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Review

Effects of mutations in Wnt/β-catenin, hedgehog, Notch and PI3K pathways on GSK-3 activity—Diverse effects on cell growth, metabolism and cancer

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ABSTRACT

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that participates in an array of critical cellular processes. GSK-3 was first characterized as an enzyme that phosphorylated and inactivated glycogen synthase. However, subsequent studies have revealed that this moon-lighting protein is involved in numerous signaling pathways that regulate not only metabolism but also have roles in: apoptosis, cell cycle progression, cell renewal, differentiation, embryogenesis, migration, regulation of gene transcription, stem cell biology and survival. In this review, we will discuss the roles that GSK-3 plays in various diseases as well as how this pivotal kinase interacts with multiple signaling pathways such as: PI3K/PTEN/Akt/mTOR, Ras/Raf/MEK/ERK, Wnt/beta-catenin, hedgehog, Notch and TP53. Mutations that occur in these and other pathways can alter the effects that natural GSK-3 activity has on regulating these signaling circuits that can lead to cancer as well as other diseases. The novel roles that microRNAs play in regulation of the effects of GSK-3 will also be evaluated. Targeting GSK-3 and these other pathways may improve therapy and overcome therapeutic resistance.

1. Introduction

GSK-3 is a kinase that phosphorylates numerous substrates on serine (S) and threonine (T) residues. For GSK-3 to phosphorylate its substrates efficiently, many substrates are first phosphorylated by casein kinase I (CKI) and other kinases depending upon the particular substrate protein. This is referred to as a priming phosphorylation event. GSK-3 was initially identified in rat skeletal muscle as an enzyme that phosphorylated and inactivated glycogen synthase (GS), the last enzyme in glycogen biosynthesis [1,2]. Thus, initially GSK-3 was thought to have key roles in metabolism but this characteristic is only the tip of the iceberg in terms of the diverse activities of the GSK-3 moonlighting enzyme [3–7]. At least forty GSK-3 substrates have been identified [7].

GSK-3 is a gene family consisting of GSK-3alpha (51 kDa) and GSK-3beta (47 kDa) [8,9]. While these two GSK-3 family members have many conserved biochemical functions, they also have unique activities and different substrates in neurological and other tissues [8–11]. GSK-

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3beta knock-out mice are embryonically-lethal indicating that wild type (WT) GSK-3alpha cannot fully compensate for GSK-3beta. GSK-3alpha mice are not embryonically-lethal but they have defects in metabolism and reduced fat mass [9]. GSK-3alpha knockout mice have enhanced glucose and insulin sensitivity and neuronal developmental abnormalities [10]. Most studies have focused on GSK-3beta, however, more recent studies are indicating unique roles for GSK-3alpha and it may play prominent roles in certain cancer stem cells (CSCs) [11,12].

Over the years, GSK-3 has been shown to be important in many physiological processes. GSK-3 has critical functions in many diseases and disorders including: metabolic disorders (diabetes, atherosclerosis, and heart disease) [13–16], neurological disorders (Parkinson's, Alzheimer's, amyotrophic lateral sclerosis [ALS], schizophrenia, bipolar disorder, and mood disorders) [17,18] and more recently, cancer and aging (cellular senescence, cancer stem cells [CSC], resistance to chemotherapy, radiotherapy and targeted therapy [19–21], control of stem cell pluripotency and differentiation) [22–24], immune disorders and other maladies [25–31]. GSK-3 is a target of lithium that for decades has been used in the treatment of patients suffering from bipolar and mood disorders [28]. Recent studies indicate that GSK-3 may be a therapeutic target for certain cancers and immunological and other diseases [12,30,31].

GSK-3 can function in regulating insulin signaling and glucose metabolism. Inactivation of GSK-3 activity results in dephosphorylation and activation of GS that leads to improved glucose tolerance. However, there are additional mechanisms that can lead to increases in GS activity and improve insulin-resistance. Insulin-resistance is very important in the development and establishment of type 2 diabetes mellitus [31]. Type 2 diabetes is increasing at an alarming rate in developed countries due to the increase in obesity, especially among children in the western world.

GSK-3 is important in glucose homeostasis. GSK-3 inhibitors stimulate GS and glucose uptake in tissue culture models and pre-diabetic obese rats [32,33]. Treatment of diabetic Zucker rats with some GSK-3 inhibitors (CT118637, CHIR98014 and CHIR99021) elicited dramatic improvement of oral glucose tolerance and insulin sensitivity. Treatment with the GSK-3 inhibitors enhanced insulin receptor substrate-1 (IRS-1) dependent insulin receptor (IR) signaling in skeletal muscle. There were improvements in dyslipidemia and whole body insulin sensitivity after treatment with the GSK-3 inhibitors [33].

2. Overview of pathology of key cancers discussed in this review with relevance to GSK-3 and associated signaling pathways

2.1. Breast cancer

Various risk factors have been associated with breast cancer including: lack of exercise, obesity, hormone replacement therapy during menopause, early age at first menstruation, having children late, or not having children, alcohol consumption, exposure to ionizing radiation and other factors including genetics. Breast cancer can develop in the cells lining the ducks that produce milk or the lobules that supply the milk to the ducts. Approximately 1 in 8 women will develop breast cancer during their life time.

There are multiple types of breast cancer. The different types were initially characterized on the presence and absence of certain receptor molecules, such as the estrogen receptor (ER+), progesterone receptor (PR+), human epidermal growth factor receptor 2+ (HER2+) and lack of expression of these three receptors (triple negative breast cancer, TNBC). ER+ and PR+ breast cancers (hormone receptor positive, HR+) have the most the most favorable prognosis. These breast cancer patients are often treated with hormonal based therapies. Resistance to hormonal based therapies can develop due to activation of the PI3K/PTEN/Akt/mTORC1/GSK-3 and other signaling pathways [34,35]. Some breast cancer patients are being treated with everolimus which targets mTORC1 [36,37].

Overexpression of the HER2 growth factor receptor, which is related to the epidermal growth factor receptor (EGFR) is present in approximately 25% of breast cancers. Due to overexpression of HER2, this class of breast cancers is sensitive to antibodies and small molecule kinase inhibitors which have been developed to target HER2 [38]. Overexpression of HER2 in breast cancer cells results in the activation of multiple signaling pathways such as PI3K/PTEN/Akt/mTORC1/GSK-3 and Raf/MEK/ERK.

The gene encoding the catalytic subunit of phosphoinositide 3-kinase (PI3K, *PIK3CA*) is mutated in approximately 40% of breast cancers and the tumor suppressor phosphatase and tensin homolog (*PTEN*) gene is mutated/deleted in approximately 25–30% of breast cancers [39]. The PI3K/PTEN interaction is a crucial Ying/Yang event in cellular biochemistry and oncology, PI3K adds the phosphate to 3 position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns), resulting in its activation and recruitment of other signaling molecules, while PTEN removes the phosphate and this results in its inactivation. *PIK3CA* mutations are activating and oncogenic in nature while PTEN is a tumor suppressor and mutations at *PTEN* usually result in its inactivation.

Approximately 5–10% of breast cancers arise from germline inheritance of mutant forms of breast cancer type 1 susceptibility protein (BRCA1) or BRCA2. The *BRCA1* and *BRCA2* genes encode proteins involved in DNA repair. In females carrying *BRCA* mutations, there is a 60 to 85% lifetime risk of those individuals developing breast cancer [40]. Although some studies have indicated that there are variations in the risk of development of breast cancer [41]. There is also a higher risk of men who carry mutant forms of *BRCA1* or *BRCA2* in developing breast cancer [42].

BRCA mutations have been detected in patients which express low levels of ER+/PR+ (HR+) breast cancer [43,44]. High levels of HER2 expression are not frequently detected in *BRCA* mutant breast cancer patients [45]. The *BRCA* genes are also mutated in certain triple negative breast cancers (TNBC) (19% in one study) [46] and other cancers such as pancreatic and ovarian cancers [47–49].

Other genes are also mutated in certain cancer families which can result in breast cancer and other cancers, they include: *TP53* (Li–Fraumeni syndrome) [50,51], *PTEN* (Cowden syndrome) [52], S/T kinase 11 (*STK11*, aka liver kinase B, *LKB* which is involved in Peutz–Jeghers syndrome) [53], checkpoint kinase 2 (*CHEK2*) [54], ataxia telangiectasia mutated (*ATM*), a S/T kinase that is activated by DNA double strand breaks and phosphorylates proteins involved in DNA damage checkpoint [55], BRCA1-interacting protein 1 (BRIP1), a protein involved in DNA repair, partner and localizer of BRCA2 (*PALB2*), a protein involved in DNA repair [56] and other genes involved in DNA repair [57]. Environmental factors may also be import for breast cancer development (e.g., exposure to carcinogens, radiation, occupational hazards) [58].

2.2. Colorectal cancer (CRC)

DNA mutations are present in key genes involved in CRC. The mutations may represent the initiating events in CRC that are followed by expansion of neoplastic clones. CRC development is characterized in three main phases, initiation, promotion and progression. Genetic mutations in key tumor suppressor genes and oncogenes have been associated with CRC. Several molecular pathways are involved in the transition from the normal mucosa to the colorectal carcinoma. Mutations and deregulation of the Wnt singling pathway member such as the adenomatous polyposis coli (*APC*) and beta-catenin (*CTNNB1*) as well as mutations/deletions/deregulation of *TP53*, *KRAS*, MutL homolog 1, colon cancer, and nonpolyposis type DNA mismatch repair genes (*MLH1* and *MLH2*), Mothers Against DPP Homolog 4 (aka *SMAD4* and deletion target in pancreatic carcinoma (DPC4) and low expression of transforming growth factor beta receptor 2 (TGFbeta-R2)) [59]. The majority of CRC are sporadic; however, at least 25% have a clear genetic (familial) background [60].

Members of the Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt/mTORC1/GSK-3 are activated/mutated/repressed in CRC. The *KRAS* gene is mutated in CRC [61]. Another component of this pathway, the *BRAF* gene has been determined to be mutated in CRC [62]. The *PIK3CA* gene is mutated in CRC [63]. More recent studies indicate the role of epigenetic alteration of the chromatin, especially of genes encoding tumor suppressors and oncogenes [64]. In addition, microRNAs (miRs) and long non-coding RNAs (lncRNAs) play key roles in CRC development [65–67].

2.3. Pathogenesis of glioblastoma

TP53 is mutated in approximately 25–40% of glioblastoma multiforme [68]. Mouse double minute 2 homolog (MDM2) is an E3 ubiquitin-protein ligase. MDM2 is amplified and overexpressed in glioblastoma [69]. MDM2 serves to negatively regulate TP53 protein half-life. In addition, the *EGFR* locus is amplified in approximately 36%, the $p16^{INK4A}$ (*CDKN2a*) locus is deleted in 31% and mutations at the *PTEN* locus occur in approximately 25% in glioblastoma multiforme [70]. Abnormal platelet derived growth factor (PDGF) signaling has been implicated in glioblastoma [71]. The receptor for PDGF (PDGFR) has been observed to be overexpressed in many glioblastomas [72]. Vascular endothelial growth factor (VEGF) is also expressed at high levels in glioblastoma [73]. Also epigenetic dysregulation has been observed in glioblastoma multiforme [74].

2.4. Hepatocellular carcinoma HCC

The major risk factors for HCC include: viral hepatitis due to infection with Hepatitis B virus (HBV) or Hepatitis C virus (HCV), alcohol abuse and consumption of aflatoxin-B1-contaminated food [75]. Various singling pathways have been shown to be deregulated in HCC including Wnt/beta-catenin, PI3K/PTEN/Akt/mTORC1, Raf/MEK/ERK, insulin like growth factor (IGF), hepatocyte growth factor (HGF)/MET, VEGF and other growth factors including EGFR and PDGF [76,77]. The multi-kinase inhibitor sorafenib has been approved to treat HCC [78].

2.5. Pathogenesis of melanoma

The *BRAF* and *NRAS* genes are frequently mutated in melanoma [79–82]. Some of the mutations at *BRAF* may be associated with occupational exposures [83]. These mutations may result in therapeutic approaches with specific BRAF inhibitors [84,85]. In contrast, mutations at the *PIK3CA* gene are rare in melanoma [86]. The $p16^{INK4A}$ (*CDKN2A*) gene is also frequently deleted in melanoma [87]. *KIT* mutations and Kit expression have been found in melanoma [88]. Amplification of cyclin D1 (*CCND1*) has been observed in acral melanoma (AM) [89]. *CDK4* and *MDM2* are amplified in melanoma [90].

