Unraveling multifaceted roles of Grainyhead-like transcription factor-2 in breast cancer

Coban, B.

Chapter 5

Limited control of EMT/MET balance and targetable vulnerabilities by GRHL2 alone in breast cancer cells

Bircan Coban[1], Zi Wang[1], Julia Star Darnold[1], Cecilia Bergonzini[1], Annelien J.M. Zweemer[1], Erik H.J. Danen[1,2]

[1]Leiden Academic Center for Drug Research, Leiden University, Leiden, The Netherlands; [2]correspondence: e.danen@lacdr.leidenuniv.nl

Summary

Cellular plasticity is a critical factor in the development of resistance to anti-cancer drugs. Epithelial-mesenchymal transition (EMT) is one of the key processes contributing to this plasticity. In this study, we aimed to investigate the relationship between plasticity and drug resistance mediated by the epithelial transcription factor GRHL2 in Luminal and Basal B subtypes of breast cancer. We employed a GRHL2 knockout system in the Luminal subtype using MCF-7 cells and examined the changes in signaling pathways triggered by GRHL2 loss. Our findings revealed that GRHL2 deletion primarily affected the TGFβ pathway but did not provoke a complete EMT. Subsequently, we investigated whether stable or inducible overexpression of GRHL2 in Basal B subtype MDA-MB-231 cells resulted in the inverse process, mesenchymal-to-epithelial transition (MET). However, GRHL2 expression in these cells did not result in significant changes in the EMT/MET balance. Taking advantage of the possibility to explore GRHL2-regulated drug vulnerabilities without being affected by changes in the EMT/MET state, we screened a series of kinase inhibitors in MDA-MB-231 cells lacking or expressing GRHL2. Overall few differential sensitivities to these compounds were detected but four kinase inhibitors were identified that selectively inhibited proliferation of GRHL2-expressing cells in the screen. However, subsequent dose-response experiments showed that these kinases did not represent actionable GRHL2-regulated targets in MDA-MB-231 cells. These findings argue against a major change in EMT/MET balance in response to altered expression of GRHL2 and do not point to GRHL2-regulated drug vulnerabilities in breast cancer.

Introduction

Breast cancer cells rewire signaling pathways that enhance cellular plasticity, enabling resistance to anti-cancer drugs.[1–3] Unraveling the mechanisms modulating the plasticity and drug responses is crucial to overcome drug resistance. This adaptive behavior of the cancer cells is often accompanied by plasticity with respect to the balance between epithelial and mesenchymal characteristics through epithelial-to-mesenchymal transitions (EMT) and mesenchymal-to-epithelial transitions (MET).[4–6]

EMT supports drug resistance, it enables cancer cells to become more motile, and these two responses may be interconnected.[7,8] Cancer cells undergoing EMT often show increased drug efflux due to the upregulation of ATP-binding cassette (ABC) transporters. These transporters pump chemotherapeutic agents out of the cells, thereby reducing their efficacy.[9–11] Moreover, EMT can result in changes in cell cycle regulation, rendering cancer cells less responsive to treatments that target rapidly dividing cells.[12,13] The mesenchymal phenotype also enhances the efficiency of DNA damage repair mechanisms, further contributing to resistance against DNA damage inducing therapies.[7,14]

The connection of EMT not only with cancer cell migration but also with drug resistance highlights the importance of understanding the underlying pathways to develop more effective therapeutic strategies.[15,16] The Grainyhead-like-2 (GRHL2) transcription factor has been shown to act as a critical epithelial suppressor of EMT.[17–20] During EMT, loss of GRHL2 results in a more mesenchymal phenotype with enhanced invasive properties.[21,22] In addition, it was shown that silencing GRHL2 expression increases the sensitivity of ovarian cancer cells to cisplatin.[23] GRHL2 is also implicated in the regulation of various signaling pathways associated with drug resistance, including the PI3K/AKT/mTOR pathway, the MAPK/ERK pathway, and the NF-κB pathway.[24,25]

Elucidating molecular mechanisms behind GRHL2-mediated drug responses and the signaling pathways regulated by GRHL2 could lead to the development of new targeted therapeutic strategies for breast cancer. To study this, we took two approaches: GRHL2 was deleted in Luminal breast cancer cells, or it was overexpressed in Basal B breast cancer cells. The effect on signaling pathways, EMT/MET balance, and drug sensitivity was explored.

Materials and Methods

Cell culture

Human breast cancer cell lines MDA-MB-231 (Basal-b subtype; triple negative breast cancer (TNBC)), MCF-7 (Luminal subtype), and the human embryonic kidney cell line HEK293T were obtained from ATCC. MCF-7 and MDA-

MB-231 were cultured in RPMI 1640 while HEK293T cells were cultured in in DMEM (Dulbecco's Modified Eagle Medium, both supplemented with 10% fetal bovine serum (FBS), 25 U/mL penicillin, and 25 µg/mL streptomycin (Fisher Scientific) and maintained in a humidified incubator with 5% CO2 at 37 °C.

Bru-seq analysis of EMT-associated genes

CRISPR/Cas9-mediated conditional knockout (KO) of GRHL2 in MCF-7 cells expressing one of two different GRHL2 sgRNAs or a control sgRNA in combination with an inducible Cas9 construct was induced using 1ug/ml doxycycline (dox) for 8 days as explained previously.[21,26] By employing the KO system, Bru-seq analysis enabled identification of GRHL2-regulated genes and pathways.[27] Based on their relationship with GRHL2, six EMT-associated genes (Occludin, Zonula Occludens-1/ZO-1, E-cadherin, Claudin-4/CLDN4, Vimentin, and Zinc finger E-box-binding homeobox 1/ZEB1) were chosen to explore signs of EMT following GRHL2 deletion. Changes in gene expression were calculated by comparing dox-treated KO-1 and KO-2 cells to the same cells without dox induction. The Bru-seq data is accessible in the Gene Expression Omnibus 477 (GEO) database, www.ncbi.nlm.nih.gov/geo (Accession No. GSE222353).

