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Review Article

Grainyhead-like 2 as a double-edged sword in development and cancer

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Abstract: Grainyhead-like 2 (GRHL2), one of the three homologs of *Drosophila* grainyhead, contributes to epithelial morphogenesis and differentiation. Dysregulation of GRHL2 has been shown to be involved in hearing loss and neural tube defects during embryogenesis. Moreover, it is well-recognized that GRHL2 suppresses epithelial-to-mesenchymal transition (EMT) that is required for migration and invasion of carcinoma, implicating, GRHL2 in carcinogenesis. Diverse mechanisms, as well as the varied roles of GRHL2 in different tumor tissues, have been elucidated. However, the functions of GRHL2 appear to be more complicated than initially thought. GRHL2, acting as either a tumor enhancer or a tumor inhibitor, depends on the type of cancer. In this review, we summarize research progress about normal physiological functions of GRHL2 including epithelial morphogenesis, neural tube closure, and hearing loss. Moreover, the mechanisms of GRHL2 in tumorigenesis, containing EMT suppression, forming a negative feedback loop with ZEB1 and miR200 family, interactions with estrogen receptor (ER)-dependent signaling pathway, regulation of telomerase reverse transcriptase and relationships with TGF-beta signaling pathway are discussed in this review in an effort to better understand the roles of GRHL2 in a variety of cancers toward the goal of GRHL2-targeted treatment in the near future.

Keywords: Grainyhead-like 2, epithelial morphogenesis, neural tube closure, tumorigenesis, epithelial-to-mesenchymal, estrogen receptor

Introduction

The Grainyhead (GRH) gene, the first member of the Grainyhead-like (GRHL) proteins family, was first reported in mutant *Drosophila melanogaster* embryos that showed a head defect: holes in large specific cuticular regions, abnormal lack of cuticular structures leading to a specific phenotype [1]. Later, more GRH family members and their specific functions were discovered [2-4]. According to differences of their biological roles, these family members are split into two different subfamilies, the LSF subfamily including CP2, LBP-1a, and LBP-9 transcription factors and the GRH subfamily, consisting of GRHL1, GRHL2, and GRHL3 transcription factors [5, 6]. The biological roles of the LSF subfamily are distinguished from those of GRH subfamily in that they widely regulate tissue development such as liver function and neural system development. Additionally, regulation of the cellular processes including cell cycle progression and cell survival are observed as well [7-9]. The LSF subfamily is systematically reviewed in the context of cancer [10]. Unlike LSF, GRHL proteins, a highly-conserved subfamily, are associated with the development and maintenance of the epithelial barrier. During murine development, GRHL proteins are expressed in the epidermis, oral cavity, gastrointestinal tract and non-ectoderm-derived tissues including the heart, the lung and the kidney [11]. Roles of GRHL proteins have been widely studied in the normal and abnormal development of the epidermis. GRHL1-null mice show defective hair anchoring, altered keratinocyte terminal differentiation and abnormal desmosomes suggesting that GRHL1 may play a key role in maturation and differentiation of epidermis [12]. It has also been shown that the loss of GRHL1 has an essential influence on the maintenance of the epidermal barrier [13]. The

GRHL3 protein is necessary for neural tube closure and wound healing, knockdown of GRHL3 results in spina bifida and severe barrier defects with death at birth [11, 14-16], relevant mechanisms are also explored and studied [17, 18].

GRHL transcription factors are classified as DNA-binding nuclear proteins containing a transactivation domain, a highly conserved DNA-binding domain (DBD), and a dimerization domain. The GRHL DBD is more structured than the transactivation domains. Despite their importance in development and tumorigenesis, their structure and DBD remain elusive. The crystallographic analysis shows that GRHL1 and GRHL2 share a highly conserved three-dimensional structure characterized by an IgG-like core. A recent report presents the first crystal structures study of mammalian GRHL1 DBD and GRHL2 DBD. Their structures are closely similar and contain an Ig-like core decorated by three α helices and a series of surface loops. The crystal structure of the GRHL1 DBD shows consensus binding sequence (AACCGGTT) which is shared by all members of the GRHL family bound to a 12-base-pair DNA duplex. Lys386 benefits the overall stabilization of the DBD-DNA complex. Arg427, Gly387, and Arg-430 are required for formation of DBD-DNA complex [19]. Interestingly, the protein fold of the GRHL1 DBD resembles the tumor suppressor p53 and their DNA-binding modes are similar suggesting cooperation of p53 and GRHL proteins during epidermal development and function [19, 20].

Recently, GRHL2 has drawn great attention for its physiological functions in embryogenesis and diseases including cancer. The GRHL2 gene is located on human chromosome 8q22. GRHL2 expression is detected in epidermis tissue, lung and kidney during murine embryogenesis. Placenta, brain, lung, salivary gland, thymus, and pancreas in human adults show relative high GRHL2 expression. Therefore, once GRHL2 is in a disorder condition, disease may follow. To date, many studies have emphasized the mechanisms and roles of GRHL2 in diseases. It is well established that GRHL2 regulates epithelial morphogenesis, neural tube closure, and hearing loss. In addition, GRHL2 contributes to the tumorigenesis via various signaling pathways such as epithelial-to-mesenchymal transition, miR200 family as well as human telomerase reverse transcriptase. Furthermore, the role of GRHL2 appears to be more complicated than we predicted. In the present review, we summarize research progress about the normal physiological functions of GRHL2 including epithelial morphogenesis, neural tube closure, and hearing loss. Moreover, the mechanisms of GRHL2 in tumorigenesis, containing EMT suppression, forming a negative feedback loop with ZEB1 and miR200 family, interactions with ER-dependent signaling pathway, regulation of telomerase reverse transcriptase and relationships with the TGF-beta signaling pathway are discussed in an effort to better understand the roles of GRHL2 in a variety of cancers toward the goal of GRHL2-targeted treatment in the near future.

Physiological functions of GRHL2 in mammals

A study by Riethdorf et al [21] revealed the expression relevance of GRHL2 in a variety of normal tissues by performing a comprehensive immunohistochemical analysis. Many studies show the irreplaceable role of GRHL2 in neural tube closure during embryogenesis as well as regulation of epithelial phenotype. Moreover, hearing loss is partially regulated by GRHL2. Thus GRHL2 may serve as an important transcription factor for embryo development and maintenance of tissues.

GRHL2 influences neural development and formation of the craniofacial skeleton during embryogenesis

Neural tube closure is necessary for the development of the central nervous system (CNS) during embryogenesis. If neural tube closure is defective, it will lead to neural tube defects (NTDs). As the most common birth defect in humans, NTDs result in malformations of the face and brain. The most common NTDs are anencephaly arising from defective closure of anterior neural tube closure, and spina bifida resulting from the failure of neural tube closure in the spinal column [22]. To date, more than 200 underlying genes have been associated with NTDs [23, 24]. It has been shown that the Grainyhead family, including both GRHL2 and GRHL3, is involved with NTDs. The upregulation of GRHL2 is observed in mutant NTDs murine embryos [25]. Discussed here, and summarized in **Figure 1**, GRHL2 deletion mice exhibit

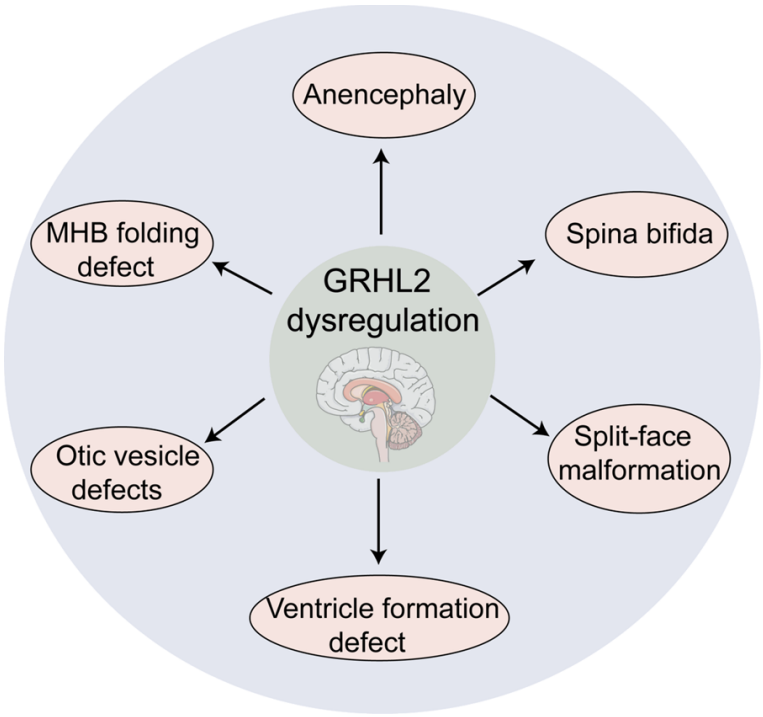

Figure 1. GRHL2 influences neural development and formation of the craniofacial skeleton during embryogenesis. GRHL2 dysregulation may exhibit severe defects during development such as anencephaly, spina bifida, split-face malformation, ventricle formation and otic vesicle defects, MHB folding defect.