2.6. Pathogenesis of non-small cell lung carcinoma (NSCLC)

Smoking is the major risk factor for lung cancer. Cigarette smoke results in DNA damage. Remaining unrepaired DNA lesions are most likely a leading cause of NSCLC. Approximately 85% of lung cancers are NSCLC [91–93].

DNA repair deficiencies contribute to NSCLC as well as other cancers. Increased incomplete repair of DNA can result in epigenetic changes. Epigenetic silencing occurs in NSCLC. This can result in the silencing of genes involved in DNA repair and other processes [94–96]. Various genes have been shown to be involved in DNA repair, epigenetics, and promoter methylation and may be altered in NSCLC [97–102].

Mutations at *EGFR* and anaplastic lymphoma kinase (*ALK*) have been associated with NSCLC. EGFR mutations can result in sensitivity to treatment with certain EGFR inhibitors (erlotinib, gefitinib and afatinib). These mutations are present more frequently in Asian patients with NSCLC (50%) than Caucasians (10%) [103,104]. Some NSCLC patients have *EML4-ALK* translocations [105,106] or *ROS1* mutations [107]. These patients may benefit from treatment with ALK inhibitors such as crizotinib which was approved by the FDA in 2011. Crizotinib inhibits ALK, ROS1 and MET [108]. However, as with other inhibitors and other cancer settings, resistance may develop [109]. NSCLC patients lacking *EGFR*, *ALK* or *ROS1* genetic alterations may be treated with bevacizumab which targets VEGF.

2.7. Pathogenesis of ovarian cancer

Some of the genes that are dysregulated in breast cancer, such as *BRCA1*, *BRCA2*, and *BRIP1* are also altered in ovarian cancer [110,111]. *TP53* mutations have been detected in early ovarian cancer, however, their prognostic significance is not clear [112]. *PI3KCA* mutations have been detected in advanced ovarian cancers [113]. HER2, EGFR, and Akt are expressed in some ovarian cancer and the PI3K/PTEN/Akt/mTORC1/GSK-3 pathway is activated [114].

2.8. Pancreatic cancer pathogenesis

Pancreatic cancer is one of the most difficult cancers to treat and drug resistance is frequently encountered [115]. Pancreatic cancer is also associated with the activation of mutant oncogenes such as *KRAS* and the inactivation of key tumor suppressor genes such as *TP53* and *BRCA2*. Mutations at *KRAS* result in its constitutive activity and growth which can result in cancerous growth [116]. Mutations at *TP53* can result in abnormal cell cycle progression and the prevention of apoptosis. Mutations in tumor suppressors such as *BRCA2* can result in abnormal DNA repair and tumor progression.

KRAS mutation occurs during the early stages of pancreatic cancer and is believed to be responsible for initiation. Pancreatic patients with *KRAS* mutations have a worse prognosis than patients with wild type (WT) *KRAS* [117–119].

Notch is also involved in pancreatic cancer [120]. High expression of Notch inhibits apoptosis and also induces activation of NF-kappaB. Cyclooxygenase 2 (Cox2) is also expressed at high levels in pancreatic cancer [121]. Cox2 is an inducible enzyme unlike Cox1 which is produced at a constitutive level. Cox2 expression is increased in pancreatic and other cancers. Crosstalk between Cox2, Ras, NF-KappaB signaling can lead to abnormal pancreatic growth as well as contribute to drug resistance [122]. Intrinsic drug resistance is a major problem with pancreatic cancer and one of the key factors in the demise of chemotherapeutic treatment of pancreatic cancer patients.

NF-kappaB is detected constitutively in pancreatic cancer [123,124]. Deregulation of NF-kappaB could result from Notch signaling. There is important cross talk between Notch and NF-kappaB in pancreatic cancer which may regulate invasion and progression [125].

The *AKT2* gene is amplified in 10–15% of pancreatic cancers [126]. The *MYB* gene is amplified in approximately 10% of pancreatic cancers [127]. Myb is a transcription factor which has previously been shown to be dysregulated in other cancers.

Cyclin D1 is overexpressed in pancreatic cancer and associated with a poor prognosis [128]. Cyclin D1 is a transcriptional target of the Wnt/beta-catenin pathway [129].

$p16^{INK4A}$ normally suppresses the activity of the Cyclin D/CDK4/6 complex which is important in the regulation of retinoblastoma protein (Rb) phosphorylation and cell cycle progression. $p16^{INK4A}$ is inactivated in approximately 95% of pancreatic cancers due to deletions (40%), mutations (40%) and hypermethylation of the promoter region [130,131].

TP53 is inactivated in approximately 50% of pancreatic cancers. Interesting mutations at *TP53* and *KRAS* are associated [132]. These observations suggest cooperatively between TP53 and KRAS in pancreatic cancer [133]. The *TP53* gene status is often associated with the sensitivity of various cancers, including pancreatic cancer, to chemotherapeutic drugs.

TP53 induces the transcription of $p21^{Cip-1}$ and other proteins important in cell cycle regulation [134]. $p21^{Cip-1}$ negatively regulates the CyclinD1/CDK2 complex which results in cell cycle arrest at G_1.

The deleted pancreatic cancer locus 4 (*DPC4* aka *SMAD4*) is a tumor suppressor gene which is mutated in pancreatic cancer [135]. Interestingly SMAD4 inactivation is reported to be always accompanied with inactivation of $p16^{INK4A}$ [136].

$p21^{Cip-1}$ is expressed at low levels in 30–60% of pancreatic cancers [137,138]. $p27^{Kip-1}$ is also detected at low levels in pancreatic cancer [138].

BCRA1, *BCRA2* and other genes involved in DNA repair (e.g., *PALB2*) are also mutated in pancreatic cancer [139,140]. Many of these same genes are also mutated in breast and ovarian cancers.

Aberrant activity of the EGFR pathway is detected in pancreatic cancer [141]. *HER2/Neu* amplifications have been detected pancreatic cancer patients [142].

High levels of activated Akt are detected in many pancreatic cancer patients which is associated with poor prognosis [143]. Inhibition of Akt was shown to suppress NF-kappaB activity and sensitized the Mia-PaCa-2 pancreatic cancer cell line to chemotherapy [144].

The hedgehog pathway (Hh) is important for embryonic development. This pathway is also regulated by GSK-3. Overexpression of the sonic hedgehog factor (Shh) is involved in pancreatic cancer [145–147]. Shh was determined to be expressed aberrantly in approximately 70% of patients with pancreatic adenocarcinoma.

Other signaling pathways such as Raf/MEK/ERK, STAT3, VEGF, and IGF also have roles in pancreatic cancer. Pancreatic cancer has many dysregulated signaling pathways which likely contribute to the difficulty of treatment of pancreatic cancer and to the development of drug resistance.

2.9. Summary of genes implicated in pathogenesis

Various genes and signaling cascades have been implicated in the development of cancer; however, we often see that many of the same culprits are involved in multiple different cancers. This could result from initial studies which have implicated them in other cancers and then scientists have then studied them in additional cancers. However, many of the implicated genes play key roles in signaling pathways and they are frequently mutated in multiple cancers. We are beginning to learn more about other genes which are not as frequently mutated in all cancers and some of these genes may play key roles in specific cancers. Furthermore, the activation/inactivation of certain receptors (e.g., HER2, ER) in certain cell types provides some specificity to the cancer types. In addition, various environmental factors such as: obesity, cigarette smoking, alcohol consumption and exposure to environmental mutates are also associated with multiple cancers and some of these factors may influence the induction of mutations at certain genes and/or changes in gene expression.

3. Regulation of GSK-3 activity by the PI3K/PTEN/Akt/mTORC1 and Ras/Raf/MEK/ERK signaling pathways

An overview of the kinases and phosphatases that regulate GSK-3 activity and a few of the downstream targets are presented in Fig. 1. Multiple signaling molecules can regulate GSK-3 activity. One of the best studied regulators of GSK-3 is Akt which lies in the PI3K/PTEN/Akt/mTORC1 pathway [148,149]. Akt is a S/T kinase that also phosphorylates many key proteins involved in the regulation of cell growth and apoptosis [148–155]. Upon ligand activation of growth factor (GF) receptors, the PI3K/PTEN/Akt/mTORC1, Ras/Raf/MEK/ERK and other pathways become activated. Akt activation can serve to phosphorylate GSK-3 that leads to its inactivation. A diagram illustrating the interactions of GSK-3 with the PI3K/PTEN/Akt/mTORC1 and Ras/Raf/MEK/ERK pathways is presented in Fig. 2.

Other kinases can phosphorylate GSK-3 to regulate its activity [156–164]. These include protein kinase A [156], mitogen activated kinase [aka extracellular regulated kinase 1,2 (ERK1,2)] [157], and $p38^{MAPK}$ [161]. In addition, GSK-3 can be regulated by tyrosine

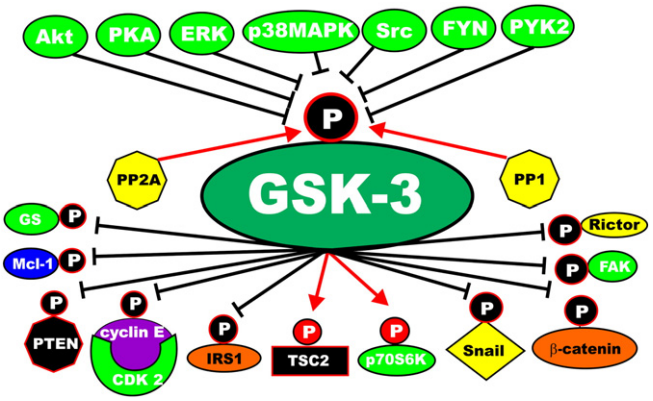

Fig. 1. Regulation of GSK-3 by phosphorylation/dephosphorylation and downstream substrates of GSK-3. Some of the kinases which phosphorylate GSK-3 and regulate its activity are indicated in green ovals with black lines pointing to a phosphorylation site on GSK-3. There is more than one regulatory phosphorylation site on GSK-3, this diagram has been simplified. For more detailed diagrams of GSK-3 phosphorylation sites and downstream substrates see [4,5]. Phosphatases which activate GSK-3 by dephosphorylation are depicted in yellow octagons and red arrows pointing to a phosphorylation site. Beneath GSK-3 are some of the various substrates which are regulated by GSK-3 phosphorylation. Black arrows indicate suppression of activity while red arrows indicate stimulation of activity. A white P in a black circle indicates inactivation of protein, while white Ps in a red circle indicate activation of a protein. This figure is presented to provide the reader the diversity of kinases and phosphatases which can phosphorylate and dephosphorylate GSK-3 and alter its activity as well as the diversity of GSK-3 target proteins.

(Y) kinases such as Src, the protein tyrosine kinase 2 beta (PYK2) [158,159] and Fyn [160]. GSK-3 can be also regulated by dephosphorylation by protein phosphatases including: protein phosphatase 2A (PP2A) and PP1 [165]. GSK-3 may also autophosphorylate itself [162]. GSK-3 can regulate many proteins including: p70S6K [166], Rictor [167] cyclin D [168,169], cyclin E [170], focal adhesion kinase (FAK) [171], Snail [172,173], myeloid cell leukemia sequence 1 (Mcl-1) [174], BCL2-Like 12 (Bcl2L12) [175], PTEN [176], insulin receptor substrate 1(IRS1) [177], and tuberous sclerosis complex 2 (TSC2) [178].

Ras can activate GSK-3beta by stimulation of the Raf/MEK/ERK cascade and activation of the v-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog 1 (ETS) transcription factor (see Fig. 2). GSK-3 can then stimulate NF-kappaB expression that influences the expression of multiple genes. GSK-3 can also phosphorylate PTEN and IRS-1 that inhibits their activity [176,177]. GSK-3 can phosphorylate TSC2 which enhances its activity and serves to suppress mTORC1 activity [178]. During cell stress, GSK-3beta can phosphorylate Rictor at serine-1235 that inhibits the binding of Akt to mTORC2. In the absence of functional mTORC2 activity, Akt is not fully activated. Thus while Akt can negatively regulate GSK-3 activity, GSK-3 can under certain circumstances return the favor and suppress Akt activation.