Measuring Pathway activity

The effect of GRHL2 loss on the functional activity of signaling pathways was assessed using the Philips Pathway Activity Profiling OncoSignal platform (https://images.philips.com/is/content/PhilipsConsumer/Campaigns/HC20140401_DG/Documents/HC06172020-2020-05_mpdx_flyerpdf.pdf). GRHL2 deletion was induced with 8ug/ml dox in MCF-7 cells and RNA isolation was performed using Trizol method. Purified RNA samples were used for the pathway analysis using the Oncosignal qPCR kit (Philips Molecular Pathway Diagnostics, Eindhoven, The Netherlands). The kit was designed to measure the activities of pathways driven by hormone receptors Androgen receptor (AR) and Estrogen receptor (ER), stem-cell related pathways (TGFβ and Hedgehog (HH), and growth factor pathways (PI3K) using several direct target genes within that pathway. PI3K pathway activity is

based on the inverse activity of the measured FOXO transcription factor activity score. The pathway activities are scored on 0-100 scale using a Bayesian computational model to determine whether the pathway is activated or not (0 score corresponds to the lowest and 100 corresponds to the highest probability of an active pathway).[28]

Establishment of GRHL2 overexpressing cells

For the stable expression of GRHL2 in MDA-MB-231 cells a pLenti-GIII-CMV-GFP-2A-Puro construct (Applied Biological Materials) containing a GRHL2 insert, and an empty control construct were kindly provided by Dr. Ruby Yun-Ju Huang (National University of Singapore). Lentiviral particles were generated using HEK293T cells as previously described[26] and used for transduction of MDA-MB-231 cells. Transduced cells were selected using 5ug/ml Puromycin and GFP-sorted. For inducible GRHL2 expression, the Lenti-XTM Tet-On 3G System (TakaraBio, 631187) was used. For this, lentiviral particles were generated as described and MDA-MB-231 cells were transduced with a pLVX-EF1a-Tet3G construct expressing a Tet-ON 3G transactivator protein, either alone (CTR) or combined with pLVX-TRE3G-Luc expressing luciferase (Luc*) or pLVX-TRE3G-GRHL2 expressing GRHL2 (GRHL2*). Transduced cells were selected with Puromycin. The asterisk indicates inducible expression. For induction, MDA-MB-231 cells were treated with 125 ng/ml dox for different time periods.

Western Blot

Cells were lysed using RIPA buffer. SDS-PAGE was run using 20µg lysates and transferred to PVDF membranes. The membranes were incubated overnight with the following antibodies; GRHL2 (1:1000, Atlas antibodies, hpa004820), E-cadherin (1:1000, Abcam, ab76055), CLDN4 (1:1000, Thermo Fisher, 329400), GAPDH (1:2000, Santa Cruz, sc-32233). HRP-linked anti-mouse and anti-rabbit secondary antibodies were used on the next day to detect protein expression with Prime ECL Detection Reagent. Membranes were detected with Amersham Imager 600 (GE Healthcare Life Sciences, the Netherlands).

Immunofluorescence

Cells were seeded in 96well plates and fixed/permeabilized with 4% formaldehyde and 0.1% Triton X100 in Phosphate-buffered saline (PBS) for 15 mins. The cells were incubated with primary antibodies recognizing GRHL2 (1:500, Atlas-Antibodies, hpa004820), ZO-1 (1:100, Cell Signaling, 13663S), Occludin (1:300, Cell Signaling, 91131S), Claudin 4 (1:100, Thermo Fisher, 329400), E-cadherin (1:1000, Abcam, ab76055), Vimentin (1:100, Abcam, ab8069), or ZEB1 (Santa Cruz, sc-515797) overnight at 4°C. After washing, AlexaFluor-488 conjugated anti-rabbit and anti-mouse secondary antibodies were incubated with Hoechst 33258 (1:10,000, Sigma Aldrich, 861405) and Rhodamin Phalloidin (1:1000, R415, Thermo Fisher) for 1 h at room temperature. Images were taken with a Nikon ECLIPSE Ti2 confocal microscope, 20x objective. The imaging data were organized using OMERO Database.

Sulforhodamine B (SRB) Assay

To examine the effect of GRHL2 overexpression in cell proliferation, MDA-MB-231 cells were seeded at a density of 3000cells/well in 96 well plates after 3 and 10 days of dox treatment. Four days later (day 7 and day 14 of GRHL2 induction), plates were fixed using 50% Trichloroacetic acid (TCA). The next day, 0.4% SRB was used to stain cells and the unbound SRB was washed away using 1% acetic acid. 10mM Tris was added to the plates and absorbance measurements were performed at 540nm using a BioTek Synergy HT plate reader (SN 269140, BioTek Instruments Inc.). The data were analyzed in Graphpad Prism, version 9.0.