split-face malformation and NTDs [26]. The mechanism regarding GRHL2 regulation in NTDs reveal that the ten-eleven translocation 1 (TET1) protein, which converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), is significantly decreased in the head of mutant NTDs murine. Furthermore, TET1 also affects the expression of GRHL2 in medullary collecting duct cells suggesting TET1 can act as an epigenetic determinant for the occurrence of NTDs [27]. Recent research demonstrates that GRHL2 influenced spinal neural tube closure depends on the regulation of surface ectoderm identity and biomechanics. Notably, not just lack of but also increased expression of GRHL2 prevents spinal neural tube closure. Both GRHL2 null and overexpressing embryos exhibit enlarged posterior neuropore (PNPs) at all stages resulting in the PNP closure failing. Failed neural tube closure in GRHL2 null embryos may arise from a possible defect in bringing together or stabilising the neural folds. Instead, GRHL2 overexpressing embryos don't have an obvious defect in elevation or apposition of the neural folds. GRHL2 null embryos show loss of epithelial character in surface ectoderm, ectopic expression of N-cadherin and Sox2 indicates the gain of neuroepithelial characteristics. Mechanistically, loss of epithelial characteristics in GRHL2 null embryos is associated with actomyosin disorganization, cell shape and thickness, and change in the balance of cell-cell junctional strength in the surface ectoderm layer. GRHL2 overexpression embryos most likely regulate the composition of apical junction complexes and increased cell-cell stress via the actomyosin network altering the biomechanical properties in the surface ectoderm [28].

GRHL2 acts as a suppressor of EMT and promotes neural tube closure [29]. Epithelial splicing regulatory protein 1 (ESRP1), sclerostin domain-containing protein 1 (SOSTDC1), fermitin family homolog 1 (FERMT1), transmembrane protease serine 2 (TMPRSS2) and laminin gamma 2 (LAMC2) are activated by GRHL2 as downstream targets through direct DNA binding and transcriptional activation. Thus, GRHL2 promotes key epithelial genes and suppresses EMT through novel downstream EMT suppressors during neural tube formation [29]. In addition, GRHL2 regulated NTDs by affecting the expression of a series of genes associated with cell adhesion and binding to the promoter of E-cadherin [30]. The GRHL3 protein is thought to have a crucial influence on neural tube closure as well [16, 31-33]. In summary, both dysregulation and abnormal expression of GRHL2 has a key impact on neural tube closure.

The midbrain-hindbrain boundary (MHB) is a crucial determinant for patterning mesencephalic-metencephalic regions of the vertebrate. GRHL2 is an important regulator for development of mesencephalon and metencephalon, contributing to both the maintenance and folding of the MHB. One orthologue of GRHL2, grhl2b in a zebrafish model is used to

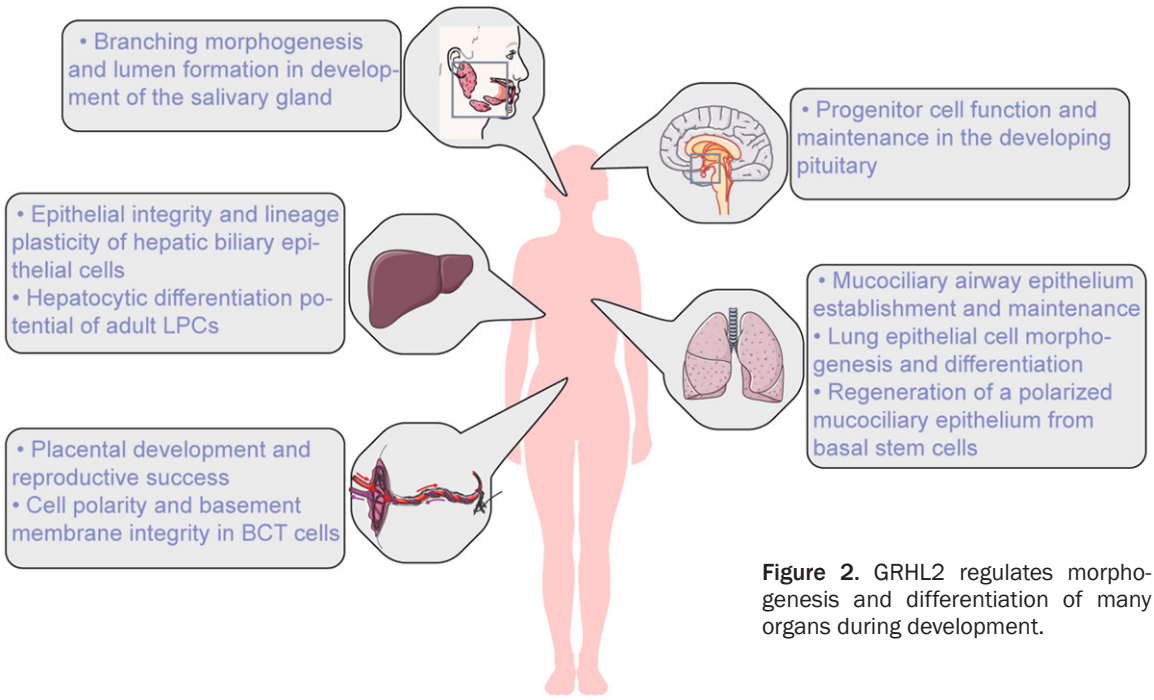

Figure 2. GRHL2 regulates morphogenesis and differentiation of many organs during development.

explore the role of the GRHL2 in vertebrate MHB morphogenesis. Loss of grhl2b leads to neural apoptosis and severe folding defects in the MHB region characterized by loss of characteristic horseshoe-shaped folding, accompanied by defective ventricle formation, and otic vesicle defects. Simultaneously, engrailed 2a (eng2a), which is a conserved target of the GRHL family and crucial feedback molecules in the maintenance of MHB, is also markedly reduced. Furthermore, neural apoptosis and expression of MHB markers are rescued by re-expression of eng2a, but the MHB folding defect persisted indicating that eng2a contributes to grhl2b-dependent inhibition of apoptosis and maintenance of MHB markers, but not to morphogenesis. Phylogenetic analysis and ChIP identify a novel direct grhl2b target spec1, which cooperates with grhl2b to regulate MHB morphogenesis proving MHB patterning and morphogenesis are regulated by diverse GRHL2-dependent pathways [34].

Formation of the craniofacial skeleton in vertebrates requires incredible synergy and complex processes of cellular proliferation, migration, homing and fusion involving multiple transcription factors, and co-ordinated signals [35]. GRHL2 is a highly-conserved genetic critical regulator of craniofacial formation in numerous species. GRHL2 involves the interaction of numerous genes associated with craniofacial development. A novel 2.4 kb enhancer element mm1286 which is 2,741 bp in length and located at mm9: chr15:37,127,220-37,129,961, drives GRHL2 gene expression in the context of normal craniofacial development. The deletion of the enhancer element in mice results in the reduction of GRHL2 mRNA expression in the craniofacial primordia instead of displaying any craniofacial phenotype. Unsurprisingly, the deletion of the enhancer element and one allele of Grhl2 display a significant predisposition to cleft secondary palate. Moreover, a functional 325-bp highly conserved region within the mm1286 enhancer element and an extremely well-conserved 12-bp sequence within this element (CTGTCAAACAGGT) are required for mediating enhancer activity of this region and substantially determining the function of the enhancer element [36].