4. Interactions of GSK-3 and the Wnt signaling pathway

GSK-3 is a critical component of the Wnt signaling pathway. The Wnt/beta-catenin signal transduction pathway is important in normal growth and development and is also frequently dysregulated in cancer and other diseases. The Wnt/beta-catenin pathway is finely tuned by both positive and negative interactions [179]. In the presence of Wnt, phosphorylation of beta-catenin by CK1 and GSK-3 is suppressed and beta-catenin forms a complex with various transcription factors (e.g., TCF/LEF) to induce the transcription of many genes. An overview of the Wnt signaling pathway is presented in Fig. 3. In the presence of Wnt, CK1 and GSK-3 can phosphorylate low-density lipoprotein receptor-related proteins (LRP5/6) that promote beta-catenin signaling.

In the absence of the ligand for the Wnt receptor frizzled (Fz), CK1 phosphorylates beta-catenin at S45. This serves as the priming phosphorylation for GSK-3 to phosphorylate beta-catenin at S41, S37

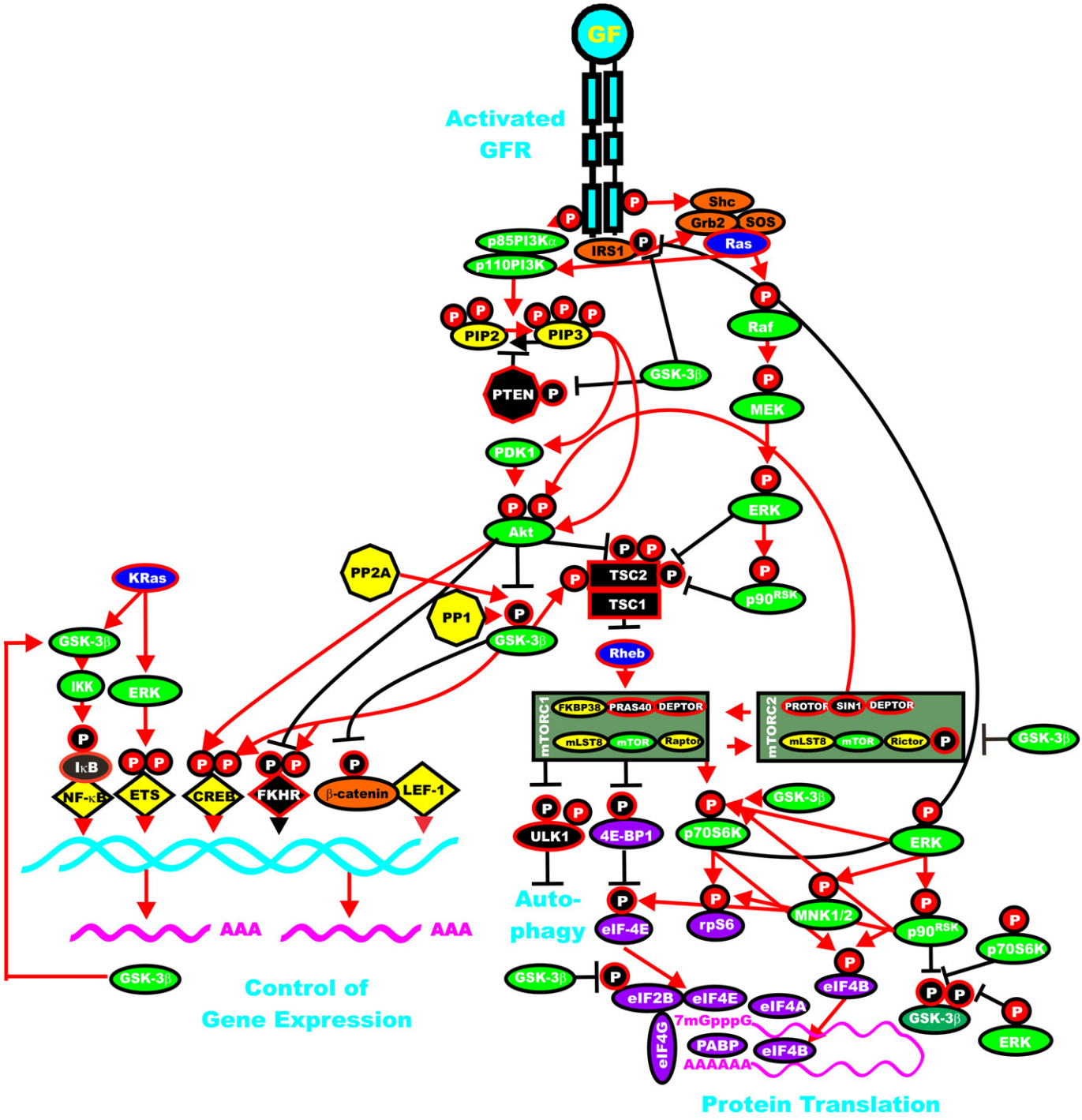

Fig. 2. Interactions of the PI3K/PTEN/Akt/mTORC1 and Ras/Raf/MEK/ERK pathways with GSK-3. Some of the regulatory interactions between GSK-3 and the PI3K/PTEN/Akt/mTORC1 and Ras/Raf/MEK/ERK pathways are indicated. A generic growth factor (GF) and the corresponding growth factor receptor (GFR) are indicated in blue. Ras and Rheb are indicated in dark blue/black ovals. IRS1, Shc, Grb2 and SOS are indicated in orange ovals. Kinases are indicated in green ovals. The p85 regulatory subunit of PI3K is indicated in a green oval. The PTEN phosphatase which dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) into phosphatidylinositol(4,5)-bisphosphate (PIP2) (yellow ovals) is indicated in a black octagon. The PP2A and PP1 phosphatases which may activate GSK-3 by dephosphorylation are indicated in yellow octagons. TSC1 and TSC2 are indicated in black squares. mTOR interacting proteins which positively regulate mTOR activity are indicated in yellow ovals. mTOR interacting proteins which negatively regulate mTOR activity are indicated in black ovals. Transcription factors activated by either ERK or Akt phosphorylation are indicated in yellow diamonds. The Foxo transcription factor that is inactivated by Akt phosphorylation is indicated by a black diamond. Beta-catenin is indicated in an orange oval. mRNA initiation factors and proteins associated with the ribosome are indicated in maroon ovals. The unc-51-like kinase 1 (ULK1) which results in the suppression of autophagy is indicated in a black oval. Red arrows indicate activating events in pathways. Black arrows indicate inactivating events in pathway. Activating phosphorylation events are depicted in red circles with Ps with a black outlined circle. Inactivating phosphorylation events are depicted in black circles with Ps with a red outlined circle. This figure is provided to give the reader an idea of the multiple interactions of GSK-3 with various signaling molecules in the PI3K/PTEN/Akt/mTORC1 and Ras/Raf/MEK/ERK pathways.

and S33. This results in destabilization of beta-catenin and ubiquitination and subsequent proteasomal degradation [3–5]. An overview of the Wnt/beta-catenin complex in the absence of Wnt is presented in Fig. 4, panel A.

The *APC* gene is a critical tumor suppressor gene. APC mutations can result in constitutive action of the Wnt/beta-catenin pathway. *APC* mutations are a critical factor in the development of CRC (Fig. 4, panel B). If

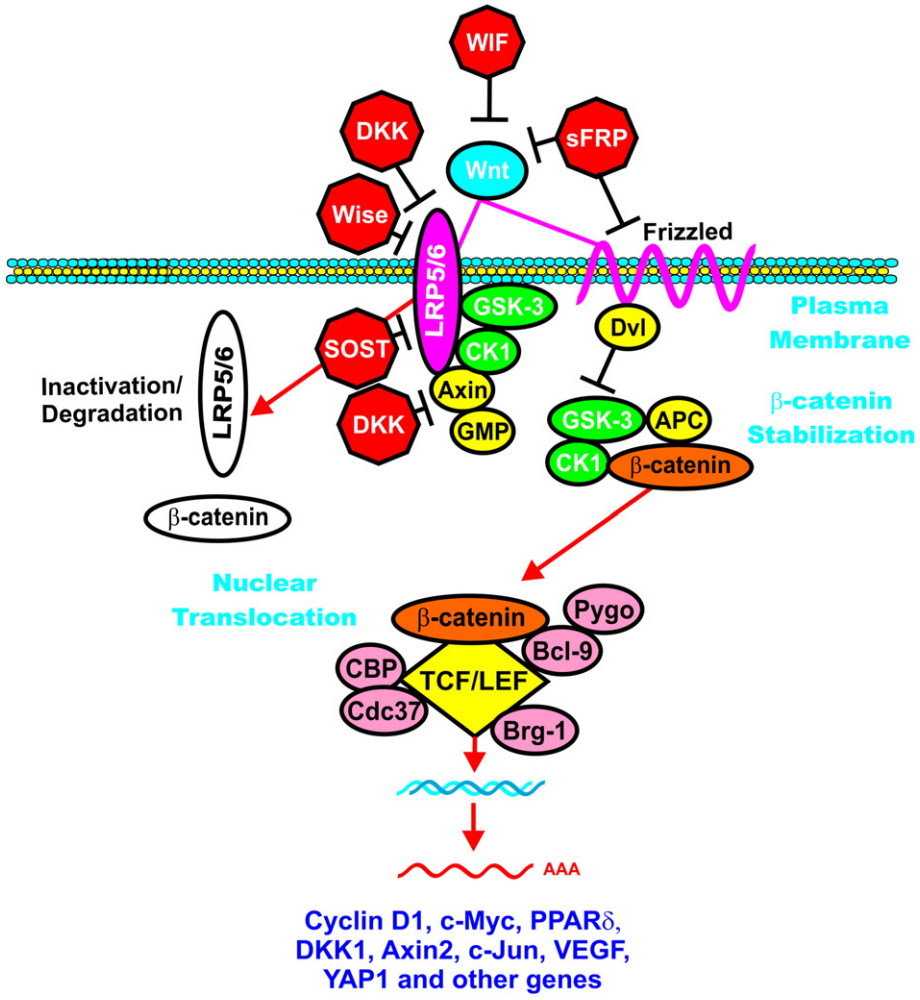

Fig. 3. Overview of Wnt/beta-catenin induced gene expression and effects of natural Wnt pathway inhibitors. In the presence of Wnt, beta-catenin is stabilized and can induce gene transcription. Wnt binds its coreceptors Frizzled (Fz) and LRP5/LRP6. The Fz transmembrane receptor is indicated by a red squiggly line to indicate its spanning the membrane seven times. The kinases GSK-3 and CK1 are indicated in green ovals. The LRP5 and LRP6 transmembrane receptors are indicated by a red oval. Beta-catenin is indicated in an orange oval. Various molecules which interact with the receptors, GSK-3 and CK1 are indicated in yellow ovals. Various Wnt and LRP5/6 inhibitors such as WIF, DKK, Wise, sFRP and SOST are indicated as red octagons. LRP5/6 and beta-catenin can be inactivated and degraded by Wnt pathway inhibitors, indicated in white ovals. When Wnt is present, beta-catenin is stabilized and able to induce gene expression by complexing with various transcription factors including TCF/LET, which is indicated by a yellow diamond. Various proteins which interact with the transcription factor complexes are indicated in pink circles. This figure is presented to provide the reader an idea of how activation of Wnt/beta-catenin can result in regulation of gene expression.

beta-catenin is mutated at the regulatory residues (S45, S41, S37 and S33), it might not be phosphorylated by CK1 and GSK-3 (Fig. 4, panel C). These mutations could be oncogenic and lead to constitutive beta-catenin signaling.

4.1. Transcriptional effects induced by Wnt pathway activation

Upon Wnt stimulation, APC dissociates from Axin. This results in beta-catenin stabilization (Figs. 3 & 4). When certain Wnts bind Fz, and LRP5 or LRP6, a signal is transmitted across the membrane and the segment polarity protein dishevelled homolog-1 (Dvl) is activated which acts to inhibit the beta-catenin destruction complex. Beta-catenin can then translocate to the nucleus as it is not phosphorylated, and bind the transcription factor TCF/LEF. Numerous transcriptional coactivators and histone modifiers are recruited to this complex that includes: Brg1, Bcl-9, CBP, Cdc47, Bcl9, histone acetyltransferases (HATs) and Pygopus (Pygo). This complex induces the expression of many genes associated with proliferation including: c-Jun, c-Myc, cyclin D1, dickkopf (DKK), vascular endothelial growth factor (VEGF) and Yes associated protein (YAP) [179]. The axis inhibition protein 2 (Axin2) gene is a transcriptional target of Wnt/beta-catenin/TCF. The levels of Axin2 are considered an indicator of Wnt pathway activation [180–182]. DKK1 is transcriptionally regulated by Wnt/beta-catenin signaling and likewise DKK1 regulates the Wnt/beta-catenin pathway.

