/β-catenin pathway, lentiviral expression vectors for either or both of these factors were introduced into MCF10A untransformed mammary epithelial cells derived from breast reduction surgery (Rak et al., 1991). Measurements of WNT gene expression revealed that each factor on its own is able to stimulate the expression of several WNT genes, however, for most genes highest expression was achieved when both GLIS1 and p110 CUX1 were co-expressed (Fig. 7A). In agreement with these results, highest levels of the non-phosphorylated nuclear β-catenin isofrom (Fig. 7B,C) and highest TOP/FOP reporter activation (Fig. 7D) were observed in MCF10A cells stably expressing both GLIS1 and p110 CUX1.

To investigate whether the observed increase in WNT genes correlates with an EMT-like, migratory and invasive phenotype, we measured cell migration and invasion using live-cell imaging and a quantitative matrix invasion assays. Measurements of cell migration indicated that overexpression of GLIS1 alone was insufficient to enhance cell motility, that overexpression of p110
CUX1 promoted a moderate increase in cell migration, whereas overexpression of both GLIS1 and p110 CUX1 led to significant increases in both speed and distance of migration (Fig. 7E,F; supplementary material Fig. S4). To determine whether the combination of CUX1 and GLIS1 could promote cell invasion through a 3D matrix, we performed inverted invasion assays where cells are plated on the bottom of matrigel/collagen coated boyden chamber and are allowed to invade towards a chemo-attractant, serum in this case. As expected based on the live cell imaging results, we saw an increase in the invasive capacity of MCF10A cells co-expressing CUX1 and GLIS1 compared to either one alone (Fig. 7G,H).

To verify whether p110 CUX1 and GLIS1 cooperate to activate the Wnt/β-catenin pathway in human breast cancer cells as well, Hs578t cells were infected with lentiviral vectors encoding either one of these factors or the combination of both. Combined expression of p110 CUX1 and GLIS1 had a significantly greater effect than either gene alone on the expression of several Wnt genes (Fig. 8A) and levels of nuclear β-catenin (Fig. 8B). TCF/β-catenin transcriptional activity was also tested using the TOP/FOP reporter system in cells co-transfected with suboptimal amounts of either the p110 CUX1 or GLIS1 effector plasmid, or the combination of both. The highest level of TOP/FOP ratio was observed in cells transfected with both effectors (Fig. 8C). Finally, a higher cell motility was measured in cells expressing both p110 CUX1 and GLIS1 (Fig. 8D,E). We conclude that p110 CUX1 and GLIS1 can cooperate in the activation of Wnt genes in human breast cancer cells.

**DISCUSSION**

Strikingly, while there is a single Wingless gene in Drosophila, there are 19 Wnt genes in mammals. We understand that duplication of Wnt genes during evolution led to the association of Wnt coding sequences with a large repertoire of regulatory sequences, a process that ultimately enabled the expression of distinct Wnt genes in specific cells at precise times in order to activate Wnt pathways. Superimposed on the complex transcriptional regulation of Wnt ligands is the combinatorial nature of Wnt receptors and the multiple control steps that can prevent or limit Wnt pathway activation. Delivery of receptors at the cell membrane or secretion of ligands can be inhibited. Moreover, secretion of non-membrane bound receptors can squelch the action of ligands that are present in the extracellular milieu. Altogether, the multiple ways by which Wnt pathways can be activated and inhibited enables precise regulation both in time and space. Yet, perturbations in one or the other control systems have been found to cause aberrant activation of the Wnt/β-catenin pathway in human cancers. The majority of cases were reported in colon cancers and involve inactivating mutations of the APC tumor suppressor or mutations that affect axin or β-catenin itself (Segditsas and Tomlinson, 2006). In addition, autocrine activation of the Wnt/β-catenin pathway was shown to occur in a sizeable fraction of cancers from multiple organs and tissues (see Introduction). While elevated Wnt gene expression and repression of secreted Fzligands have been reported, we know very little about the transcription factors that play a role in the regulation of these genes.