GRHL2 regulates epithelial morphogenesis and differentiation

Epithelial differentiation depends on a variety of transcriptional factors including GRHL2 which are critical for cell adhesion, epithelial polarity, and cytoskeleton remodeling. As summarized in **Figure 2**, knockout of GRHL2 inhibits human basal cells from differentiating into the mucociliary epithelium and reduces related

genes expression including notch and ciliogenesis genes [37]. Moreover, the morphological changes in lung epithelial cells are associated with down-regulation of GRHL2 and NK2-Homeobox1 transcription factor (NKx2-1). GRHL2 binds to the Nkx2-1 promoter and activates its transcription. In turn, NKx2-1 also binds to GRHL2 intronic regions and increases transcription forming a positive loop that maintains lung epithelial cells morphogenesis and differentiation [38]. Other research verifies that GRHL2 controls normal lung morphogenesis by tightly regulating the activity of distal tip progenitor cells through the direct regulation of Elf5 [39]. In addition, GRHL2 is necessary for the establishment and maintenance of barrier functions including cell morphogenesis, adhesion, and motility in human mucociliary airway epithelium [40]. Cholangiocytes (biliary epithelial cells) isolated from the neonatal liver can differentiate into functional hepatocytes, whereas adult cholangiocytes fails. Researchers have discovered that GRHL2 is continuously expressed in adult cholangiocytes and overexpression of GRHL2 inhibits hepatocytic differentiation. Thus, GRHL2 and GRHL2-associated molecular mechanisms may influence the plasticity of epithelial cells [41]. A recent study by Tanimizu et al [42] explores probable roles of GRHL2 in hepatocytic differentiation of neonatal and adult liver progenitor cells (LPC) which can form bipotential colonies containing albumin (ALB+) hepatocytes and CK19+ cholangiocytes with shared surface markers including epithelial cell adhesion molecule (EpCAM). They found that GRHL2 expression is maintained in colonies of adult LPCs, whereas both GRHL2- cells and GRHL2+ cells are observed in neonatal LPCs. Moreover, more ALB+ hepatocytes exist in the GRHL2- cells colonies and are restricted in the GRHL2+ colonies. Thus, GRHL2 may be associated with the hepatocytic differentiation potential of LPCs. GRHL2 is highly expressed in chorionic trophoblast cells which are essential for healthy placental development. GRHL2-deficient murine embryos show cell polarity disruption, defects of the basement membrane and labyrinth branching morphogenesis required for placental development. Furthermore, GRHL2-dependent gene target networks are identified to improve placental development and reproductive success [43]. GRHL2 null mice perturb labyrinth branching morphogenesis and display a severe disruption of cell polarity and basement membrane integrity in basal chorionic trophoblast (BCT) cells suggesting the key role of GRHL2 in placental development. ChIP-seq identified GRHL2 binding sites of the serine protease inhibitor Kunitz type 1 (SPINT1) in placental tissue, which regulates trophoblast branching morphogenesis [43]. A recent study also revealed that GRHL2 regulates E-cadherin and SPINT1 expression controlling salivary gland development [44]. GRHL2 contributes to progenitor cell function and maintenance associated with Notch signaling in the developing pituitary. GRHL2 is localized in pituitary progenitor cells and GRHL2 knockdown display progenitor cell number defects in the developing pituitary [45].

Epithelial tissues form a selective barrier through the formation of intercellular protein junctions to regulate the selective movement of small molecule substances. These intercellular protein junctions include tight junction, adherens junction, desmosome, and gap junction. The formation of intercellular protein junctions is crucial for the maintenance of cell polarity and differentiation of epithelial tissues. At present, the functions of tight junction are the most diverse among these intercellular barriers. Structurally, tight junctions are composed of occludin, the claudin family proteins (Cldn), tricellulin, junctional adhesion molecules (JAM) and other transmembrane-associated components. Tight junctions are closely linked with epithelial differentiation, gene expression, and cell proliferation. GRHL2 also has an important influence on the regulation of tight junction-associated components during epithelial morphogenesis and differentiation (**Figure 3**). Outlined in relevant research by Werth et al [46], GRHL2 specifically associates with cis-regulatory elements at the Cldn4 core promoter and within intron 2 of the E-cadherin in several types of epithelia, and GRHL2 mutant mice result in defective proteins of tight junctions. When GRHL2 is deleted in collecting duct cells, low expressions of tight junction-associated barrier components are observed. Moreover, Grhl2-deficient mice have diabetes insipidus leading to a susceptibility to prerenal azotemia. These results indicate that GRHL2 plays a unique role in collecting duct epithelial barrier [47]. Another recent research also demonstrates that GRHL2 could contribute to the formation of the mucosal epithelial barrier through

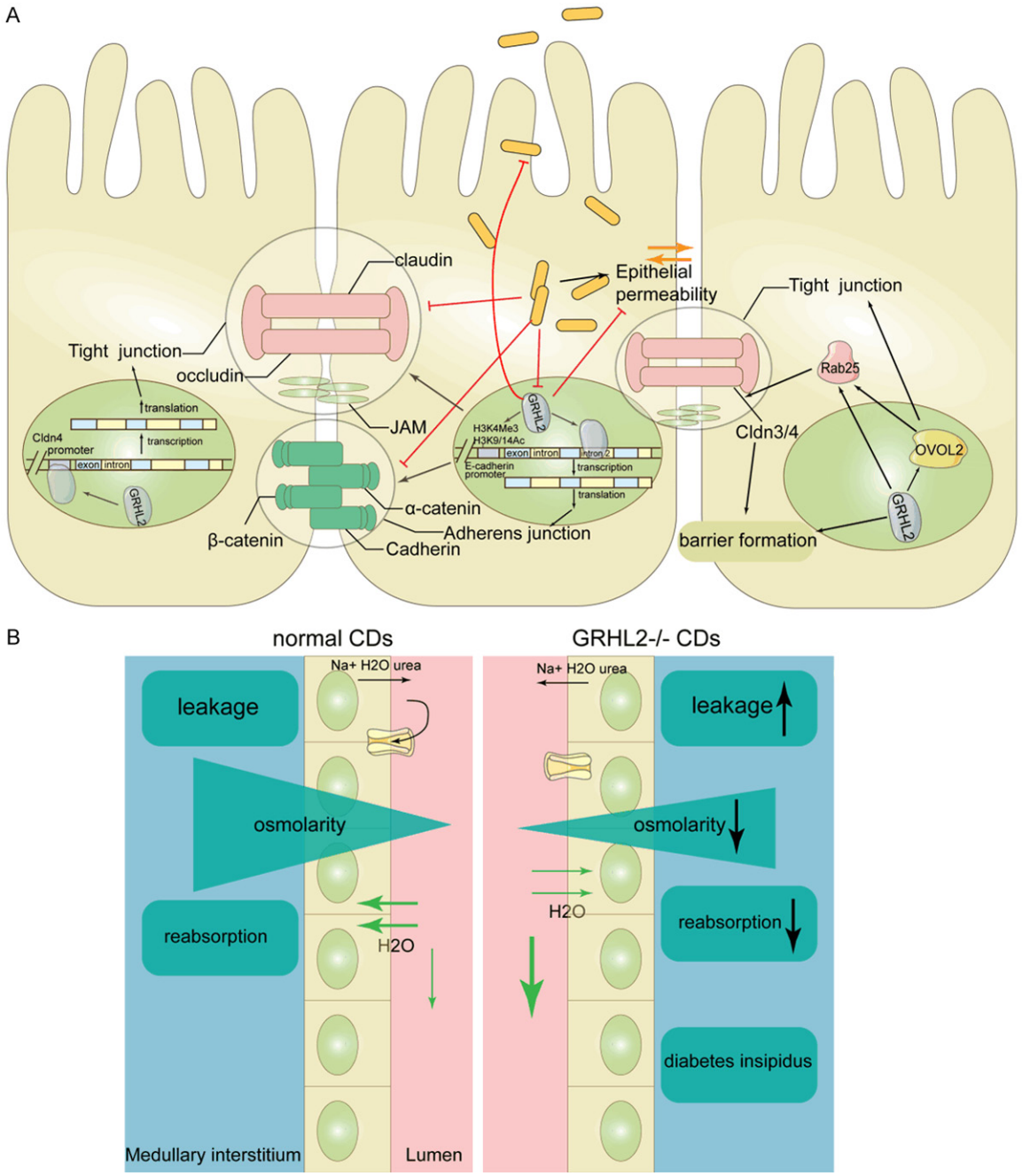

Figure 3. GRHL2 has an important influence on the regulation of cell-cell junction associated components during epithelial morphogenesis and differentiation. A. GRHL2 binds and transactivates the Cldn4 promoter, followed by the regulation of tight junction; there exists physical interaction between the intron 2 region of E-cadherin and GRHL2, GRHL2 increases H3K4Me3 and H3K9/14Ac to activate transcription of E-cadherin; Pg exposure inhibits expression of GRHL2, adherens complex, and tight junction proteins. GRHL2 overexpression inhibits Pg LPS-induced epithelial permeability indicating that Pg impairs epithelial barrier by targeting GRHL2 and GRHL2 is required for the maintenance of the epithelial barrier; GRHL2 transactivated a group of genes including Ovol2, as well asclaudin 4 (Cldn4), and Rab25 forming a GRHLI2/Ovol2 network controlling Cldn4 and Rab25 expression that regulates barrier formation. B. GRHL2 knockdown leads to reduced collecting duct transepithelial resistance, and defective renal medullary accumulation of osmolytes characterized by increased paracellular flux of sodium, chloride, and urea. Grhl2-deficient mice had diabetes insipidus generating a direct functional link between GRHL2 and collecting duct epithelial barrier function.