4.2. Wnt signaling specificity

The Wnt family is composed of at least nineteen family members in humans. Different Wnt molecules affect various properties involved in growth control. In ovarian cancer cells, Wnt7A promoted beta-catenin-dependent transcription [183] while Wnt7A inhibited the growth of certain leukemias, potentially by a beta-catenin-independent mechanism [184]. This difference in outcomes induced by Wnt7A may result from the expression of different receptors expressed in the different cell types [185]. These observations indicate that one cannot easily predict the effects of a given Wnt on different cell types.

4.3. Wnt signaling independent of beta-catenin

There are also Wnt signaling events that are independent of beta-catenin. In some cells, signaling involved in cell migration and mobility is associated with Wnt1 and Wnt5a but beta-catenin is not stabilized

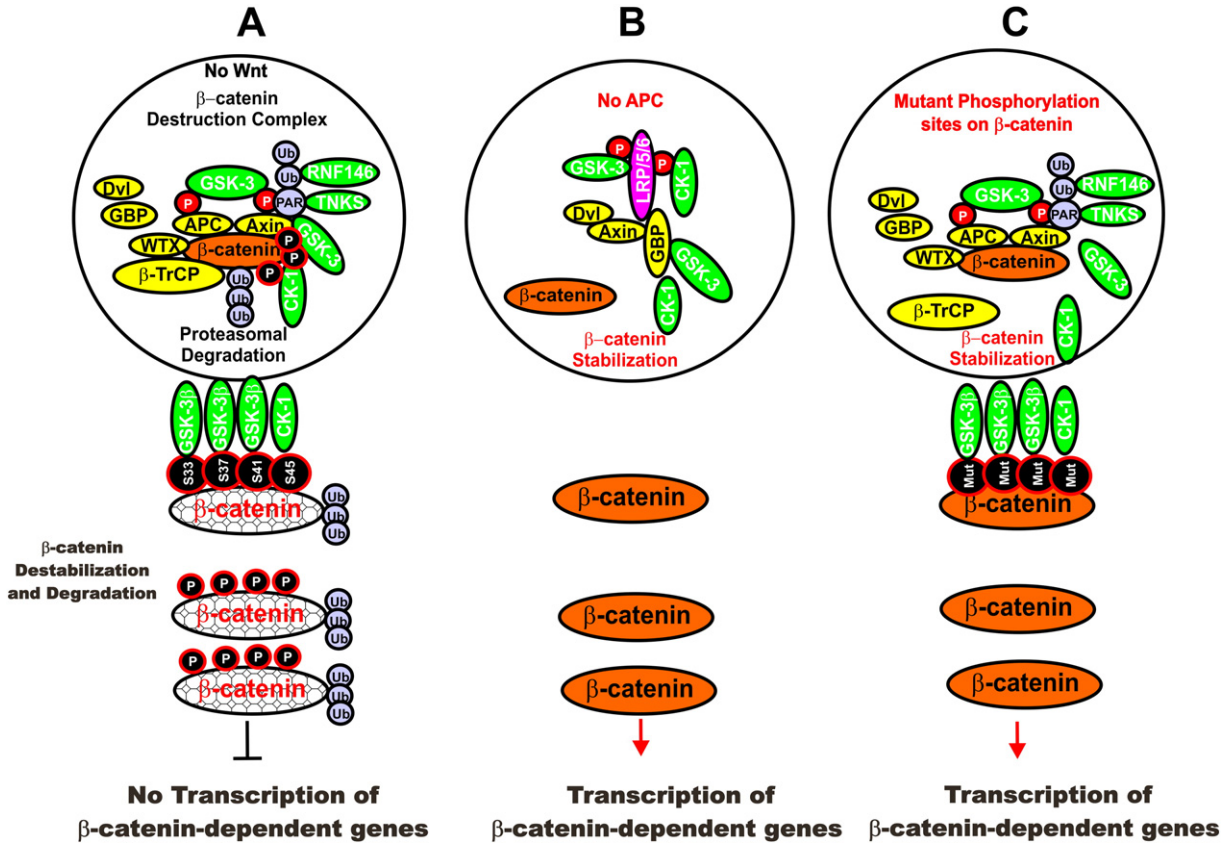

Fig. 4. Comparison of formation of beta-catenin destruction complex vs. scenarios which result in beta-catenin-dependent transcription in the absence of Wnt. Panel A) When Wnt is absent, beta-catenin is targeted for proteasomal degradation. The GSK-3 binding protein (GBP = frequently rearranged in advanced T-cell lymphomas, FRAT2), indicated in a yellow oval, is displaced from GSK-3. CK1 and GSK-3 are able to phosphorylate beta-catenin which results in its ubiquitination by the beta-TrCP complex which is indicated in a yellow oval. Ubiquitination is indicated by Ub in purple circles and results from the ring finger protein 146 (RNF146) E3 ubiquitin-protein ligase which is indicated in a green oval. Axin is also poly-ADP-ribosylated by tankyrase (TNKS), which is indicated by a PAR in a purple circle, which subsequently leads to its proteasomal degradation. CK-1 phosphorylates S45 of beta-catenin which is the priming phosphorylation event for GSK-3 which subsequently phosphorylates beta-catenin on S41, S37 and S33 which results in proteasomal degradation of beta-catenin. Panel B) In cells where there is no APC, there is no formation of the beta-catenin destruction complex and beta-catenin is stabilized even in the absence of Wnt and transcription of beta-catenin-dependent genes occurs. Panel C) In cells containing mutations at the CK-1 or GSK-3 phosphorylation sites, CK-1 or GSK-3 cannot phosphorylate beta-catenin and there is no formation of the beta-catenin destruction complex and beta-catenin is stabilized even in the absence of Wnt and transcription of beta-catenin-dependent genes occurs. This figure is presented to provide the reader an idea of how beta-catenin turnover can be regulated by GSK-3 and CK1 phosphorylation.

[186]. In those cases, Fz and Dvl may be involved. Alternatively, Wnt may associate with receptor tyrosine kinases (RTKs) present at the cell membrane, such as related to receptor tyrosine kinase (RYK) and receptor tyrosine kinase-like orphan receptor (ROR2). RYK and ROR2 can transmit their signals to Src and Jun N-terminal kinase (Jnk), respectively. In addition, these RTKs can interact with Wnt, Fz, and Dvl and induce cell division control protein 42 (CDC42) and Ras-related C3 botulinum toxin substrate 1 (Rac) activity. Dvl can also associate with dishevelled-associated activator of morphogenesis 1 (DAAM1), Ras homolog gene family, member A (RhoA) and Jnk. These pathways are often linked with signaling occurring at the cytoskeleton. The activity of activator protein-1 (Ap-1) and nuclear factor activated T cell (NF-AT) is also modulated by Jnk activity. Fz and Dvl can also associate with phospholipase C (PLC) that results in mobilization of intracellular Ca^{++}. This can stimulate PKC and Ca^{++}/calmodulin-dependent protein kinase (CaMKII). This can result in NF-AT activation in the nucleus. In contrast to the "classical" conception about Wnts stimulating growth, Wnt5a can suppress proliferation and induce cellular senescence. In addition, Wnt5a can have tumor suppressor effects in certain cancer types by antagonizing beta-catenin-dependent transcription [185–187]. B cell lymphomas and chronic myeloid leukemia (CML) can arise in certain mouse models due to Wnt5a suppression. Thus Wnt-5 has tumor suppressor activity in certain hematopoietic lineages, at least in mice [188]. These studies point to the paradoxical effects of Wnt-5a, as in other cells, Wnt-5a signaling promotes cell growth.

4.4. Proteins that serve to inhibit the Wnt pathway

Upon Wnt binding to its receptor, APC is dissociated and Axin is removed from the Axin/GSK-3/APC complex. Axin's interaction with the Wnt co-receptor LRP5/6 causes APC to dissociate from Axin and beta-catenin is stabilized, translocates to the nucleus, interacts with transcription factors such as TCF/LEF which in turn induces gene expression and causes cells to proliferate. In contrast, natural antagonists of Wnt, such as the DKK family of proteins, suppress Wnt signaling (Fig. 3) [189, 190]. DKK1 antagonizes Wnt signaling by binding the LRP5/6 proteins and suppressing their activity. DKK1 acts as an inhibitory ligand and prevents the cells from receiving the Wnt signal. DKK1 binding to LRP5/6 results in Kremen binding that promotes clathrin-mediated internalization of LRP5/6. Kremen is a homolog of DKK1 and can cooperate with DKK1 to suppress Wnt signaling. Other possible mechanisms by which DKKs inhibit Wnt signaling are by their ability to induce the release of Axin from LRP5 that permits Axin to destabilize beta-catenin. There are other DKKs, DKK2, DKK3 and DKK4. Some DKKs (e.g., DKK2) can serve as inhibitors or activators of the Wnt/beta-catenin pathway depending upon the cellular context [191].

There are also additional secreted proteins that antagonize Wnt such as secreted Frizzled-related proteins (sFRPs) and Wnt inhibitory proteins (WIFs). WIFs and sFRPs can competitively displace certain Wnt molecules from their receptors (Fig. 3). In some myelomas and breast cancers, which are dependent on Wnt signaling, increases in

sFRP levels can attenuate autocrine growth mediated by Wnt [192–195]. The effects of the sFRPs on cancer growth are not universal, as in other cancer types; the sFRPs can actually enhance cancer growth. WIFs can also inhibit growth of many cell lines by beta-catenin-dependent and -independent mechanisms. The effects of sFRPs and WIFs may be difficult to predict as they both interact with multiple Wnts and other signaling pathways. There are other Wnt inhibitors such as Wise and SOST that bind to and inactivate LRP5/6 [196,197].

WIF-1 functions by interacting with Wnt ligands and by binding Frizzled receptors. WIF-1 can also interact with DKK1. WIF-1 and DKK1 can interact and suppress Wnt signaling and increase TP53 and $p21^{Cip-1}$ expression. WIF-1 suppressed tumor growth via inhibition of angiogenesis. This suppression occurred by inhibition of the PI3K/PTEN/Akt/mTORC1 pathway [198]. A diagram depicting these interactions is presented in Fig. 5.

Knock-down of the natural Wnt inhibitor WISP2 was shown to increase the invasion and motility of Caco-2 CRC cells. Upregulation of MMP-7 was observed in Caco-2 cells that had WISP2 knocked down. In contrast, the levels of MMP-2 and MMP-9 did not appear to change. The authors suggested that WISP2 regulation of invasion might involve the Wnt pathway [199].

When the promoter region of WIF-1 is methylated, the expression of the WIF-1 promoter is suppressed and Wnt signaling is increased (see Fig. 6). This has been studied in NSCLC and other cancers [200]. The miR-29 family (miR29 a, b and c) can suppress the DNA methyl transferases DNMT3A and DNMT3B. A positive association between miR-29 and WIF-1 expression was observed in NSCLCs. miR-29 was determined to regulate WIF-1 expression by preventing the methylation of the WIF-1 promoter. Suppression of miR-29 was shown to decrease beta-catenin expression, proliferation and induce apoptosis. A diagram depicting these interactions is present in Fig. 6.

miR-31 may target and suppress DKK1 in lung cancer cells. Knock-down of miR-31 was demonstrated to increase DKK1 and inhibit growth. Overexpression of miR-31 was shown to increase proliferation and tumorigenicity of lung cancer cells [201].

Certain Wnt inhibitors are regulated by promoter hypermethylation in CRC. sFRPs, WIF-1 and DKK1 are regulated by promoter CpG hypermethylation. The expression of DKK1 could be induced by treatment with the DNA-demethylating agent 5-aza-2-deoxycytidine. DKK1 was hypermethylated in 17% of primary CRC samples examined [202].