In this study, we presented evidence from transgenic mouse models, tissue culture systems and human breast cancer databases implicating the CUX1 and GLIS1 transcription factors in the autocrine activation of the Wnt/β-catenin pathway. A fraction of mammary tumors from MMTV-p75 and p110 CUX1 transgenic mice exhibit higher Wnt gene expression (Cadieux et al., 2009). Using inhibitors that prevent activation of the FRP-LRP5/6 receptor in a mouse mammary tumor-derived cell line, we demonstrated that elevated Wnt gene expression leads to autocrine activation of the Wnt/β-catenin pathway (Fig. 1). CUX1 knockdown in six human tumor cell lines that display autocrine activation of the Wnt/β-catenin pathway showed that CUX1 is required for maximal expression of WNT genes (Fig. 2). In turn, ectopic expression of p110 CUX1 in three human cell lines led to increased β-catenin expression (Fig. 3). Moreover, stimulation of TCF/β-catenin transcriptional activity in this context was dependent on FRP-LRP5/6 receptor activation. Together these results clearly establish a causal link between CUX1 transcriptional function, the expression of Wnt genes, the activation of FRP-LRP5/6 receptors and the stimulation of TCF/β-catenin transcriptional activity. Note that we cannot exclude that Wnt ligands produced by a given cell in a culture dish can bind and activate a FZD/LRP receptor present on a neighboring cell, a type of signaling that could be described as paracrine. However, the term paracrine stimulation is reserved for cells of different types. Since cell lines maintained as monolayers in a culture dish are composed of one type of cells only, the term autocrine loop is more appropriate.

We note that distinct sets of Wnt genes were regulated in response to CUX1 knockdown or overexpression in different cell lines and tumors (Figs 2 and 4). For example, CUX1 did not stimulate the expression of WNT5A in MCF10A cells, although this gene was upregulated in two MMTV-CUX1 mammary tumors and was previously reported to be activated by CUX1 in the PANCI and HT1080 cell lines (Figs 4 and 6) (Ripka et al., 2007). Clearly, CUX1 is neither sufficient nor required in all cell lines or tumors for the expression of all Wnt genes. A few points are worth considering. First, ChIP-chip assays in 8 different cell lines indicated that CUX1 binds to the promoter of only 6 Wnt genes: Wnt 1, 2, 4, 6, 8b and 10a (Cadieux et al., 2009; Harada et al., 2008). Although we cannot exclude that CUX1 may regulate other Wnt genes from a distant binding site, it is likely that CUX1 directly regulates only a subset of Wnt genes. The effect of CUX1 on other Wnt genes must be mediated indirectly through other transcription factors or co-regulators. We envision that precise regulation of each Wnt gene must rely on distinct combinations of transcription factors and co-regulators such that the ultimate
effect of CUX1 on a particular Wnt gene will vary depending of the status of these factors in each cell line.

The fact that one third or less of mammary tumors from MMTV-CUX1 transgenic mice exhibited elevated Wnt gene expression indicated that CUX1 alone is not sufficient to activate this pathway and provided an opportunity to identify other transcription factors that cooperate with CUX1 in this process. Expression profiling of microdissected mammary epithelial tumor cells revealed that Glis1 is overexpressed in tumors that display elevated Wnt expression (Fig. 4). Meta-analysis of human breast cancer datasets showed that elevated WNT gene expression also correlated with high levels of CUX1 and GLIS1 (Fig. 5; supplementary material Fig. S3; Table S1). High expression of GLIS1 in cancers is particularly striking considering that GLIS1 expression in mouse fibroblasts (Maekawa et al., 2011). GLIS1 has been shown to cooperate with Oct3/4, Sox2 and Klf4 (OSK) in the activation of several Wnt genes and in the reprogramming of mouse fibroblasts (Maekawa et al., 2011). Expression profiling analysis indicated that GLIS1 cooperates with OSK to increase the expression of several Wnt ligands (Wnt3, Wnt6, Wnt8a and Wnt10a) in p53–/– MEFs. We also observed that that GLIS1 increased the expression of many Wnt genes in MCF10A cells: Wnt3, Wnt6, Wnt8a and Wnt10a in p53–/– MEFs. We also observed that that GLIS1 increased the expression of many Wnt genes in MCF10A cells: Wnt3, Wnt4, Wnt5b, Wnt6, Wnt9a, Wnt10a and Wnt110b (Fig. 7A). We did not, however, observe the upregulation of stem or progenitor cell markers in mammary tumors that otherwise displayed Glis1, CUX1 and Wnt expression (supplementary material Fig. S2). Hence, we conclude that Glis1 is not sufficient but requires OSK factors to activate stem cell markers. One functional class of genes whose regulation in mammary epithelial cells correlated with that of Wnt, Cux1 and Glis1 are those associated with epithelial-to-mesenchymal transition (EMT). Both in mouse mammary tumors and in human breast cancers, we observed elevation of Vim, Cdh2 (N-cadherin), Snail and Twist1 and downregulation of Cdh1 (E-cadherin) and Ocln (Figs 4, 5; supplementary material Fig. S3). These findings are in agreement with previous studies showing that CUX1 knockdown inhibits cell motility (Michl and Downward, 2006; Michl et al., 2005) and that ectopic CUX1 expression can stimulate cell migration and invasion (Kedinguer and Nepveu, 2010; Kedinguer et al., 2009). Our results in MCF10A human mammary epithelial cells revealed that GLIS1 cooperates with CUX1 to simulate cell migration and invasion. Altogether, the findings that GLIS1 stimulates both the production of induced pluripotent stem cells by OSK factors and the activation of EMT markers by CUX1 indicate that GLIS1 can regulate distinct sets of transcriptional targets depending on cell context. Overall, results from these two independent studies
reveal an important role of GLIS1 in reprogramming gene expression profiles.