regulating expression of the junction proteins. Within 15 minutes of exposure of human oral keratinocytes to porphyromonas gingivalis (Pg), rapid suppression of GRHL2 and the junction

proteins including E-cadherin, claudins, and occludin are detected. Moreover, increased epithelial permeability is observed when keratinocytes are exposed to purified LPS from Pg, while GRHL2 overexpression restores the Pg LPS-induced epithelial permeability. GRHL2 cKO mice reveal downregulation of junction proteins, increased alveolar bone loss and microbial loads in the blood suggesting the crucial role of GRHL2 in oral mucosal barrier and paracellular penetration [48].

The overexpression of GRHL2 increases Cldn3, Cldn4, and Rab25, which form a molecular network contributed to epithelial morphogenesis and differentiation [49]. Furthermore, GRHL2 is identified as a transcriptional activator of ovol2, E-cadherin, Cldn4 and the small GTPase Rab25 in collecting duct epithelial cells, and closely related to histone H3 lysine 4 trimethylation. Ovol2 re-expression partially rescued epithelial barrier formation and lumen expansion caused by GRHL2 impairment demonstrating GRHL2/Ovol2 network in regulating Cldn4 and Rab25 expression and facilitating lumen expansion and barrier formation in collecting duct epithelial cells [50].

GRHL2 impairment is involved in hearing loss

Age-related hearing impairment (ARHI) and noise-induced hearing loss (NIHL) is a complicated disease arising from complex regulation of environmental and genetic factors. The functions of both environmental and genetic factors have been studied. GRHL2 single nucleotide polymorphisms (SNP) have been identified as a strong candidate for the regulation of ARHI [51]. Epidemiological investigation in a Chinese population found that polymorphisms of GRHL2 might be involved in the etiology of NIHL as an NIHL susceptibility gene [52-55]. However, two additional studies have showed that there is no significant relationship between GRHL2 polymorphisms and ARHI, or with non-syndromic hearing loss (NSHL), respectively [56, 57]. They speculate population differences may contribute to the etiology of hearing loss [58]. Zebrafish mutant embryos with the knockout of GRHL2 expression reduce the expression of Cldnb and EpCAM, apical junction complexes are also abnormal in otic epithelial cells of mutant embryos. The reasons discussed above cause mutant phenotypes of hearing loss including malformed semicircular canals, insensitiveness to sound stimulation and imbalanced swimming motion [59]. The expression patterns of about 20 hearing loss related genes including GRHL2 were examined in primates and distributed differently in primates and in rodents [60]. In summary, the etiology of several types of hearing loss in genetics needs to be further studied.

Roles of GRHL2 in cancer

GRHL2 is also implicated in tumor development including lung cancer, oral cancer, breast cancer, and gastric cancers. However, the function of GRHL2 seem to be complex and controversial in the context of cancer, varying with cancer type. Diverse dysregulation of GRHL2 expression in different tumor tissues using tissue microarrays were tested [21]. A summary of the roles and functions of GRHL2 in various cancers is shown in **Table 1**.

GRHL2 mutations appear to be seen in almost all types of cancer including esophagus adenocarcinoma, breast carcinoma and skin basal cell carcinoma [61-63]. We observed that missense mutations of GRHL2 are the most common (64.00%) followed by synonymous substitution (25.33%) and nonsense substitution (5.78%) from the database (https://cancer.sanger.ac.uk). These mutations suggest the significance of regulation in tumorigenesis by GRHL2.

The majority of GRHL2 roles are shown to be implicated with carcinogenesis action. GRHL2 expression is higher in oral cancer cells than the normal control group, knockdown of GRHL2 leads to inhibition of oral cancer proliferation and colony formation, loss of tumorigenicity and an epithelial phenotype is also observed [64]. A study by Chen et al [65] applying in vivo and in vitro experiments indicated that GRHL2 contributes to oral tumor development by interacting with HPV-16 and directly binding with the promoter region of forkhead box M1 (FoxM1B) which is related to cell cycle processes. Similarly, a role for GRHL2 is demonstrated in tumors-promoting action in ovarian cancer and esophageal cancers, similar to the results of oral cancer [66]. However, it appears that both low GRHL2 expression and high HIF-1α expression indicate poor prognosis in esophageal cancers [67]. Unlike esophageal cancers, high-

Table 1. The roles and functions of GRHL2 in various cancers

Cancer	Expression	Function	Mechanism	Ref
Oral cancer	Up-regulated	Pro-tumor	(1) Directly bind and regulate the FoxM1B gene promoter activity induced by HPV16 E6.	[65]
			(2) Inhibit the occurrence of EMT and regulate the expression of the ZEB/miR-200 family through directly binding to miR-200 and Oct-4 promoter.	[64]
			(3) Promote the hTERT promoter activity.	[105]
Breast cancer	Down-regulated	Anti-tumor	(1) Inhibit EMT through GRHL2/ZEB1 reciprocal feedback loop and Wnt and TGF-β pathway activation.	[85, 87]
	Up/down-regulated	Pro-tumor	(2) Regulation of ERBB3; form a double-negative feedback loop with ZEB1; inhibit the occurrence of EMT.	[74]
Lung cancer	Up-regulated	Pro-tumor	(1) Suppress metastasis and promote growth via regulation of transcriptional activity of RhoG.	[68]
			(2) Inhibit EMT through miR-200/ZEB and miR-145/ZEB feedback loop.	[89]
Gastric cancer	Down-regulated	Anti-tumor	(1) Reduce the expression of MMP-2, MMP-7, and MMP-9; antagonize TGF-β-induced EMT.	[71]
	Up-regulated	Pro-tumor	(2) Unclear.	[70]
Ovarian cancer	Up-regulated	Pro-tumor	Bind to the intron 2 of E-cadherin and the promoters regions of RAB25 and ERBB3; repress ZEB1 by directly binding to the promoters of miR-200 family to regulate EMT; affect histone mark of E-cadherin and miR200.	[66, 82]
Cervical cancer	Down-regulated	Anti-tumor	Unclear.	[72]
Colorectal cancer	Up-regulated	Pro-tumor	(1) Regulate cell cycle and proliferation proteins expression; inhibit the proliferation by targeting ZEB1.	[69, 79]
			(2) Promote proliferation and inhibit apoptosis by activating the PI3K/Akt pathway.	[111]
Prostate cancer	Up-regulated	Pro-tumor	(1) Promote cancer cell proliferation by colocating with AR at specific sites and enhancing AR signaling.	[75]
			(2) Suppress EMT and invasion of prostate cancer.	[75]
Renal cancer	Down-regulated	Anti-tumor	May be associated with the expression of the von Hippel-Lindau (VHL) gene.	[73]

er GRHL2 expression in non-small lung cancer (NSCLC) predicts poor prognosis. GRHL2 promotes cell growth and colony formation whereas inhibits cell migration and invasion. GRHL2 plays a regulatory and dual role in cell proliferation and metastasis via directly binding to the promoter region of RhoG which regulates cell shape, attachment, and motility [68]. Similar conclusions have been reported in colorectal cancer [69] and gastric cancer [70].