The vitamin D metabolite, 1alpha,25-dihydroxyvitamin D3 (1,25(OH)2D3) inhibits beta-catenin transcriptional activity. This occurs by 1,25(OH)2D3 inducing vitamin D receptor (VDR) binding to

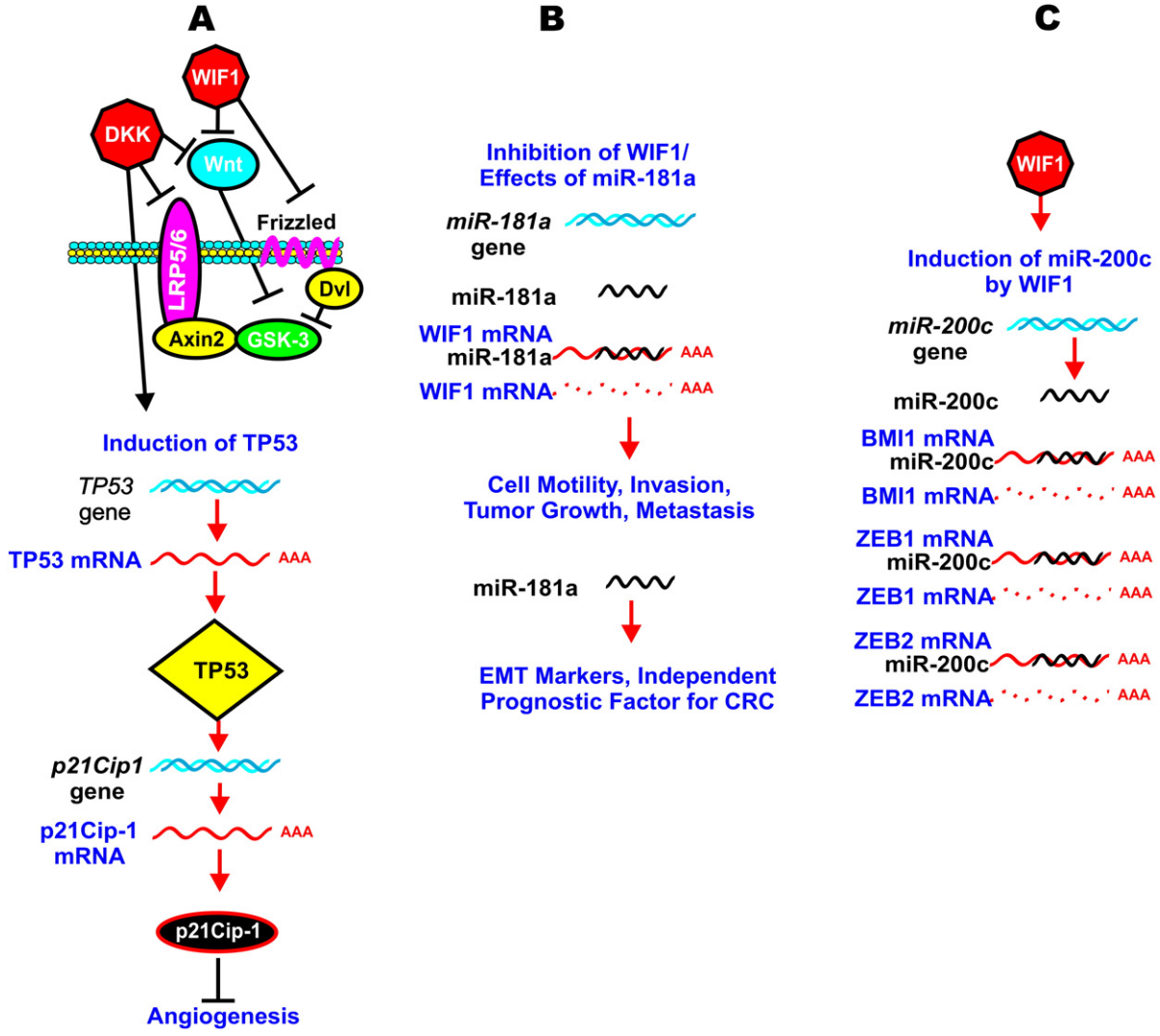

Fig. 5. Effects of WIF1 on TP53 and genes involved in EMT. Panel A) Effects of WIF-1 on the induction of TP53 and $p21^{Cip-1}$ and prevention of angiogenesis. Panel B) Effects of inhibition of WIF1 by miR-181a and subsequent induction of cell motility, invasion, tumor growth and metastasis. Panel C) Effects of WIF-1 on induction of miR-200c and the suppression of mRNAs involved in metastasis. This diagram is presented to provide the reader the concept of the effects of WIF-1 on TP53 and genes involved in EMT and metastasis.

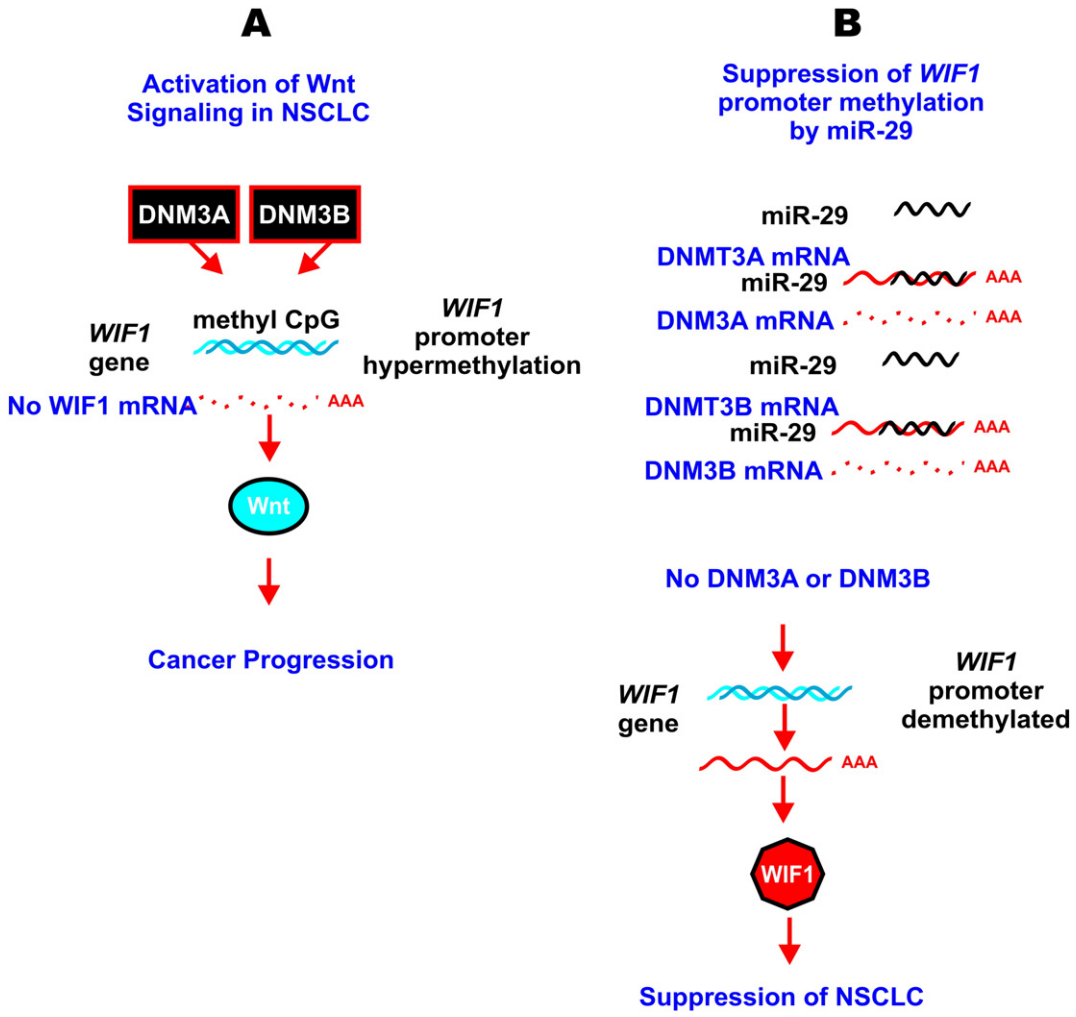

Fig. 6. Interactions between miRs and DNA methylation in regulation of Wnt signaling. Panel A) DNA methyl transferases (DNMT) can methylate the WIF1 promoter region which suppresses its expression but allows Wnt activity which may result in cancer progression. Panel B) miR-29 can suppress DNMT3A and DNMT3B mRNA transcripts which can lead to WIF1 expression and suppress certain cancers such as NSCLC. This diagram is presented to provide the reader of the effects of miRs on DNA methyltransferases on WIF1 and Wnt gene expression.

beta-catenin which results in the induction of E-cadherin expression. 1,25(OH)2D3 can regulate DKK1 and DKK4 expression. 1,25(OH)2D3 regulated positively DDK1 expression but modified negatively DKK4 expression. DKK1 functions as a tumor suppressor in CRCs which have mutations in the Wnt/beta-catenin pathway. In contrast, DKK4 is a target of the Wnt/beta-catenin pathway and increases migration, invasion and angiogenesis [203].

WIFs have also been shown to regulate the induction of certain miRs that can negatively-regulate certain molecules associated with the epithelial mesenchymal transition (EMT) in cancer progression [204]. WIFs are also the targets of miRs. miR-181a targets WIF-1 mRNA in CRC sells. Transfection of CRCs with miR-181a was shown to promote cell mobility, invasion, tumor growth and metastasis. miR-181a promoted EMT and the mesenchymal marker vimentin and decreased the epithelial markers E-cadherin. miR-181a was correlated with advanced stage and distant metastasis of CRCs and was determined to be an independent prognostic factor for CRC [205] (see Fig. 5, panel B).

4.5. Wnt pathway interaction with miR-200 family members and ZEB1/ZEB2 altering EMT, stemness and chemosensitivity

WIF-1 can also induce the expression of miR-200c that can control the expression of many genes involved in EMT such as: B lymphoma Mo-MLV insertion region 1 homolog (BMI1), zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2 (Fig. 5, panel C). Inhibition of these genes will suppress EMT and metastasis [206,207]. miR-200 family members are important in regulating EMT, chemosensitivity, cell growth and apoptosis and often have tumor suppressor functions [206,207]. Lower levels of miR-200b expression are observed in certain cancers (e.g., glioma and others) and associated with a poor prognosis [208].

The ZEB transcription factors can also regulate the expression of the miR-200s which have been shown to be important in: cell division, apoptosis, EMT, CSCs and chemosensitivity. The miR-200 is a gene family which consists of miR-200a, miR-200b, miR-200c, miR-141 and miR-429 [206,209]. The miR-200 family is located on two different chromosomes, 1p36 and 12p13. miR-200 is downregulated during EMT, invasion and metastasis. In contrast, increased miR-200 family member expression resulted in a reversal of EMT in various cancers [209]. miR-200 expression has been correlated with tumor progression in various cancer types [206,209]. A reciprocal relationship between miR-200 and ZEB1 expression has been observed in certain cancers. These interactions are important in EMT and invasion [206,209,210].

The miR-200 family can also regulate the expression of ZEB transcription factors [211,212]. The miR-200 family and ZEB1/ZEB2 expression are also associated with chemotherapeutic drug sensitivity and the response to drugs such as paclitaxel and progression-free survival in ovarian cancer [213].

ZEB1 has been shown to be important in EMT. ZEB1 inhibits the expression of miR-200 family members. miR-200 family members are inducers of epithelial differentiation. ZEB1 was demonstrated to

be important for the tumor-initiating properties of CRC and pancreatic cancer cells. miR-203 is repressed by ZEB1. miR-203 normally inhibits stemness. miR-200 also regulates the stem cell factors including Sox2 and Klf4. The group of miRs including miR-183, miR-200c and miR-203 was determined to suppress certain stem cell factor gene expression. Thus ZEB1 may repress multiple miRs which normally suppress stemness at least in certain tumor types [214].

miR-203 has also been shown to be involved in chemosensitivity. The drug resistance induced by ZEB1 can be reversed by treatment with the histone deacetylase (HDAC) inhibitor mocetinostat. miR-203 is a target gene of ZEB1 and epigenetic inhibition of ZEB1 with mocetinostat resulted in miR-203 expression, inhibited stemness and restored sensitivity to chemotherapy. These studies point to the possibility of combining epigenetic drugs and chemotherapeutics [215].

Chemotherapeutic drugs such as doxorubicin will induce reactive oxygen species (ROS). ROS will induce miR-200 that will subsequently inhibit ZEB1 expression [216]. Altered, often decreased miR-200 levels have been associated with resistance of various cancers to various chemotherapeutic drugs including cisplatin, docetaxel, doxorubicin, EGFR inhibitors (erlotinib), 5-flurouracil, gemcitabine, hydroxycamptothecin, oxaliplatin, paclitaxel, tamoxifen and vincristine. The chemotherapeutic and targeted therapeutic resistance has been observed in: bladder cancer [217], breast cancer [218,219], cholangiocarcinoma [220], CRC [221,222] gastric cancer [223], lung adenocarcinoma [224], NSCLC [225,226], ovarian cancer [227–229], and pancreatic cancer [230].