Kinase inhibitor (KI) screening and validation

MDA-MB-231-Luc* and MDA-MB-231-GRHL2* cells were treated with dox for 10 days, seeded at a density of 3000cells/well in 96 well plates and exposed for 4 days to 760 KIs from the L1200 library (Sellcheckchem, Munich, Germany). KIs were dissolved in 0.1% DMSO or water to a final concentration of 1uM. Cisplatin (1uM) served as a positive control. After 4 days, the cells were fixed and analyzed with SRB assay as described. & proliferation was obtained by normalizing the data to DMSO or water treated cells for each treatment plate. The screen was performed in single technical replicates and two

independent biological replicates were performed. KIs of interests (Torkinib, Mirin, A-674563 and LDC-4297) chosen based on the two biological replicates were tested together with some known DNA damaging agents (Cisplatin, Gemcitabine and Docetaxel) in a dose response curve. MDA-MB-231 cells were seeded as explained above and treated with increasing doses (0.1, 0.3, 1, 3, 10uM) of these six drugs for 4 days and processed for SRB.

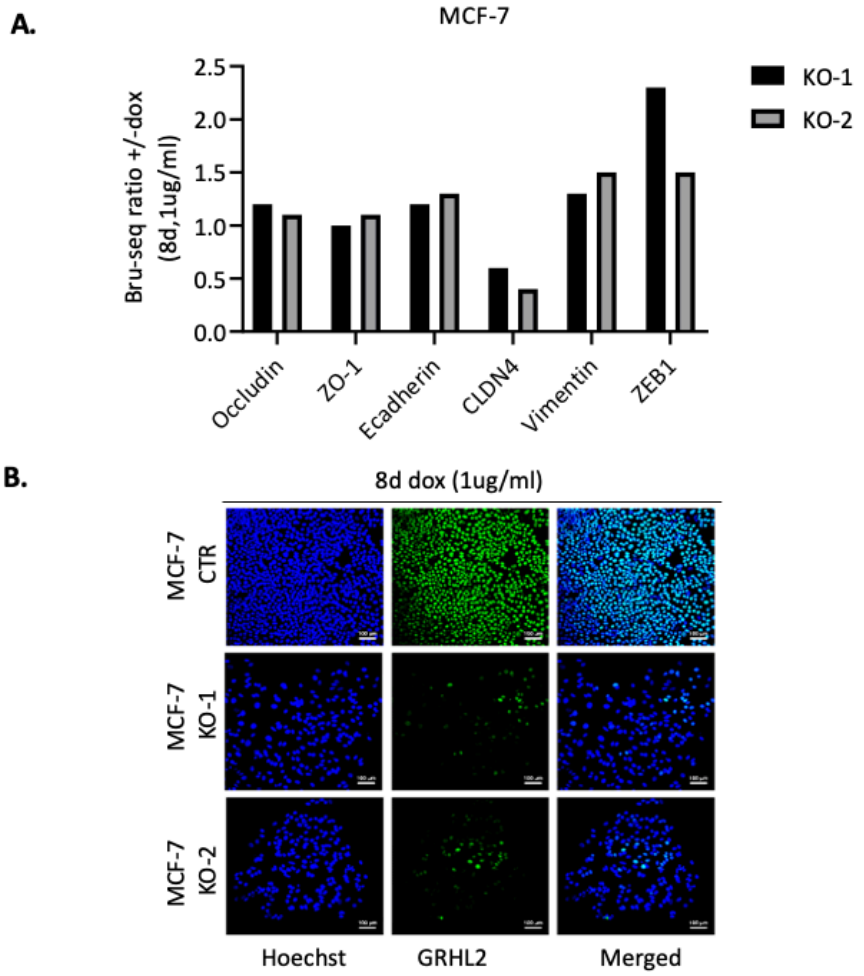
Statistical analysis

GraphPad Prism 9 was used to perform one-way ANOVA with Tukey's multiple comparison test for statistical analysis.

Results

GRHL2-controlled signaling pathway activities in Luminal-like breast cancer cells

We have previously identified GRHL2-controlled gene networks in MCF-7 cells.[27] To investigate the effect of GRHL2 deletion on EMT progression, we employed Bru-seq data of MCF-7 cells in which GRHL2 KO was induced. We chose a timepoint of 8 days treatment with dox be in line with a subsequent experiment where changes in signaling were explored in the same system. A panel of EMT-associated genes was analyzed: epithelial markers Occludin, ZO-1, E-cadherin, CLDN4 and mesenchymal cell markers Vimentin and Zeb1. Overall, mRNA expression levels were similar for both KOs although CLDN4 was downregulated in GRHL2 KO cells (Fig. 1A). The change in CLDN4 did not reach statistical significance as compared to CTR cells at this time-point but it was significantly downregulated at other timepoints (see chapter 3). This suggested that deletion of GRHL2 alone is insufficient to trigger an EMT in Luminal-like breast cancer cells, in contrast to the EMT-related changes described previously. [29,30]


A.
MCF-7
Bru-seq ratio +/-dox (8d,1ug/ml)
2.5
2.0
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0.0
KO-1
KO-2
Occludin
ZO-1
Ecadherin
CLDN4
Vimentin
ZEB1
B.
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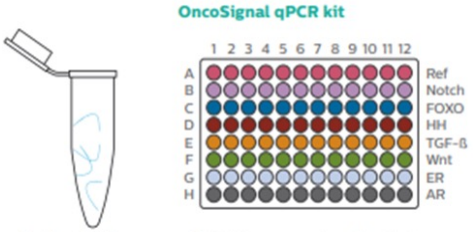
C.