However, there are some contradictory reports regarding the anti-tumor roles of GRHL2 in some cancer types. As above mentioned, GRHL2 may function as a pro-tumor factor in gastric cancer, but contradictory research reports that GRHL2 may have a key influence on tumor suppression in gastric cancer, the results show the expression levels of GRHL2 are significantly down-regulated and GRHL2 overexpression indeed suppresses proliferation and increases apoptosis [71]. In addition, similar research reports that expression of GRHL2 decreased in cervical cancer tissues compared to cervical tissues without lesions. Loss of GRHL2 expression seems to be a characteristic of cervical cancer [72]. GRHL2 is down-regulated in clear cell renal cell carcinoma (ccRCC) and associated with the expression of the von hippel-lindau (VHL) gene, which is one of the most famous tumors suppressors [73]. Interestingly, GRHL2 appears to exhibit dual roles in breast cancer [74]. GRHL2 expression is associated with growth-promoting activities in breast cancer cells by inducing morphological changes and increasing cell proliferation. However, loss of GRHL2 expression is detected in a proportion of primary breast cancer at the invasive front and is closely related to increased tumor stages and lymph node metastases. Similar evidence is verified in prostate cancer [75]. Paltoglou and co-workers reveal GRHL2 has a dual oncogenic/anti-tumor role in prostate cancer: (1) GRHL2 is required for cell proliferation by colocalizing with AR at specific sites and enhancing AR signaling; (2) alternatively, it also suppressed EMT and invasion of prostate cancer closely involved in progression and metastasis in prostate cancer.

GRHL2 acts as new prognostic biomarkers

It was firstly proposed that GRHL2 might be a prognosis biomarker by Tanaka et al [76]. Using Gene Chip oligonucleotide arrays and RNA interference approaches, they demonstrated that GRHL2, as a predictive marker, played a key role in the early recurrence of hepatocellular carcinoma (HCC). In order to identify efficient markers for early detection of ccRCC, the researchers used an integrative bioinformatics system approach and revealed that GRHL2 is involved in disease relapse and predicts prognosis. Therefore, GRHL2 can act as a potential biomarker involved in ccRCC pathogenesis [77]. Another study using a novel strategy of relative expression analysis with gene-set pairs (RXA-GSP), discovered that GRHL2 is able to effectively predict breast cancer metastasis together with several GRHL2-mediated marker candidates including mesenchymal genes as a poor prognosis marker and epithelial genes as good prognosis markers [78]. In colorectal cancer, a retrospective cohort study with a five-year follow-up showed that GRHL2 is an independent prognostic factor correlated with both overall survival and recurrence-free survival. GRHL2 is also positively correlated with tumor size and TNM stage indicating the prognostic value of GRHL2 in colorectal cancer [79]. In short, there is great potential for GRHL2 being a promising biomarker in cancer prognosis.

GRHL2 suppresses EMT

As a highly conserved cellular process, EMT, the epithelial-to-mesenchymal transition, coverts epithelial cells characterized by immotility and polarity to motile mesenchymal cells. EMT is widely observed in a variety of biological activities such as embryogenesis, epithelial morphogenesis, fibrosis and wound healing [80]. Similarly, cancer progression, metastasis, chemoresistance, and phenotypic plasticity are governed by EMT.

Transcription factors are involved in complex associations with regulatory loops among EMT inducers and EMT suppressors. There is some obvious overlap in functions of EMT and GRHL2. Therefore, GRHL2 is probably well connected with EMT in mechanisms of biological processes (**Figure 4**). Recently the signaling pathways between EMT and GRHL2 have attracted considerable interest by researchers. In different cancer cell lines, higher GRHL2 expression is seen in cancers with lower EMT scores; whereas cancers with mesenchymal features have reduced GRHL2 expression. In cultured human colorectal cancer cells, overex-

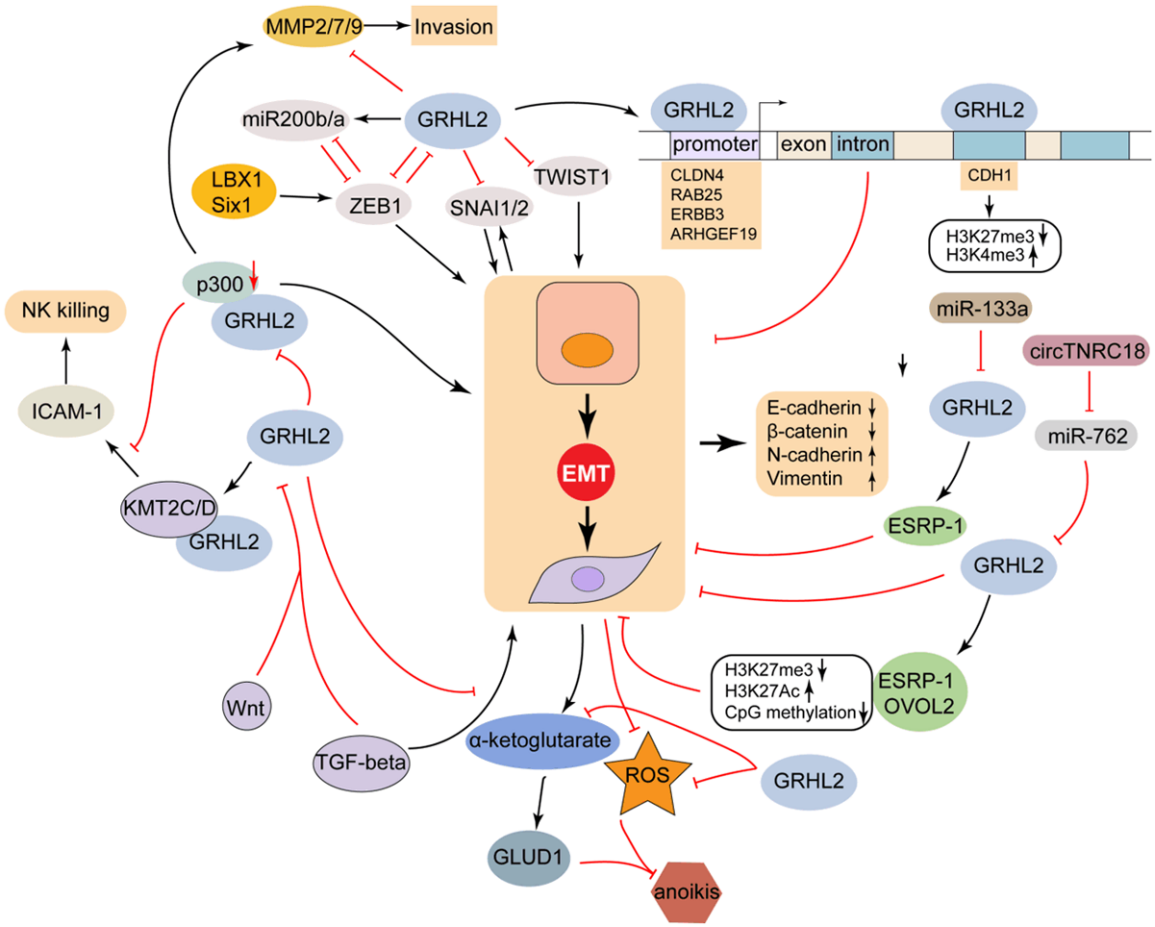

Figure 4. Mechanisms of EMT inhibition by GRHL2. GRHL2 is directly suppressed by ZEB1, which in turn is a direct target for repression by GRHL2, suggesting that GRHL2 and ZEB1 form a double negative regulatory feedback loop. Furthermore, GRHL2 directly bound to the miR-200 promoter to regulate the expression of ZEB1 and the occurrence of EMT. GRHL2 bound to the intron 2 of E-cadherin and the promoter regions of RAB25, CLDN4 and ERBB3. GRHL2 changes H3K4me3 and H3K27me3 at the GRHL2 binding site of CDH1 (intron 2). GRHL2 inhibits SNAI2, TWIST1 and TGF-beta/Wnt-induced EMT. GRHL2-KMT2C/D interactions and inhibition of p300 inhibit EMT, promotes NK-sensitization enhancing the sensitivity of NK killing and ICAM-1 expression. MiR-133a induces spontaneous EMT of airway epithelial cells via down-regulation of GRHL2 causing down-regulation of epithelial splicing regulatory protein 1 (ESRP1). CircTNRC18 elevates GRHL2 protein level by sponging miR-762 and inhibiting miR-762 activity leading to inhibition cell migration and EMT. EMT enhances mitochondrial oxidative phosphorylation accompanied by the overall declined level of ROS and increased GLUD1 expression which is restored by GRHL2.