In contrast, increased miR-200 expression can also result in increased chemosensitivity and decreased invasiveness of certain cancers, e.g., breast cancer [231], and lung cancer [232]. Upregulation of miR-200 by natural products results in reversal of EMT in gemcitabine-resistant pancreatic cancer [233]. In contrast, clinical studies have shown that prostate cancer patients which did not respond to docetaxel had high pre-treatment levels of miR-200 family members documenting the complexity of the miR-200 feedback pathways in certain cancers [234]. The ZEB/miR-200 interaction may be dysregulated in glioblastoma and responsible for chemoresistance [235].

4.6. Interactions between miR-200 family and anti-apoptotic molecules, Akt and chemosensitivity

The miR-200 family has been shown to regulate major two anti-apoptotic molecules, the B cell lymphoma-2 (BCL-2) protein and X-linked inhibitor of apoptosis protein (XIAP). Restoration of miR-200bc/miR429 cluster eliminated the vincristine-resistance of gastric cancer cells and suppressed the cisplatin-resistance in lung cancer cells [236]. miR-200 may be differently regulated by Akt1 and Akt2 in breast cancer cells. This may be important in CSCs and EMT [237]. miR-200 expression has been postulated to be regulated by the balance between the Akt1 and Akt2 isoforms [238].

In studies with esophageal squamous cell carcinomas (ESCC), loss of miR-200b activity was determined to promote Akt and invasion. Recently miR-200b was determined to suppress the integrin beta1 Akt pathway. This occurred by targeting Kindlin-2. When miR-200b activity was decreased, the Kindlin-2/integrin beta1/Akt pathway was induced which promoted ESCC invasion. Kindlin-2 expression was correlated with PI3K/Akt and integrin signaling in ESCC patient samples [239].

Suppression of activated Akt has been observed to result in upregulation of miR-200 in gastric cancer cells. These studies observed that expression of Snail1, Twist1 and ZEB1 was inversely correlated with miR-200 expression while they correlated with activated Akt-1 expression. In these studies, inhibition of activated Akt resulted in enhanced miR-200 expression. Upregulation of the miR-200 family in gastric CSCs resulted in decreased Twist1 and ZEB1 expression and impeded invasion and migration [240].

miR-200 can activate PI3K/Akt signaling by targeting friend of GATA 2 (FOG2) miR-200 can activate PI3K/Akt signaling [241]. FOG2 can bind the p85alpha regulatory subunit. This can result in activation of Akt which normally suppresses GSK-3 activity. miR-200 was determined to be detected at elevated levels in lung adenocarcinomas in comparison to normal lung cells. miR-200 had oncogenic effects as it promoted spheroid growth of lung adenocarcinoma cells. Depletion of FOG2 did not affect Akt activation, thus the authors proposed that miR-200 induced Akt and lung cancer growth through a FOG2-independent mechanism in these cells. These studies document the complexity of the effects of miRs in various cell types [242].

4.7. Interactions between miR-200, ZEB and stem markers

miR-200 expression may also affect the expression of stem cell markers after modified natural product treatment. Difluorinated-curcumin (CDF) is a modified curcumin. The effects of CDF in pancreatic cancer cells were determined to be regulated by miR-200, miR-21 and PTEN. CDF inhibited the sphere forming capacity of the pancreatic cancer cells and altered the expression of the CD44 and EpCAM molecules on the cell surface on gemcitabine-resistant MIA-PaCa-2 cells. CD44 and EpCaM are important protein involved in cancer stem cells [115,116]. In the pancreatic sphere forming population, miR-21 expression was increased while miR-200 expression was decreased. In contrast, miR-200 and PTEN expression were increased by CDF treatment while miR-21 was decreased by CDF treatment in the tumors [243].

ZEB1 has been shown to increase CD44 expression. EMT has been shown to repress the expression of epithelial splicing regulatory protein 1 (ESRP1). ESRP1 can control the alternative processing of CD44. Repression of ESRP1 can result in a shift from the variant form of CD44 (CD44v) to the standard form of CD44 (CD44s). CD44s also can regulate the expression of ZEB1 which can lead to CD44s expression. Activation of the CD44s/ZEB1 feedback loop has been shown to be important in maintaining EMT, tumor initiating cells, drug resistance and stemness [244].

The marker proteins CD133 +/CD326 + are expressed on certain lung adenocarcinoma (LAD) CSCs. The expression of these proteins is negatively regulated by miR-200b. miR-200b is an important molecule in the docetaxel-resistant LAD. The level of expression of miR-200b is lower in docetaxel-resistant CD133 +/CD326 + LAD CSC. Restoration of miR-200b could reverse the docetaxel-resistance of the LAD CSC. In these studies, suppressor of zeste-12 [Suz-12, a component of the polycomb repressive complex 2 (PRC2)] was determined to be a target of miR-200b. HDAC1 interacted with miR-200b. HDAC1 overexpression repressed miR-200b in the LAD CSCs via specificity protein 1 (Sp1). Inhibiting HDAC1 expression suppressed LAD CSCs and their drug resistance via Suz-12-E-cadherin. Suz-12 was determined to be upregulated in docetaxel-resistant LAD cells in comparison to docetaxel-sensitive cells and inversely correlated with miR-200b levels [245].

4.8. Mutations of Wnt/beta-catenin pathway components and their involvement in cancer and other diseases

Components of the Wnt/beta-catenin are mutated at different frequencies in various cancers. The most famous genetic lesion in this pathway is germline mutation in the tumor suppressor *APC* gene which causes familial adenomatous polyposis (FAP) [246–248]. A diagram indicating some of the mutations in the Wnt signaling pathway is presented in Fig. 7. FAP patients are normally heterozygous at *APC* and then subsequently the second allele is lost in individual cells in early adulthood that leads to colon adenomas (polyps). Sporadic colorectal cancer frequently results from loss of both alleles of *APC* [249]. Loss of *APC* results in stabilization of beta-catenin activation of TCF-mediated transcription [250]. *APC* is mutated at high frequency in CRC [251] but not in other cancers such as HCC [252,253] or melanoma [254]. *APC* mutations can have many phenotypes. This is due to its diverse roles, such as: a component of the beta-catenin destruction complex, a microtublin-binding protein and as a guardian of the genome integrity [247–254]. Deletions and truncation mutations in the tumor

suppressor *AXIN1* gene are detected in HCC and CRC [255,256]. Mutations that render *AXIN* inactive have been observed in HCC [257]. Mutations in *AXIN2* (conductin) have been observed in rare CRCs that remain WT for *APC* [258].

In contrast, activation of beta-catenin-TCF signaling occurs more frequently in CRCs by mutations in *APC* and *CTNNB1*. Some of the mutations in the regulatory N-terminal S/T residues of beta-catenin result in constitutive activity [259]. Mutations that potentially disrupt the phosphorylation and degradation of beta-catenin (*CTNNB1*) have been detected in HCC [260,261], medulloblastoma [262] and ovarian cancers [263]. Beta-catenin mutations have been observed in melanomas and other cancers [264,265]. Gain of function mutations at some of the above proteins (especially beta-catenin) have been found in the colon, liver, skin and prostate cancer [266].

Mutations that inactivate the TCF family member *LEF1* have been observed in sebaceous skin tumors [267]. Global exome-sequencing has revealed that the *TCF4* gene (Tcf7L2) was fused with the vesicle transport through interaction with t-SNAREs homolog 1A (*VTI1A*) gene in some rare cases [268]. The fusion lacks the TCF4 beta-catenin-binding domain. The authors found that some CRC lines which contained the fusion were dependent on VTI1A-TCF7L2 for anchorage-independent growth.

WTX (aka *FAM123B*) has tumor suppressor activities and is an X-linked gene. *WTX* mutations are usually found in Wilms tumors [269,270]. Wtx upregulates transcriptional activation by the Wilms tumor protein and interacts with beta-catenin, APC, Axin1 and Axin2. Mutations at *WTX* are also detected in CRC and predicated to activate Wnt/beta-catenin signaling. Loss of function mutations in *WTX* has been reported to result in beta-catenin (*CTNNB1*) stabilization and *osteopathia striata congenita* with cranial sclerosis (OSCS), a disease characterized by excessive bone disposition and hardening [271].

4.9. Interactions of Wnt/beta-catenin with other signaling pathways to promote oncogenesis

The Wnt/beta-catenin pathway can interact with other signaling pathways to promote oncogenesis. In a mouse model of lung cancer, overexpression of beta-catenin, by itself, did not result in lung tumors. However, overexpression of mutant *KRAS* (*KRASG12D*) induced lung cancers and Wnt/beta-catenin (*CTNNB1*) overexpression accelerated

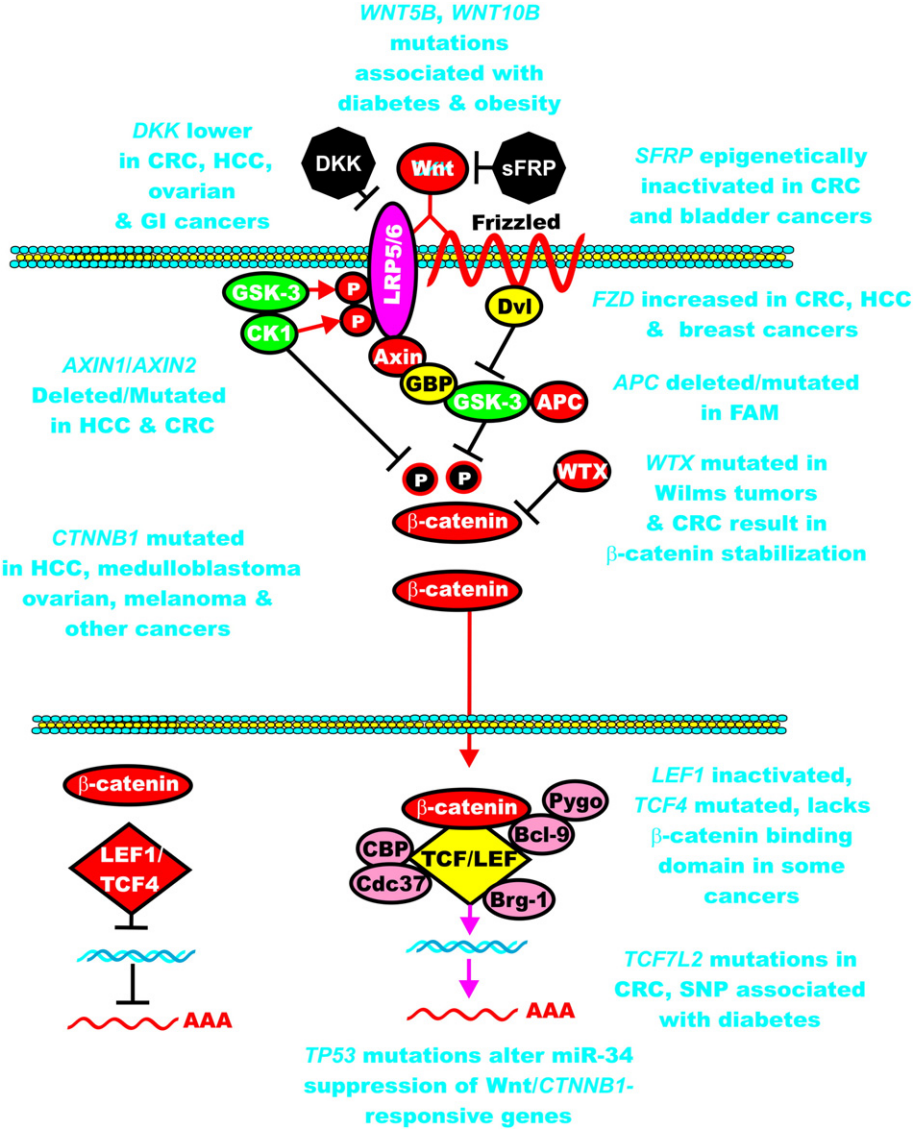

Fig. 7. Sites where mutations alter activity of Wnt/beta-catenin signaling pathway. In this figure, the genetic sites which when mutated or epigenetically silenced alter the activity of the Wnt/beta-catenin pathway are indicated. Activating mutations are indicated in red, inactivated or decreased signaling is indicated in black octagons. Brief descriptions of mutations are indicated in blue text to the sides of mutations. This figure is presented to provide the reader of the consequences of mutations in this pathway to activation/inactivation of various proteins.

lung tumorigenesis by stimulating an embryonic progenitor phenotype [272,273]. Activated beta-catenin can also interact with activated *NRAS* by suppressing cyclin-dependent kinase inhibitor 2A (*CDKN2A* = $p16^{INK4a}$) to induce melanocyte immortalization. This interaction promoted melanoma formation in mice [274,275]. While introduction of constitutively-active beta-catenin oncogene or the HPV16 E7 viral oncogene by themselves resulted in cervical tumors with a long latency, when both oncogenes were introduced into mice, cervical tumors were observed faster [276]. The core protein of HCV can also synergize with beta-catenin. This resulted in increased HCC cell engraftment in mice [277]. The Wnt/beta catenin pathway can interact with various cellular and viral oncogenes and have effects on tumor formation including: immortalization, onset and engraftment.