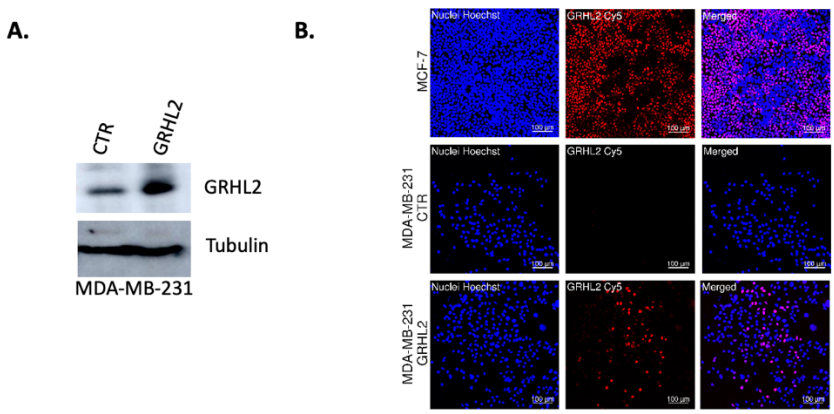
	Pathway Activity Score				
Sample ID	**AR**	**ER**	**HH**	**TGFB**	**PI3K**
MCF-7 CTR	21	56	15	19	50
MCF-7 CTR + dox	15	53	15	22	52
MCF-7 KO-1	12	56	17	20	49
MCF-7 KO-1 + dox	13	60	12	**35**	**67**
MCF-7 KO-2	12	54	15	16	52
MCF-7 KO-2 + dox	15	62	15	**35**	**66**

Figure 1: Signaling pathways affected by GRHL2 deletion in luminal cells. (A) Bru-seq analysis of EMT-associated genes in MCF-7 cells with GRHL2 KO-1 and KO-2 ; induced by 1ug/ml dox for 8 days. Graph representing fold change of transcription in response to GRHL2 deletion. (**B)** Immunofluorescence images showing GRHL2 expression after 1ug/ml dox exposure for 8 days for MCF-7 CTR, KO-1 and KO-2 cells ; Hoechst (blue), and GRHL2 Ab (green). (**C)** Cartoon explaining the Oncosig-nal qPCR platform to measure signaing pathway activities. Pathway activities were evaluated in RNA isolated from MCF-7 cells with or without dox induction (1ug/ml, 8 days). Scores range from 0-100 after normalization to house-keeping genes.

Following this, we sought to elucidate changes occurring in signaling path-ways in response to GRHL2 deletion. For this, we made use of the qPCR-based Philips Oncosignal platform. A complete loss of GRHL2 was achieved in KO-1 and KO-2 cells using 8 days of dox (Fig. 1B). The activities of five different pathways that play an important role in (breast) cancer growth and progres-sion were evaluated. No differences were observed in the activities of the ER, AR, or HH signaling pathways (Fig. 1C). Activities of the PI3K and TGFβ path-ways were elevated in both KOs as compared to CTR cells. Together, these data indicate that despite upregulation of TGFβ signaling (which is a major EMT inducing pathway[31,32] in response to GRHL2 deletion, this is not suffi-cient to trigger an EMT in Luminal-like breast cancer cells.

Stable overexpression of GRHL2 in MDA-MB-231 cells does not trigger an MET

We next performed an inverse experiment where GRHL2 was stably overexpressed in Basal-B cells. These cells express little or no GRHL2 as compared to Luminal-like breast cancer cells and have a mesenchymal phenotype. Western blot analysis confirmed that GRHL2 cDNA expressing MDA-MB-231 cells had higher GRHL2 protein expression as compared to CTR cells expressing an empty vector (Fig. 2A). This result was confirmed by immunostaining and showed that GRHL2 cDNA expression levels were comparable to the endogenous expression in MCF-7 cells, but not all MDA-MB-231-GRHL2 cells expressed the cDNA (Fig. 2B). We analyzed changes in protein expression of selected EMT/MET-associated genes induced by GRHL2 overexpression using immunostaining. Based on the expression of GRHL2 in a subset of cells, a change in the expression pattern may be expected in a subset of the cells for these markers. However, no obvious downregulation of the mesenchymal markers Vimentin and ZEB1 was observed (Fig. 2C). Moreover, no enhanced expression of the epithelial genes E-cadherin, Occludin, or ZO-1 was observed and expression of CLDN4, encoded by the established direct GRHL2 target gene *CLDN4*, was not affected (Fig. 2C). These results demonstrate that stable expression of GRHL2 in this TNBC cell line is insufficient to induce MET-associated changes in gene expression.