pression of GRHL2 recovers the epithelial-like shape from a spindle-like shape that displays front-to-back polarity. Consistent with this, vimentin, the mesenchymal marker, is significantly down-regulated and the expression of E-cadherin, β-catenin, ZO-1 are increased [81]. Knockdown of GRHL2 induces EMT and alters the molecular subtype of ovarian cancer cell lines [82, 83]. A recent study uncovered that TGFβ-induced EMT is inhibited by GRHL2, preventing invasion and migration of gastric cancer. In turn, inhibition of TGF-β signaling pathways increased GRHL2 expression [84]. It has also been shown that GRHL2 significantly inhibits TGFβ-induced, Twist-induced and spontaneous EMT in breast cancer [74, 85]. Microarray analysis identified the epidermal growth factor receptor family member Erbb3 considered to be a relevant target of GRHL2. GRHL2 knockdown induces down-regulation of Erbb3 gene expression as well as reduces cell proliferation, resulting in EMT-like morphological changes. Further mechanistic studies reveal that GRHL2 is directly suppressed by ZEB1, which is a direct target for repression by GRHL2, suggesting that GRHL2 and ZEB1 form a double negative regulatory feedback loop in breast cancer cells. Mooney et al [86] using a mathematical model

to investigate the relationship between GRHL2 and EMT regulation, modelled results demonstrate a negative feedback loop between GRHL2 and ZEB that suppresses EMT and tumor progression. Other research supplements detailed mechanisms about the reciprocal feedback loop. First, GRHL2 suppresses EMT through inhibiting ZEB1 promoter transactivation which depend on homeodomain proteins including Six1, LBX1, and HoxA5 which directly interacted with ZEB1 promoter. Alternatively, the combination of TGF-β and Wnt activation down-regulated the expression of GRHL2 via ZEB1 directly interacting with the GRHL2 promoter which resulted in EMT [87]. The role of GRHL2 in the regulation of EMT is also seen in pancreatic cancer [88]. Notably, GRHL2 also maintains epithelial plasticity and stemness including self-renewal capacity and anoikis resistance. In oral cancer, GRHL2 is shown to maintain the stem-like characteristics and determine the epithelial plasticity of oral cancer cells. Overexpression of GRHL2 reduces expression of the mesenchymal cell markers and enhances expression of epithelial cell markers whereas knockdown of it has opposite result. In addition, decreased expression of ZEB1 and ZEB2 and increased expression of Octamer-binding transcription factor 4 (Oct-4) and all members of the miR-200 family are observed in oral cancer cells transfected with GRHL2. Further experiments reveal that GRHL2 directly binds to the miR-200 promoter and the proximal region of the Oct-4 gene promoter to regulate the expression of ZEB and the occurrence of EMT [64]. However, although knockdown of GRHL2 induces EMT accompanied with decreased E-cadherin and increased ZEB1 expression in ovarian cancer, ChIP-seq data shows that GRHL2 bound to the intron 2 of E-cadherin and the promoter regions of RAB25 and ERBB3 but does not bind to the ZEB1 promoter. Furthermore, GRHL2 represses ZEB1 by directly binding to the promoters of the miR-200 family (miR-200B/200A/429) gene cluster. Additionally, GRHL2 has an impact on the levels of histone mark of E-cadherin and miR-200B/200A/429 promoters to regulate the occurrence of EMT [82]. The reciprocal feedback miR-200/ZEB1 loop was also demonstrated in lung cancer. With the exception of miR-200, miR-145 also inhibited ZEB2 and was inhibited by ZEB2 inducing EMT and forming another reciprocal feedback loop which may be associated with GRHL2. In addition, OVOL was also shown to suppress EMT [89, 90].

A study by Pifer et al [91] demonstrates that GRHL2 prevented EMT by inhibiting the histone acetyltransferase (HAT) coactivator p300 and inhibiting its HAT activity and transcriptional activation of target genes including matrix metalloproteases. Moreover, a small 13-amino acid region of GRHL2 is required for the inhibition of p300 and suppression of EMT. To identify additional transcription factors that interact with GRHL2, the interaction of GRHL2 protein with KMT2C/D proteins was confirmed performed by a yeast-two hybrid assay followed by co-immunoprecipitation. GRHL2-KMT2C/D (MLL3/4) interactions as well as inhibition of p300 promote mesenchymal-epithelial transition, NK-sensitization enhancing the sensitivity of NK killing and ICAM-1 expression which is anti-correlated with EMT, suggesting novel mechanisms connecting the epithelial phenotype with target cell susceptibility to NK killing induced by GRHL2 [92].

A new study indicates that GRHL2 is more subject to epigenetic remodeling by DNA methylation and histone modifications. EMT induced by GRHL2 knockdown leads to global epigenetic remodeling, resembling the changes observed in ovarian cancer cells with progressive EMT phenotypes. Specifically, GRHL2 knockdown results in CpG methylation gain, increased H3K27me3 and reduced H3K27ac of target epithelial genes (ESRP1 and OVOL2) suggesting epigenetic remodeling in EMT plasticity induced by GRHL2 [93].

EMT enhances mitochondrial oxidative phosphorylation accompanied by the overall declined level of ROS and increased glutamate dehydrogenase 1 (GLUD1) expression. GLUD1 is important for suppressing hydrogen peroxide and protecting against anoikis. GRHL2 overexpression suppresses EMT-induced metabolism shifts, accumulation of ROS, downregulation of GLUD1 and cell sensitization to anoikis demonstrating a role for GRHL2 in EMT through metabolic alterations [94].

Furthermore, GRHL2 is required for the regulation of spontaneous EMT by some non-coding RNAs. For example, miR-133a induces spontaneous EMT of airway epithelial cells via down-regulation of GRHL2 causing down-regulation

of epithelial splicing regulatory protein 1 (ESRP1) and isoform switching of adherens junction-associated protein p120-catenin [95]. CircTNRC18 has also been identified as a regulatory circular RNA (circRNA) of GRHL2 that elevates GRHL2 protein level by sponging miR-762 and inhibiting miR-762 activity leading to inhibition of trophoblast cell migration and EMT [96].

GRHL2 influences the promoter of human telomerase reverse transcriptase (hTERT)

Telomeres, located in chromosomal ends, form special heterochromatin complexes with repetitive sequences and protect chromosome structures from degradation and damage. Without telomere protection, it would increase the great risk of chromosome instability and oncogenesis [97]. Telomeres shortening is age-related, and is observed in many human proliferative tissues [98]. The replication and maintenance of telomeres are critically regulated by telomerase that is a reverse transcriptase by adding G-rich repetitive sequences to the single-stranded overhang of telomeres according to RNA template sequence [99]. Scientists have revealed a high-level expression of telomerase in many human malignant cells, while there is a very low or even undetectable level in normal cells [100]. Therefore, telomerase dysregulation may be associated with the occurrence of various tumors, many studies also focus on the regulation of telomerase to explore the mechanisms and functions of telomerase in tumor development [101-103]. The telomerase is composed of telomerase reverse transcriptase (TERT) as the catalytic core subunit, a noncoding RNA as the reverse transcription template, dyskerin as a regulator for stability and function of telomerase [104] and other proteins associated with telomerases [99]. TERT has drawn great interest as an important signaling target for mechanisms of tumorigenesis and would be a molecule target for anticancer treatment. Furthermore, accumulating evidence about detailed TERT-associated molecule mechanisms showed there is a close relationship between TERT and GRHL2. Knockdown of some genes including GRHL2 and hnRNPs notably reduces telomerase activity and TERT mRNA expression in several types of oral cancer cell lines and GRHL2 seems to be more critical than other factors. In addition, researchers using chromatin immunoprecipitation and promoter magnetic precipitation (PMP) assays have shown GRHL2 bound near the transcription start site (in the region 1 (-144 to +5) containing the transcription start site) of the hTERT promoter suggesting a specific role of GRHL2 in regulation of hTERT and telomerase activity in oral cancer [105]. A similar research has also shown GRHL2 bound with TERT promoter and confirms the binding region at -53 to -13 of the promoter with the three nucleotides from -21 to -19 are critical for GRHL2 binding. Furthermore, overexpression of GRHL2 leads to hypomethylation of the hTERT promoter occurred with hTERT expression inhibition suggesting GRHL2 regulates hTERT expression by altering the methylation status of the binding promoter [106]. A recent study, has shown that GRHL2, as a novel trans-regulator of the hTERT gene, exerts great influence in the coding of the catalytic subunit of the human telomerase and expression of hTERT expression and telomerase activity [107]. Additional significant mechanisms or pathways about how GRHL2 regulates telomeres and telomerase still remain elusive and require further study. The significance of how GRHL2 affects hTERT will be elaborated in the future.