4.10. Wnt receptors in cancer development

Fz receptors have been implicated in cancer. FZD7 promotes proliferation of cancer cells and it is often overexpressed in cancer cells in comparison to normal cells. FZD7 is detected at increased levels in CRC, HCC and TNBC [278,279]. Suppression of FZD7 expression has been shown to inhibit growth and may be involved in the sensitivity of certain cells to chemotherapeutic drugs [279,280].

miR-199a has been implicated in the transformation process of many different types of cancer including HCC and correlated with poor prognosis. FZD7 was shown to be a target of miR-199a. Increased expression of miR-199a leads to decreases in the expression of FZD7 target genes (e.g., beta-catenin, c-Jun, cyclin D1 and Myc [281].

The deltaNp63 isoform of the Trp63 transcription factors increases the expression of FZD7 that enhances Wnt signaling and normal mammary stem cells activity. DeltaNp63 induced FZD7/Wnt signaling which also regulated basal breast cancers [282].

miR-100 targets FZD-8 and suppresses Wnt/beta-catenin signaling in certain breast cancer cells. miR-100 is important in the regulation of migration and invasion. Overexpression of miR-100 decreased key components of the Wnt pathway including: FZD8, beta-catenin, MMP-7, TCF-4, and LEF-1. In contrast, overexpression of miR-100 increased the expression of both GSK-3beta and phosphorylated-GSK-3beta in MDA-MB-231 TNBC cells. In human breast cancer tissues, miR-100 was negatively correlated with FZD8 levels [283]. miR-100 also has been shown to inhibit breast cancer self-renewal and tumor development [284].

miR-203 has been shown to antagonize FZD2 expression. The tetraspannin family member CD82/KAI1 inhibits metastasis. CD82/KAI1 has tumor suppressor functions and its expression is regulated by TP53. CD82/KAI1 suppresses several FZD isoforms which inhibit Wnt signaling. miR-203 was upregulated by CD82/KAI1. miR-203 was shown to downregulate FZD2 expression and suppressed cancer metastasis [285].

miR-124 has been shown to be a tumor suppressor. miR-124 was downregulated in drug resistant Caki-2 renal cell carcinoma cells. The drug resistant Caki-2 cells expressed increased levels of FZD5 and MDR1 (P-gp) than the drug-sensitive parental cells. Thus miR-124 expression is implicated in drug resistance [286].

The miR-199a-5b regulates FZD6 in CRC cells. FZD6 is detected at elevated levels in CRC tumor samples as compared to adjacent non-cancerous tissue [287].

Inhibition of the histone deacetylase (HDAC) SIRT1/2 resulted in decreased levels of FZD7. Beta-catenin and c-Jun normally occupy a seven Kb upstream region of the *FZD7* gene. Inhibition of SIRT1 decreased the occupancy of beta-catenin and c-Jun in the FZD7 upstream region [288].

4.11. TP53, GSK-3 and Wnt signaling

GSK-3beta has been shown to regulate DELTANp63alpha and CD82 expression. DELTANp63alpha is a potential tumor suppressor protein and a member of the TP53 gene family. CD82 is a metastasis suppressor. Suppression of GSK-3beta led to decreased DELTANp63alpha and CD82 expression which resulted in increased invasion. These effects were beta-catenin-independent [289].

GSK-3 can phosphorylate the RNPC1 RNA-binding protein (aka Rbm38) which can lead to TP53 mRNA translation. Upon inhibition of the PI3K/PTEN/Akt/mTORC1 pathway, GSK-3 is active which leads to more TP53 expression via RNPC1 [290]. A diagram of these interactions is presented in Fig. 8.

GSK-3beta can phosphorylate the Kruppel-like transcription factor-6 (KLF6) which is often inactivated in human cancer. KLF6 is involved in TP53-independent transactivation of $p21^{Cip-1}$. CRC and HCC often have *KLF6* mutations which lead to loss of $p21^{Cip-1}$ activation and growth suppression. Some of the *KLF6* mutations in these cancers may be present in the GSK-3beta phosphorylation sites which inhibit the ability of GSK-3beta to phosphorylate and suppress KLF6 [291].

GSK-3beta is involved in the drug resistance of HCT116p53KO CRC cells. Silencing GSK-3beta was shown to inhibit the drug resistance of the cells. Studies with fifty chemotherapy-treated stage II CRC patients demonstrated that GSK-3beta was upregulated in the drug-resistant vs. drug-sensitive cells [292].

TP53 has been shown to regulate GSK-3 in CRC. This can occur by TP53-induced miR-34 expression which results in Axin2 suppression [293]. Suppression of Axin2 by either TP53 or miR-34 resulted in increased GSK-3beta expression in the nucleus. Increased GSK-3beta expression was observed to lead to decreased expression of Snail. Thus, at least in CRCs, TP53/miR-34 may play key roles in regulating GSK-3 expression and Wnt signaling via interactions with the non-coding UTR of Axin2 [293].

Another mechanism of activation of TP53 is by GSK-3 mediated phosphorylation of the histone acetyltransferase Tip60. This results in activation of TP53 by acetylation [294]. A diagram of some of the interactions between GSK-3, TP53, miR-34 and Snail is presented in Fig. 9.

4.12. TP53, miRs and Wnt signaling

One target of miR-7 in CRC lines is the transcription factor ying-yang-1 (YY1). YY1 can inhibit TP53 and activate Wnt signaling through beta-catenin, survivin and fibroblast growth factor 4 (FGF4). miR-7 can act as a tumor suppressor by inhibiting YY1 [295].

Recently miR-504 has been shown to be an obesity-responsive negative regulator of TP53 in mouse models of breast cancer. miR-504 has been postulated to be a regulator of EMT and a novel target for breast cancer which may result from obesity [296].

miR-34 is downstream of TP53. miR-34 targets conserved sites in the 3′UTRs of many genes involved in the Wnt signaling pathway including: Wnt1, Wnt3, LRP6, Axin2, beta-catenin, LEF and Snail. By targeting these genes, TCF/LEF transcription as well as EMT was suppressed (see Fig. 9). Loss of TP53 was shown to increase Wnt signaling and resulted in Snail-dependent EMT. Loss of TP53 and miR-34 may also lead to hyperactivation of Wnt signaling which is important for metastasis [297]. The c-Met proto-oncogene, which is a receptor for hepatocyte growth factor, is a target of miR-34. c-Met is important in the regulation and invasion of osteosarcoma cells [298].

4.13. Wnt/beta-catenin and suppression of cellular senescence

In some cell systems, the Wnt/beta-catenin pathway is important in the prevention of cellular senescence. Studies in primary human fibroblasts have indicated that loss of Wnt2 resulted in an increase in expression of senescence markers while treatment with Wnt3 prevented senescence [299]. In addition, these investigators also determined that inhibiting GSK-3 delayed oncogene-induced senescence. The role of Wnts in cellular senescence is complicated as overexpression of Wnt5 suppresses ovarian cancer by inducing senescence [300].

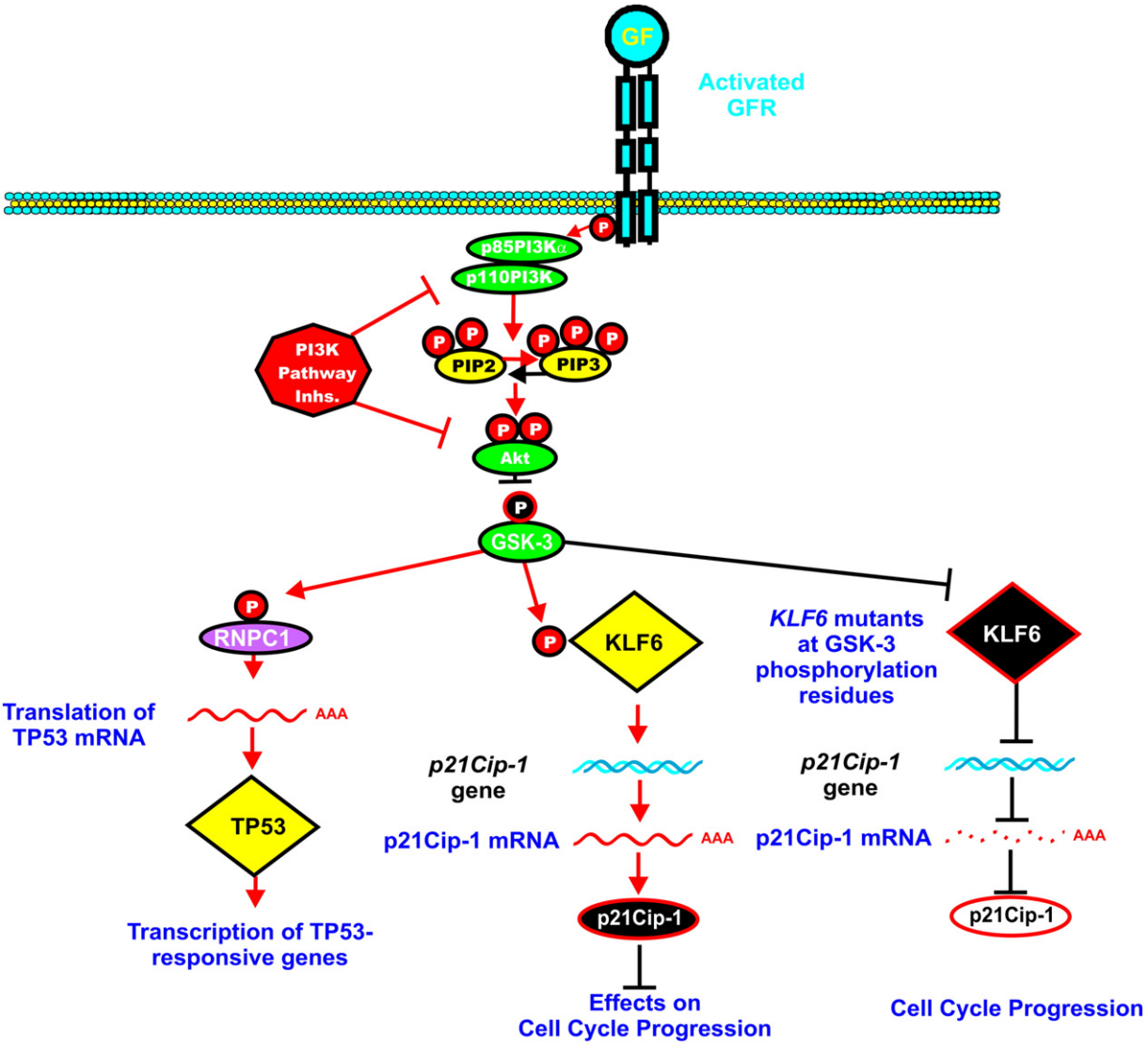

Fig. 8. Interactions of PI3K/PTEN/Akt/mTORC1 pathway and GSK-3 in regulation of TP53 and KLF6 activity. Blocking the PI3K/PTEN/Akt/mTORC1 pathway can result in GSK-3 activity which can lead to phosphorylation of RNPC1 and the enhanced translation of TP53 mRNA transcripts and transcription of TP53-responsive genes. GSK-3 can also phosphorylate KLF6 which can induce the expression of $p21^{Cip-1}$ and suppress cell cycle progression. In contrast, in certain cancers, the GSK-3 phosphorylation sites on the *KLF6* gene may be mutated which results in a mutant protein which cannot be phosphorylated by GSK-3. This diagram is present to provide the effects of GSK-3 on TP53 and KLF6 activity.

The GSK-3 inhibitors SB-216763 and 6-bromoindirubin-3-oxime (BIO) delayed cellular senescence in umbilical cord blood-derived mesenchymal stem cells (MSC). Likewise, the addition of Wnt-3a conditioned medium, which activated Wnt signaling, delayed the progression of cellular senescence. These delays in senescence were determined to occur by decreasing TP53 and pRb expression as well as decreasing senescence-associated beta-galactosidase activity and altering telomerase activity. Treatment with DKK1 or transfection with siRNAs to beta-catenin promoted senescence in the MSCs [301].