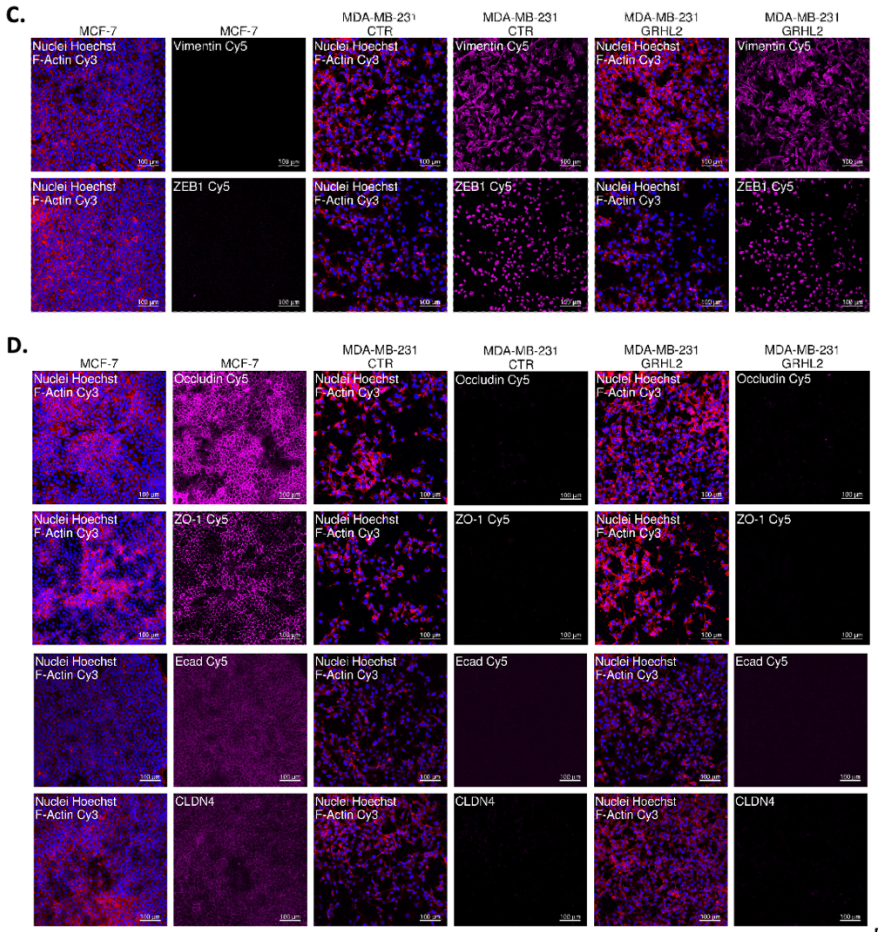
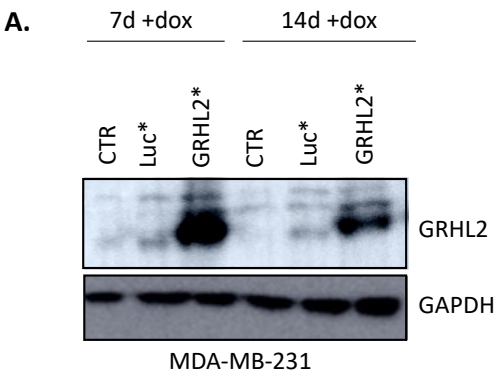


Figure 2: Effect of stable GRHL2 overexpression in Basal-b cells on EMT-associated genes. (A, B) GRHL2 protein expression detected by western blotting (A) and Immunoflorescence (B) in CTR and GRHL2-overexpressing MDA-MB-231 cells. Immunofluorescence analysis of GRHL2 protein expression in MCF-7 cells serves as control for endogenous expression level of GRHL2 protein. Blue, Hoechst; Red-Cy5 GRHL2 Ab. **(C, D)** Immunostaining of mesenchymal markers Vimentin and ZEB1 (C) and epithelial markers Occludin, ZO-1, E-cadherin and CLDN4 (D) in MCF-7, MDA-MB-231 CTR, and MDA-MB-231-GRHL2 cells. Blue, Hoechst; Cy5, Abs recognizing EMT /MET-associated genes; Cy3, Phallodin.

No MET-associated changes are observed upon inducible GRHL2 overexpression in MDA-MB-231 cells

We next sought to investigate the effect of induced, strong expression of GRHL2 in MDA-MB-231 cells. Therefore, we utilized a dox-inducible GRHL2 overexpression system to investigate possible early, but transient signs of MET triggered by GRHL2. MDA-MB-231 cells were analyzed by Western Blot after 7 and 14 days of dox treatment (Fig. 3A). A clear induction of GRHL2 was observed at both time points in GRHL2* overexpressing cells but not in CTR or Luc* expressing cells. We further validated this system by immunostaining of GRHL2, localized in the nucleus, upon 7 and 14 days of dox induction (Fig. 3B). Then, we examined the changes in the expression of EMT/MET associated genes after GRHL2 overexpression but, again, no induction was observed for E-cadherin or CLDN4 upon expression of GRHL2 (Fig. 3C). Altogether, these findings show that overexpression of GRHL2 by itself does not trigger MET in MDA-MB-231 cells, contradicting previously reported findings, which demonstrated GRHL2-mediated phenotypic and genetic changes in these cells.[33,34]