The relationships between GRHL2 and TGF-β signalling pathways

TGF-β is secreted by many cells types that binds to the TGF-β receptors (types I/II) forming tight complexes leading to activation of Smad2 and Smad3 by phosphorylation, p-Smad2/3 combines with cytoplasmic Smad4 and translocates to the nucleus to regulate target genes transcription [108]. Additionally, TGF-β also interacts with some notable Smad-independent signal pathways, including the MAPK pathways (ERK1/ERK2, p38 MAPK, and JNK) and the phosphoinositide 3-kinase (PI3K)/Akt pathways. It is well-recognized that TGF-β signal pathways play complex and inconsistent roles in the regulation of growth and development. During the early stages of carcinogenesis, several studies have proved TGF-β principally acts as a tumor suppressor. However, TGF-β is converted into a pro-tumor factor in advanced stages, promoting cell migration and metastasis through EMT and reprogramming of cellular microenvironments [109].

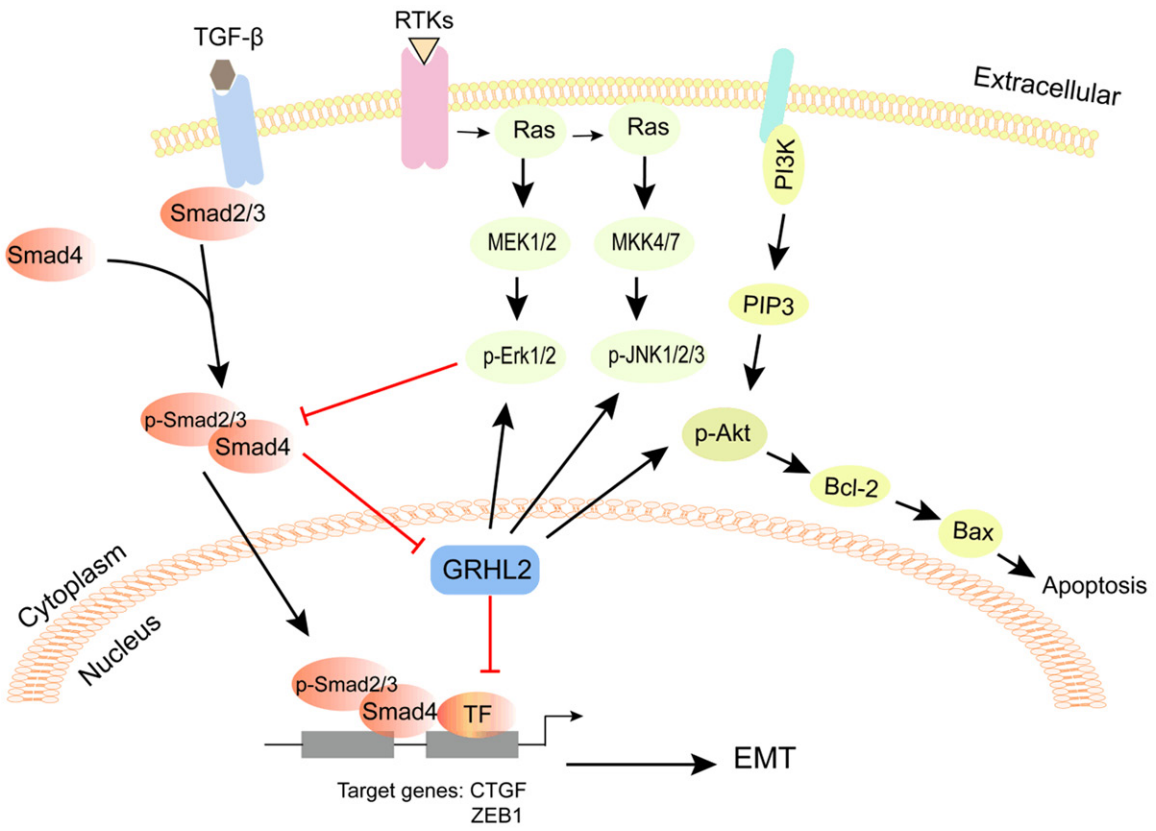

Figure 5. The relationships between GRHL2 and TGF-β/MAPK signal pathways. GRHL2 suppresses TGF-β-induced EMT, TGF-β/Smad mediated transcription of target genes including CTGF and ZEB1 in part via direct repression of the ZEB1 promoter; GRHL2 suppresses TGF-β signaling through activating the MAP kinases, GRHL2 is also down-regulated by Wnt and TGF-β signaling; GRHL2 promotes proliferation and inhibits apoptosis by activating the PI3K/Akt pathway.

Recently, several studies have shown that GRHL2 is involved in the regulation of TGF-β pathways (**Figure 5**). GRHL2 suppresses TGF-β-induced EMT, Smad-mediated transcription and upregulates the TGF-β receptor antagonist, BMP2 in mammary epithelial cells indicating a relationship of GRHL2 with the TGF-β pathway in breast cancer [85]. However, the mechanisms underlying the functional interaction between GRHL2 and TGF-β are still elusive. A recent study gives evidence of both interactions. Chen and colleagues demonstrate that GRHL2 knockdown induces and stimulates TGF-β signaling while GRHL2 overexpression suppresses TGF-β signaling and MAPK pathways (p-Erk1/2 and p-JNK MAP kinase) showing the opposite result. Further mechanisms show inhibition of MAP kinase signaling induced by treatment with various MAP kinase inhibitors diminishes the suppressive effect of GRHL2 on TGF-β signaling indicating that GRHL2 suppresses the TGF-β signaling by activating MAP kinases [110]. The current studies provide evidence to support the functional interaction of GRHL2 with TGF-β signaling and the MAPK pathways. In addition, it is been shown that GRHL2 promotes proliferation and inhibits apoptosis by activating the PI3K/Akt pathway in colorectal cancer [111]. Details about the GRHL2 controlling the Erk and JNK MAP kinases and PI3K/Akt pathway will be elucidated in the future. These data suggest that the functions of GRHL2 are complex and controlled by a network of important regulatory factors, some of which have been identified.

GRHL2 contributes to the estrogen receptor signaling

Estrogen receptor (ER) is a nuclear hormone receptor that is triggered by binding of its ligand, estrogen (E2). ER is responsible for the growth and is also a major target of therapy in many types of breast cancers [112]. Recent evi-