Activation of Wnt signaling has been associated with an EMT gene expression signature in a number of studies, notably in basal-like breast cancers (DiMeo et al., 2009). Whether activation of Wnt signaling is sufficient by itself to promote cell motility is not clear. We can envisage a scenario where both Wnt signaling and cell motility would be stimulated by a common set of transcription factors. Indeed, the present study strongly suggests that GLIS1 and CUX1 play an important role in the promotion of both the Wnt signaling and the EMT phenotype in many cancer cells.

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Competing interests

The authors declare they have no conflict of interest.

Author contributions

C.V., M.P., M.H. and A.N. conceived and designed the experiments. C.V., P.S., C.V.R., R.L., L.H., S.D. and C.C. performed the experiments. C.V., P.S. and C.V.R. analysed the data. C.V. and A.N. wrote the paper.

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References


**Fig. S1.** BreSAT sorting of MMTV-CUX1 Mammary Tumors. Heatmap representation of mammary tumors ranked according to Wnt expression using the BreSAT algorithm.

**Fig. S2.** Activation of the Wnt/β-Catenin Pathway in MMTV-CUX1 Mammary Tumors is independent of Frzd receptor and β-Catenin transcript levels. (A) Heatmap of mammary tumors clustered according to Wnt gene expression, with corresponding expression of Frzd receptor genes, β-Catenin and markers of progenitor cells. (B) Boxplot representation of β-Catenin expression in normal mammary epithelial cells, “non-Wnt” tumors and “Wnt” tumors.
Fig. S3. Expression of CUX1, Glis1 and EMT Markers in Human Breast Cancer Datasets. (A) Expression of indicated genes in the top 25% (High Wnt) and bottom 25% (Low Wnt) samples sorted according to Wnt genes expression from the Glück et al. human breast cancer dataset from Oncomine (Glück et al., 2012). * indicates p<0.05, **<0.01, ***<0.001 on a Welch-corrected student’s T test. (B) Analysis as in A for the TCGA Dataset (Cancer Genome Atlas Network, 2012).

Fig. S4. Tracking Plot of Cell Migration and Sample Images. MCF10A cells over expressing Glis1, p110 CUX1 or both were imaged by time-lapse video microscopy (Fig. 7). Ten representative cells from each cell line were randomly selected and their migratory track relative to the origin is depicted using the X and Y coordinates (um). Representative phase contrast images of the cells are also shown for each cell line. MCF10A cells expressing vector, Glis or p110 CUX1 alone had an elongated morphology with very little to no ruffles and no defined leading edge. Whereas in the presence of Glis and p110 CUX1, cells form large lamellapodial ruffles at the leading edge. Scale bars: 100 µm (middle), 10 µm (bottom).
**Table S1. Correlation between Wnt, CUX1 and GLI gene expression in human tumor datasets**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Breast cancer datasets</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUX1</td>
<td>3 of 3 (100%)</td>
</tr>
<tr>
<td>Glis1</td>
<td>7 of 8 (88%)</td>
</tr>
</tbody>
</table>

The table shows the number of human breast cancer datasets retrieved from Oncomine in which the top 25% of samples ranked according to Wnt gene expression display significantly higher expression of CUX1 or GLIS1 than the bottom 25% of samples, as tested by a Welch corrected T Test with P < 0.05.