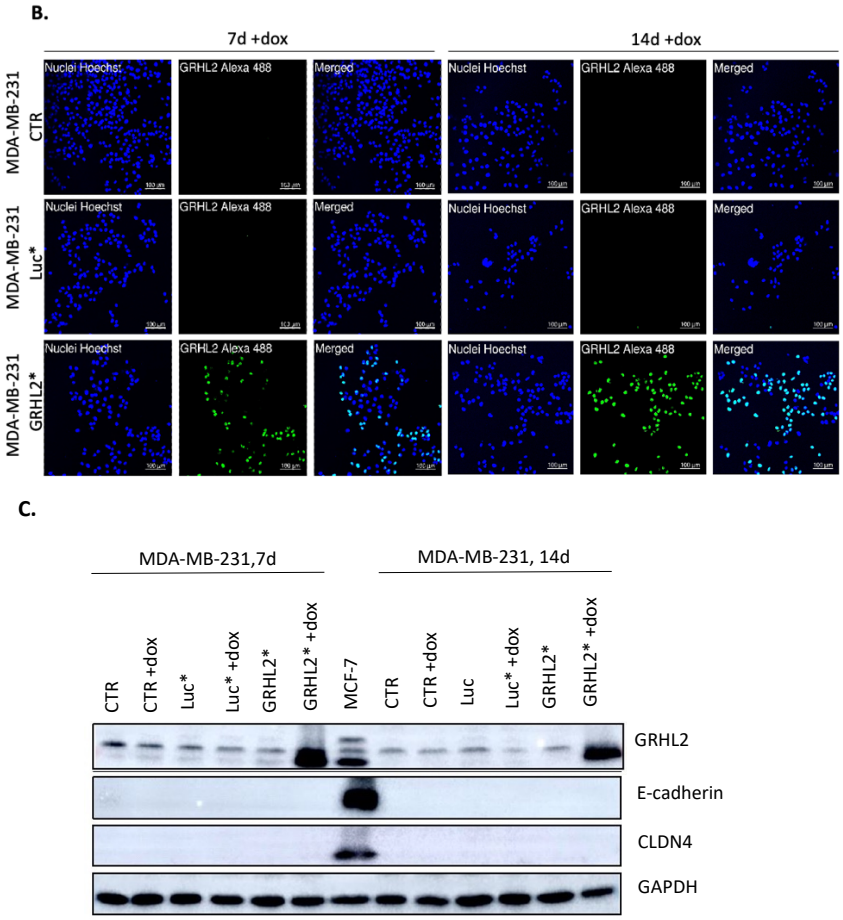


Figure 3: Inducible GRHL2 overexpression does not confer MET-associated changes in Basal-b cells. (A, B) Western blot (A) and immunofluorescence (B) analysis showing GRHL2 protein expression after 7 and 14 days dox induction (125ng/ml) in CTR, Luc*, and GRHL2* expressing MDA-MB-231 cells. Blue, Hoechst; Green, GRHL2 Ab. **(C)** Western blot showing alterations in protein expression of GRHL2 and epithelial markers CLDN4 and E-cadherin in CTR, Luc*, and GRHL2* expressing MDA-MB-231 cells with or without 7 and 14 days of dox (125ng/ml) exposure. MCF-7 was used to show endogenous levels of GRHL2 protein in Luminal cells.

GRHL2 overexpression does not affect MDA-MB-231 cell growth

Given the impact of GRHL2 deletion on cell survival and proliferation in luminal breast cancer cells,[21,27] we assessed the impact of GRHL2 overexpression on growth of MDA-MB-231 cells. In MDA-MB-231-GRHL2* cells treated for 7 days with dox GRHL2 expression was detected while it was absent in MDA-MB-231-Luc* cells (Fig. 4A). The cells were seeded after 7 days dox exposure, and cell viability was assessed after an additional 4 days growing in absence of dox using an SRB assay. This experiment did not show any significant difference in growth potential between MDA-MB-231-Luc* or MDA-MB-231-GRHL2* cells in absence or presence of dox (Fig. 4B).

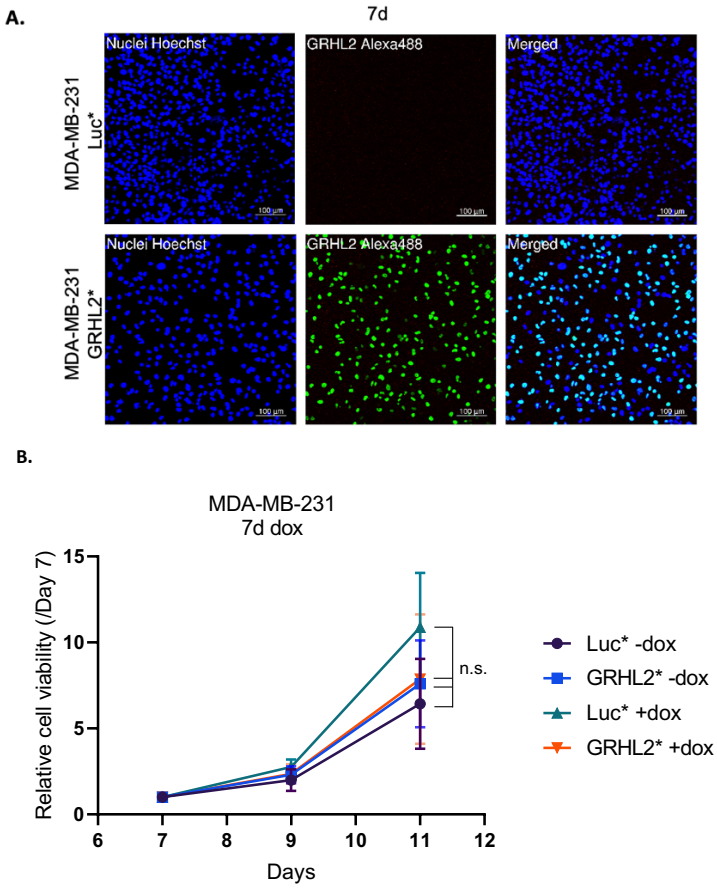


Figure 4: Cell growth is not affected by GRHL2 overexpression in MDA-MB-231 cells. (A) Immunostaining showing GRHL2 expression in MDA-MB-231-Luc* and MDA-MB-231-GRHL2* cells exposed to 125ng/ml dox for 7 days. Blue, Hoechst; Green, GRHL2 Ab. **(B)** Cell growth analyzed by SRB assay in MDA-MB-231 cells without and with 7 days dox induction of GRHL2 overexpression in MDA-MB-231-Luc* and MDA-MB-231-GRHL2* cells. Data was normalized to day 7. Mean ± SD of three biological replicates is shown. ns, non-significant.

Kinase inhibitor library screening identifies novel GRHL2-mediated vulnerabilities

The results thus far demonstrated that MDA-MB-231-GRHL2* cells provided a model in which the impact of GRHL2 overexpression in Basal-b TNBC cells could be determined on drug vulnerabilities without confounding effects on baseline growth or EMT/MET balance. Therefore, we adopted a kinase inhibitor screening approach. First, we induced GRHL2 overexpression with 9 days of dox treatment in MDA-MB-231-GRHL2* cells and used identically treated MDA-MB-231-Luc* cells as control. These cells were exposed to 760 kinase inhibitors at 1uM final concentrations for four days in two biological replicates and cell viability was determined using an SRB assay (Fig.5A). 1uM Cisplatin served as a positive control since its effect on MDA-MB-231 cell viability has been studied.[35,36] Two biological replicates were performed. In both replicates MDA-MB-231-GRHL2* cells were somewhat more sensitive to Cisplatin than MDA-MB-231-Luc* cells (Fig. 5B). In addition, four kinases of interest (Torkinib, A-674563, LDC4297, Mirin) were identified that caused a reduction to <50% cell growth in MDA-MB-231-GRHL2* cells while growth of MDA-MB-231-Luc* cells was considerably less affected. Interestingly, the PI3K/AKT pathway, which was identified as a GRHL2-regulated signaling pathway (Fig. 1C) was a target of two of these inhibitors.