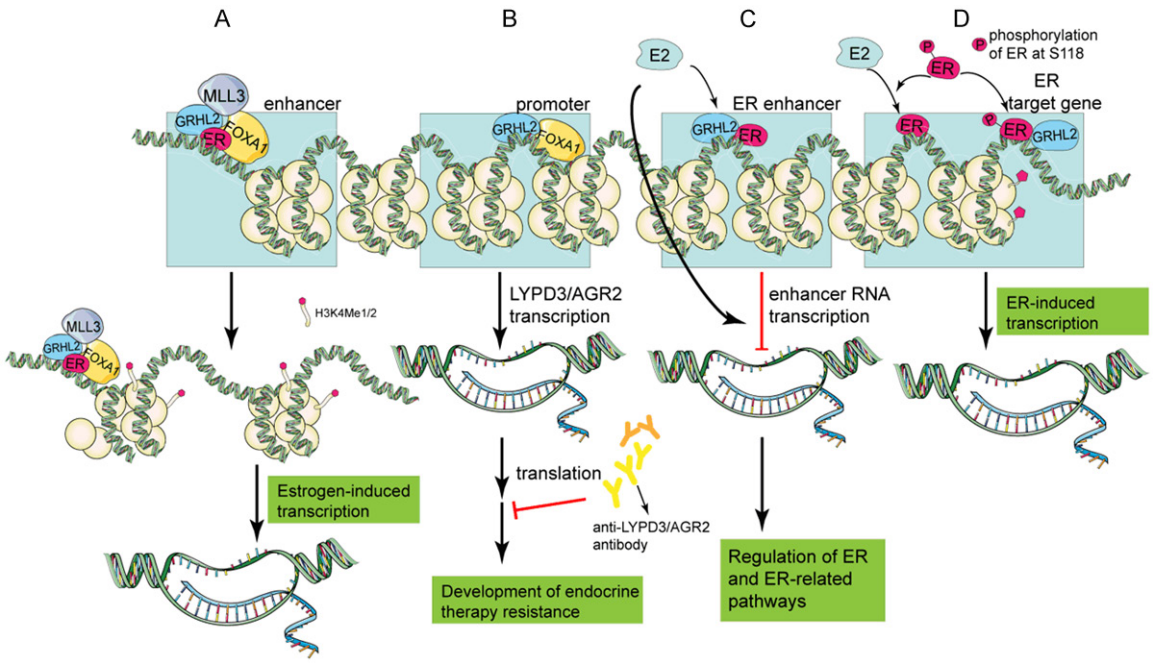

Figure 6. GRHL2 contributes to the estrogen receptor signaling. A. FOXA1 promotes H3K4me1/2 to activate enhancers via recruitment of MLL3 to chromatin. GRHL2 binding sites are overlaid with MLL3, FOXA1 and ERα and the complex activates estrogen-induced transcription. B. FOXA1 collaborates with GRHL2 to increase the expression and activity of LYPD3/AGR2 to promote endocrine therapy resistance in breast cancer. Antibodies against LYPD3 and AGR2 prevent progression of advanced endocrine therapy-resistant in breast cancer. C. GRHL2-ER complex is involved in transcription at ER enhancer sites by E2 stimulation; in contrast, eRNA transcription was largely independent of E2 stimulation when the entire GRHL2 cistrome was considered. D. pS118-ER is necessary for E2-stimulated ER transactivation and ER-DNA binding. pS118-ER is present at the promoters of certain ER target genes preferentially associated with GRHL2 to activate ER-induced transcription. pS118-ER also associates with acetylated H3K27 (H3K27ac), a mark of active enhancers, and upregulation of nearby genes.

dence indicates that GRHL2 is involved in the transcriptional activity of ER in the context of estrogen stimulation (**Figure 6**). GRHL2 overexpression is involved with ER+ breast cancer and is predictive factor for worse survival of ER+ breast cancer. Further ChIP-seq dataset analysis identified an overlap of the acetylated H3K27 histone modification and ER binding at the GRHL2 locus in breast cancers [113]. A recent GRHL2 ChIP-seq in MCF-7 cells revealed GRHL2 binding sites are overlaid with MLL3, FOXA1 and ERα suggesting a functional interaction between FOXA1 and GRHL2 [114]. FOXA1 (Forkhead box protein A1) is a pioneer factor that binds to enhancer regions that are enriched in H3K4me1 and H3K4me2 and contribute to chromatin opening to allow binding of other transcription factors and facilitate binding of ER in breast cancer. MLL3 ChIP-seq shows that the binding of FOXA1 and H3K4-me1/me2 was overlapped with the MLL3 binding sites. FOXA1 depletion decreases H3K4me1 and H3K27ac on ER-dependent enhancers leading to a global decrease in MLL3 binding indicating that FOXA1 promotes H3K4me1 to activate enhancers via recruitment of MLL3 to chromatin by FOXA1. MLL3 knockdown results in inhibition of ER-induced transcription. One study identified that FOXA1 cooperates with GRHL2 upregulating the membrane receptor LYPD3 which acts downstream of FOXA1, and the LYPD3 ligand, AGR2 to drive resistance to endocrine therapy in breast cancer [115]. Interestingly, grainyhead is also a pioneer TF for epithelial cells that binds closed chromatin and nucleosomal DNA making their target regions accessible and allowing other proteins to bind [116]. Estrogen virtUaL ChIP-seq analysis through networks (VULCAN) overlaying networks from data of public databases onto ChIP-seq data, a powerful predictive tool, was applied to analyze estrogen receptor activation in breast cancer to identify potential co-regulators of the estrogen receptor's transcriptional response.

GRHL2 was identified as a strategic co-regulator of ERa. QPLEX-RIME and Co-IP identified a significant increase in the interaction between ER and GRHL2 in the context of estrogen stimulation. In addition, transcription of enhancer RNAs (eRNAs) are strongly increased by E2 stimulation and involved in the regulation of GRHL2. Specific ER eRNA transcription at GREB1 and XBP1 enhancers is moderately reduced after overexpression of GRHL2 demonstrating that GRHL2 constrains specific ER enhancer transcription. Further, knockdown of GRHL2 led to a significant increase of H3K27ac marks around the XBP1 and GREB genes supporting a partial inhibitory role for GRHL2 within the ER regulation [117].

Protein phosphorylation has been shown to regulate diverse biological processes, including enzymatic activity, cellular localization, and degradation. Unsurprisingly, ER activity also is affected by phosphorylation. Multiple phosphorylation sites have been identified in ER. Phosphorylation of ER at serine 118 (pS118-ER) occurs in response to multiple stimuli and is necessary for ER-dependent gene transcription [118, 119]. The pS118-ER cistrome was identified with ChIP-seq on pS118-ER and ER with estrogen stimulation. Interestingly, pS118-ER sites display an increase in the H3K27ac signal as an active enhancer mark associated with gene activation compared to that of ER sites. Further motif analysis reveals enrichment of GRHL2 motif in the pS118-ER sites relative to the ER sites and GRHL2 occupancy increases in response to E2 at pS118-ER sites indicating that E2 induces an increase in GRHL2 binding at pS118-ER sites [120].

Conclusion

Clearly, GRHL2 acts as an irreplaceable regulator either in organogenesis or in cancer development. Beyond the above-mentioned roles of GRHL2, other promising evidence has been seen for the molecular mechanisms of GRHL2 in different aspects of cancer. For instance, GRHL2 suppresses non-small cell lung cancer metastasis via regulation of the RhoG/Cdc42 signaling pathway. RhoG, as a member of the signaling pathway acts as a switch of the signal transduction cascades, promotes cytoskeleton recombination and regulates cell shape, attachment, and motility. Furthermore, GRHL2 inhibited activation of the RhoG/Cdc42 signaling pathway by directly binding to the RhoG promoter region indicating GRHL2 is likely to form an extremely complicated molecular network with related signaling pathways regulating tumorigenesis [68]. In addition, molecular targets of GRHL2 overlap with other signaling pathways such as the hippo signaling pathway which similarly regulates EMT [121] and interacts with the miR200 family [122] suggesting the two signaling pathways may share some part of molecular targets. More studies are needed to uncover the complex molecular network of GRHL2. Research has shown that GRHL2 may maintain progenitor cell function as a novel progenitor cell marker in the developing pituitary [45].

As noted above, GRHL2 dually acts as either oncogene by facilitating tumorigenesis and metastasis or tumor suppressor by inhibiting EMT and forming a negative feedback loop. Functions of GRHL2 in assorted types of tumors are so complicated that at present we are unable to apply GRHL2 to clinical diagnosis and targeted therapy. Though GRHL2 has not been fully elucidated, scientists are making efforts to better understand how GRHL2 affects tumor changes. Additional studies will propel our understanding of the molecular mechanisms of GRHL2. We are convinced that research about GRHL2 will contribute to clinical therapy of cancer.

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Disclosure of conflict of interest

None.

Abbreviations

ARHI, Age-related hearing impairment; EC, Esophageal cancers; EMT, Epithelial-to-mesenchymal transition; Eng2a, Engrailed 2a; EpCAM, Epithelial cell adhesion molecule; ER, Estrogen receptor; eRNAs, Enhancer RNAs; ESRP1, Epithelial splicing regulatory protein 1; E2, Estrogen; FERMT1, Fermitin family homolog 1; FOXA1, Forkhead box protein A1; HAT, Histone acetyltransferase; HCC, Hepatocellular carcinoma; hTERT, Human telomerase reverse transcriptase; GLUD1, Glutamate dehydrogenase

1; GRHL2, Grainyhead-like 2; JAM, Junctional adhesion molecules; LAMC2, Laminin gamma 2; LPC, Liver progenitor cells; MHB, midbrain-hindbrain boundary; NIHL, Noise-induced hearing loss; NKx2-1, NK2-Homeobox1 transcription factor; NSCLC, Non-small lung cancer; NTD, Neural tube defect; Oct-4, Octamer-binding transcription factor 4; Pg, Porphyromonas gingivalis; PMP, Promoter magnetic precipitation; SNP, Single nucleotide polymorphisms; SOSTDC1, Sclerostin domain-containing protein 1; TET1, Ten-eleven translocation 1; TMPRSS2, Transmembrane protease serine 2; VHL, Von Hippel-Lindau; VULCAN, VirtUaL ChIP-seq analysis through; 5hmC, 5-hydroxymethylcytosine.

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