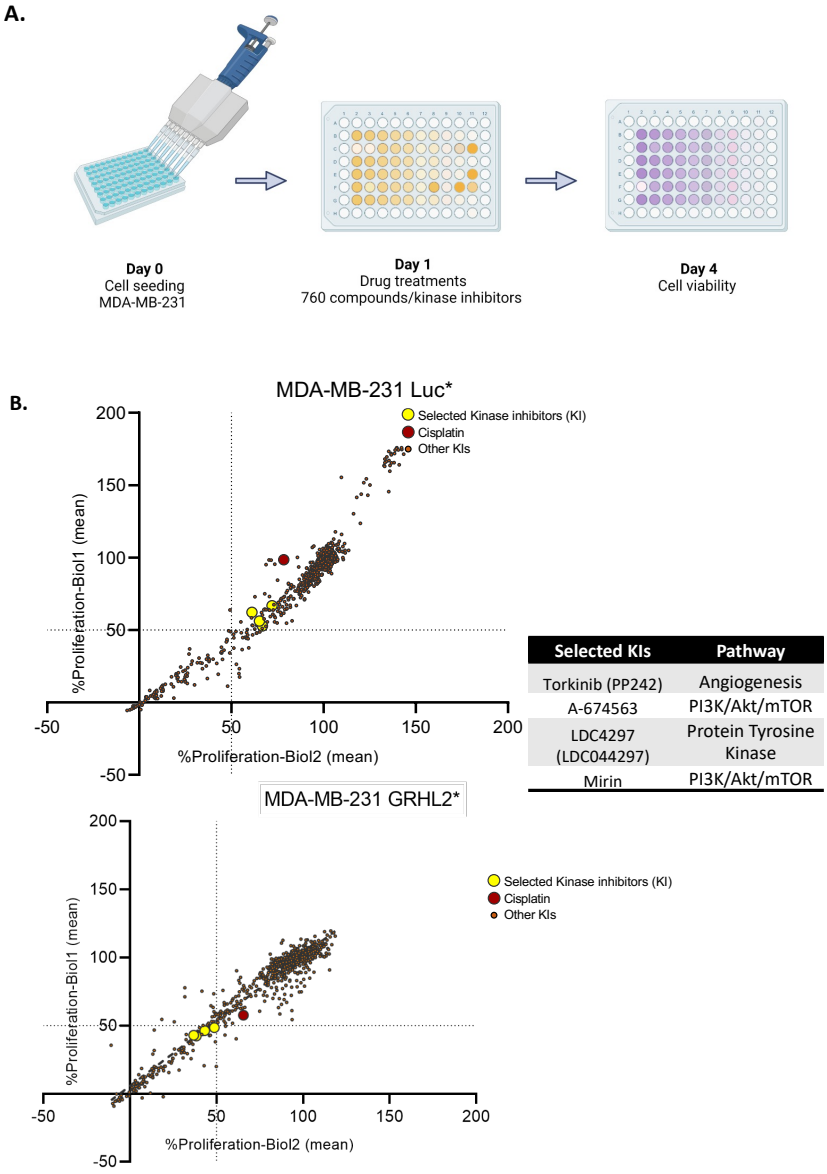


Figure 5: Kinase inhibitor library screening in control and GRHL2 overexpressing Basal-b cells. (A) Schematic representation of the kinase screen approach. Following the dox induction (125ng/ml) for 7 days in MDA-MB-231-Luc* and MDA-MB-231-GRHL2* cells, cells were treated 4 days with 1µM of 760 kinase inhibitors, Cisplatin, or vehicle (DMSO) followed by SRB assay. **(B)** Effect of kinase inhibitors

on growth of dox-induced MDA-MB-231-Luc* and MDA-MB-231-GRHL2* cells. The percentage growth is relative to DMSO condition. Two biological replicates, each performed in single technical replicates are plotted against each other. Four kinases of interest (%growth in GRHL2* < %growth in Luc*) are marked yellow. Cisplatin is marked red.

We subsequently analyzed the effect of concentration ranges of the 4 selected kinase inhibitors and included two additional DNA damaging chemotherapeutics, Docetaxel and Gemcitabine.[37,38] In this experiment, using the same strategy for the induction of GRHL2 or Luc, Cisplatin did not affect cell growth while Docetaxel and Gemcitabine strongly inhibited cell growth of MDA-MB-231-GRHL2* as well as MDA-MB-231-Luc* cells (Fig. 6). A674553, LDC4297, Torkinib, and Mirin inhibited cell growth in a concentration-dependent manner that was similar for MDA-MB-231-GRHL2* and MDA-MB-231-Luc* cells. These data demonstrate that induced expression of GRHL2 in Basal-b cells, in absence of effects on baseline growth or EMT/MET balance, does not affect sensitivity for chemotherapy or kinase inhibition.

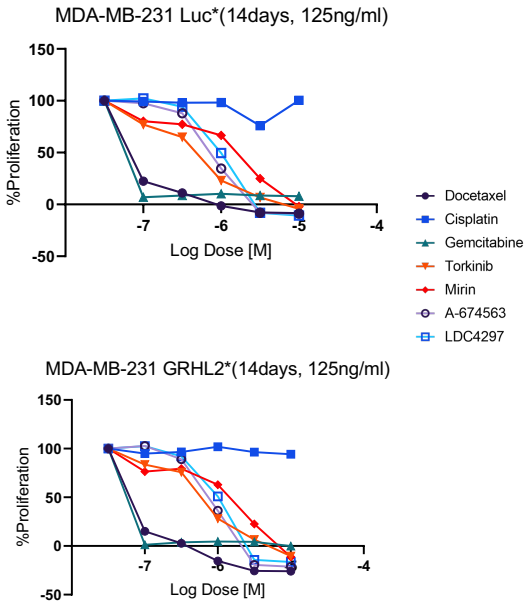


Figure 6: Effect of selected kinase inhibitors and DNA damaging drugs on growth of control and GRHL2 overexpressing Basal-b cells. MDA-MB-231-Luc* and MDA-

MB-231-GRHL2* cells were treated for 7 days using 125ng/ml dox. Induced cells were exposed to the indicated concentrations of selected kinase inhibitors Torkinib, Mirin, A-674563, or LDC4297 or DNA damaging drugs Docetaxel, Cisplatin, or Gemcitabine for 4 days. The percentage growth was determined with SRB assay and expressed relative to DMSO. Mean of three technical replicates for one experiment is shown.

Discussion

Modulating cellular plasticity holds the potential to augment the sensitivity of cancer cells to therapies and improve patient outcomes. Prior research has highlighted the influence of EMT transcription factors (TFs) on inducing or suppressing EMT, thereby controlling anti-cancer drug resistance.[8,39] GRHL2, functioning as a master regulator of the epithelial phenotype, serves to inhibit more invasive and aggressive phenotypes, thereby fostering sensitivity to anti-cancer therapies.[40,41] Our results show that GRHL2 is not solely sufficient to disrupt the balance in EMT process. The interaction of GRHL2 with other transcription factors/genes may be required to facilitate EMT/MET. Indeed, GRHL2 operates within a network controlling gene expression of other EMT-TFs. A negative feedback loop between GRHL2 and ZEB1 has been previously reported.[22,42] Ultimately, EMT suppression mediated by GRHL2 requires downregulation of E-cadherin.[25,43]

Similar to an earlier study using MDA-MB-231 cells overexpressing GRHL2,[33] our study revealed no discernible impact of GRHL2 on cellular growth of this model. However, that same study showed that overexpression of GRHL2 triggers MET-like phenotypical and molecular changes (induced expression of E-cadherin) in MDA-MB-231 cells.[33] Our study does not support these findings. In our experiments MET-like alterations were evaluated using the mesenchymal markers Vimentin and ZEB1, and the epithelial markers Occludin, ZO-1, E-cadherin, and CLDN4. No significant changes were observed in their expression levels when GRHL2 was overexpressed. EMT progression by GRHL2 knockdown has been linked to epigenetic remodeling including histone modifications and DNA methylation in ovarian cancer.[44] In that study, removal of epigenetic marks on the histones using 5-azaticidine together with GRHL2 overexpression induced MET in ovarian cancer cells. It is possible that the

MDA-MB-231 cells used in our study and that of Werner et al[33] vary epigenetically. It is also possible that the level of GRHL2 overexpression in our experiments was less strong as compared to that achieved in the study by Werner et al. However, we confirmed that we reached GRHL2 expression levels that were similar to the endogenous level present in MCF7 Luminal breast cancer cells. Lastly, the inability of GRHL2 to stimulate MET in MDA-MB-231 cells could be explained by the lack of ER alpha (ERα) signaling in MDA-MB-231 cells. Although we have demonstrated that GRHL2 rarely acts in a complex with ERα,[27] there is evidence that GRHL2 cooperates with the ERα/FOXA1/GATA3 complex[45] and the absence of ERα signaling may prevent MET induction or effects on proliferation by GRHL2.

GRHL2 regulates multiple signaling pathways (MAPK, TGFβ) that determine the anti-cancer drug response.[46,47] Basal-like breast cancer cells that survived after the therapy have been linked to lack of histone acetylation by H3K27ac, a well-known transcription enhancer, at regulatory sites of GRHL2.[48] To identify GRHL2-mediated drug vulnerabilities, we exposed CTR and GRHL2 overexpressing MDA-MB-231 cells to small molecule kinase inhibitors and chemotherapeutic agents. Interestingly, we find that besides TGFβ signaling, PI3K signaling is increased upon deletion of GRHL2 in luminal breast cancer cells, and 2/4 small molecule kinase inhibitors that appeared to selectively affect GRHL2 overexpressing MDA-MB-231 cells target PI3K signaling. Nevertheless, follow up experiments did not indicate significant vulnerabilities that are controlled by GRHL2. This implies that additional factors or mechanisms are at play in determining treatment sensitivity. The combined absence of an induction of MET and enhanced therapy sensitivity in response to GRHL2 in our study, indicates that the ability of GRHL2 to affect drug responses in breast cancer cells reported in other models may be strictly linked to its ability to shift the EMT/MET balance towards MET.

In conclusion, we find that depletion of GRHL2 in Luminal breast cancer cells or induction of GRHL2 in in Basal b breast cancer cells does not necessarily lead to a shift in the EMT/MET balance. The impact of changes in GRHL2 expression must be context dependent, which also leads to apparently distinct

effects on therapy sensitivity in different models. The engagement of GRHL2 in multifaceted regulatory networks must be distinct in different breast cancer cell models thereby making a general prediction of the outcome of GRHL2 manipulation impossible.

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