The Wnt/beta-catenin pathway has also been shown to play important roles in the regulation of senescence of bone marrow derived mesenchymal stem cells (BM-MSCs). The Wnt/beta-catenin and TP53/$p21^{Cip-1}$ pathways were hyperactivated in BM-MSCs from systemic lupus erythematosus (SLE) patients. DKK1 treatment was shown to reverse the senescence characteristics of SLC BM-MCS cells [302].

Recently, it has been shown that rapamycin may be an appropriate drug to treat human ovarian endometrioid patients which have dysregulated Wnt/beta-catenin and PI3K/PTEN/Akt/mTORC1 pathway activation. Mice with a beta-catenin mutation, which results in dysregulated beta-catenin expression in ovarian surface epithelium (OSE) cells have been determined to develop indolent, undifferentiated tumors. These tumors had epithelial and mesenchymal traits. In tumors which had also lost PTEN, they were more aggressive, and TP53 was inhibited as well as cellular senescence. mTOR and downstream p70S6K expression were detected in indolent and aggressive tumors. These mouse tumors that had gain of function mutations in beta-catenin and PTEN mutations were inhibited by rapamycin [303].

The WIF1 protein has been shown to have tumor suppressor activity in glioblastomas. It is downregulated in 75% of glioblastomas. Part of this tumor suppressor activity is due to its ability to induce cellular senescence. Genetic mechanisms that lead to decreased WIF1 expression include: gene deletion and epigenetic silencing by promoter hypermethylation [304].

4.14. Wnt/beta-catenin signaling in metastasis

Wnt1 and Wnt3 have been determined to promote the migration of primary myeloma cells isolated from patients [305]. This same group also demonstrated that Wnt3 signaling in the bone inhibited myeloma tumor growth [306]. Altered expression of Wnt antagonists such as

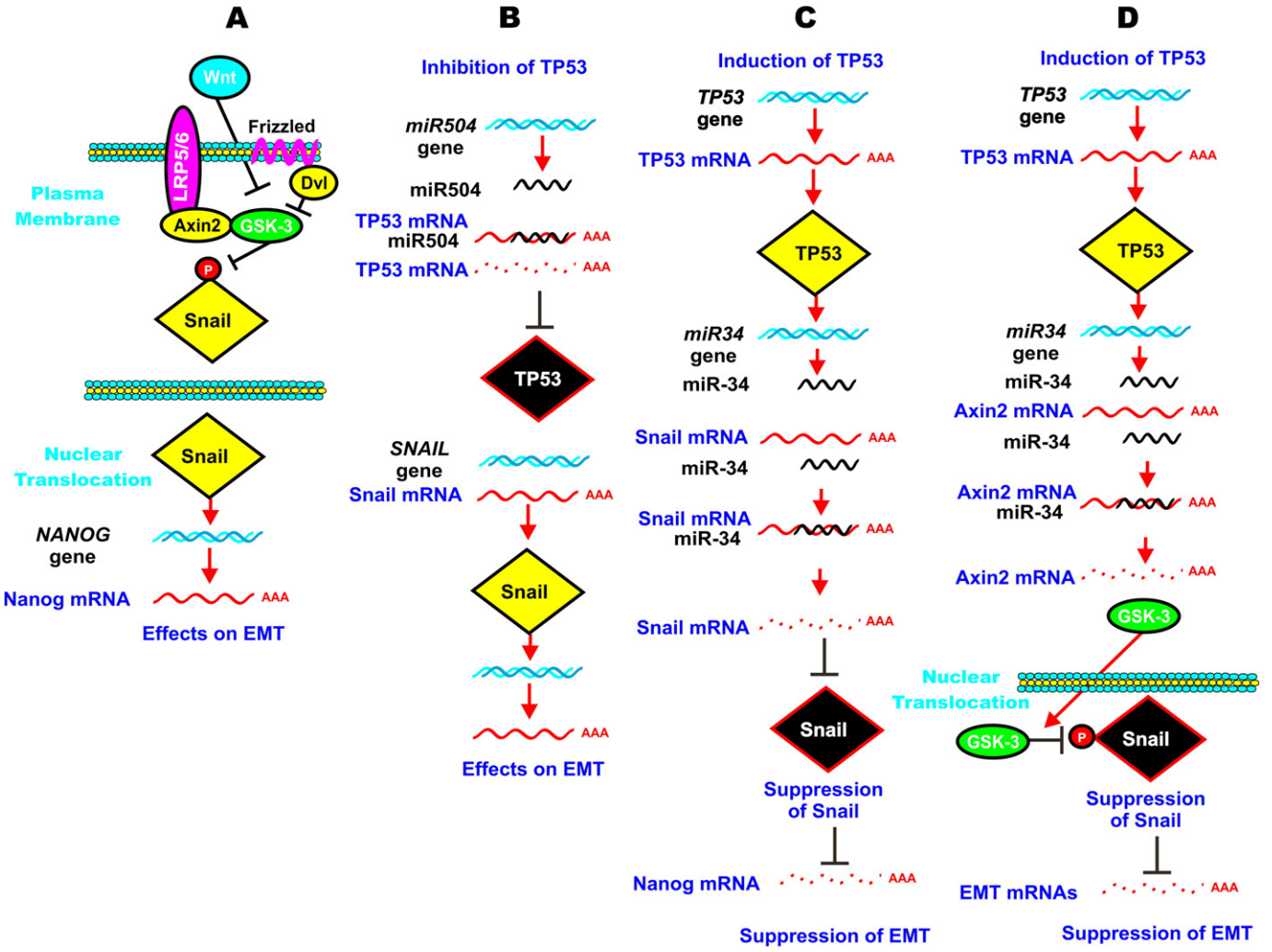

Fig. 9. Effects of GSK-3, TP53 and miRs on Snail activity. GSK-3, TP53 and miRs can regulate the activity of the transcription factor Snail. Panel A) When GSK-3 is active, it can result in the phosphorylation of Snail which results in its inactivation. In contrast, if the Wnt signaling pathway is active, Axin2 and Dvl can result in the suppression of GSK-3 and Snail is not phosphorylated. Panel B) Another mechanism by which Snail can be regulated is through miRs. miR504 can regulate TP53 mRNA which will affect Snail expression. Panel C) TP53 can also regulate mi-34 which inhibits Snail mRNA and EMT. Panel D) TP53 can also regulate miR-34 which regulates Axin2 mRNA and leads to increased GSK-3 activity which results in suppression of Snail and EMT. This figure is presented to give the reader a brief overview of some of the mechanisms by which GSK-3 and p53 can regulate the activity of important transcription factors such as Snail.

DKK1 or sFRP was shown to inhibit motility and invasion of some cancer cells. The Wnt/beta-catenin pathway also induced the expression of MMPs that are important in regulation of the extracellular matrix [307]. The Wnt/beta-catenin pathway is involved in regulation of cell morphology, polarity and motility and these processes are critical for migration and invasion. The non-canonical Wnt pathway is also important in the above-mentioned processes. Some of the events may involve activation of additional interacting signaling cascades.

Overexpression of Wnt1 in MCF-7 breast cancer cells increased their invasiveness in a beta-catenin dependent fashion [308]. Canonical Wnt signaling resulted in dedifferentiation of MCF-7 cells. The invasiveness of MCF-7 cells was mediated by an Axin2-dependent pathway that resulted in stabilization of the Snail1 zinc-transcription factor, a key regulator of EMT. Snail1 contains beta-catenin-like structural motifs that allow its GSK-3beta-dependent phosphorylation, beta-TrCP-directed ubiquitination and proteasomal degradation. Wnt signaling was determined to suppress Snail phosphorylation and increased Snail protein levels and activity. Knockdown of Snail suppressed EMT [309]. Axin2 was shown by this group to regulate EMT by acting as a nucleo-cytoplasmic chaperone for GSK-3beta, which was determined to be the dominant kinase responsible for controlling Snail1 protein turnover and activity. A diagram of these interactions is presented in Fig. 9.

4.15. Role of Wnt/GSK-3 signaling in regulation of mTORC1 pathway

Wnt signaling can also regulate the mTORC1 pathway via GSK-3 by a beta-catenin-independent mechanism [310]. GSK-3 can phosphorylate TSC2 after it has been phosphorylated (priming phosphorylation) by AMPK. Wnt signaling suppresses GSK-3-mediated phosphorylation of TSC2 and hence the mTORC1 pathway is activated. Inhibition of Wnt-mediated activation of mTORC1 by rapamycin suppressed Wnt-mediated cellular proliferation. Likewise, activation of AMPK by metformin might also suppress Wnt-mediated cellular proliferation [311]. In addition to GSK-3, other Wnt signaling components such as DKK1, Dvl and Axin regulate the effects Wnt has on regulation of mTORC1 and cellular proliferation. A diagram of these interactions is presented in Fig. 10.

4.16. Wnt/GSK-3 signaling in the regulation of SMAD1

SMAD1 is a mediator of the transforming growth factor-beta (TGF-beta)/bone morphogenetic protein (BMP) superfamily of ligands. Both

MAPK and GSK-3 were determined to phosphorylate SMAD1 in its linker region that resulted in its polyubiquitinylation and transport to the centrosome where it was degraded by proteasomes. The priming phosphorylation mediated by MAPK (ERK) to phosphorylate SMAD1 was most likely required for GSK-3 activity [312]. Wnt signaling decreased GSK-3 phosphorylation of SMAD1. Thus, the Wnt/GSK3/SMAD1 pathway plays an important role in embryonic pattern formation. A diagram of these interactions is presented in Fig. 10.

4.17. Wnt/beta-catenin involvement in metabolism

It has become apparent that pathways that were often considered primarily metabolic pathways also play key roles in cancer. Polymorphisms (single nucleotide polymorphisms, SNP) of Wnt5B and Wnt10B have been associated with increased risk for type II diabetes mellitus and obesity [313,314]. Wnt5b inhibits canonical Wnt signaling and this may promote adipogenesis [314]. The *C256Y* mutation present in *WNT10B* prevented its ability to activate canonical WNT signaling and block adipogenesis. SNP have also been detected at the *TCF7L2* gene that is associated with metabolic diseases [315–318]. The level of expression of Tcf7l2 has been determined to play key roles in regulating glucose tolerance. Altered expression of Tcf712 was shown to be associated with increased risk of type II diabetes [319]. Tcf712 was demonstrated to be an important regulator of glucose metabolism [320]. Disruptions in the *TCF7L2* cis-regulatory sequences were associated with an increased risk for the development of type II diabetes mellitus [321].

5. GSK-3 and mTORC1 regulation of autophagy

GSK-3 and mTORC1 are critical regulators of autophagy, a lysosome-dependent degradation pathway that results in recycling of damaged or unnecessary cytoplasmic components consisting of lipids, proteins and organelles [3–5,322–328]. This leads to the efficient production of metabolic precursors necessary for macromolecular biosynthesis or ATP generation. Autophagy can play dual roles in cancer cells as it can have both tumor-promoting and tumor-suppressing properties. Autophagy can result in the prevention of necrosis and inflammation. Autophagy is also important in tumor progression as it can provide energy as a result of its inherent recycling mechanism that can occur under unfavorable metabolic conditions that happen frequently during tumor growth and metastasis.

Metabolic stress will also induce autophagy. This has been investigated by nutrient deprivation of breast cancer cells. Overexpression of the aurora kinase A (Aur-A) can confer resistance to autophagic cell death and result in the survival of breast cancer cells undergoing metabolic stress. In these studies, Aur-A overexpression enhanced mTORC activity by antagonizing GSK-3beta activity. In breast cancer clinical samples, an inverse correlation was observed between Aur-A expression and autophagic levels [322].

Other studies have shown that GSK-3 can suppress autophagy through mTORC1. Overexpression of GSK-3alpha and GSK-3beta activated mTORC1 and suppressed autophagy in MCF-7 breast cancer cells. Overexpression of GSK-3 increased the number of autophagosomes and suppressed autophagic flux. Treatment of the cells with GSK-3 inhibitors inhibited mTORC1 activity and increased autophagic flux and lysosomal activation. These authors have proposed that GSK-3 inhibitors may target mTORC1 and lysosomal acidification [323].

Autophagy is involved in leukemia and may be regulated by epigenetic mechanisms [324–329]. In certain drug resistant cells, autophagy may be deregulated [327]. TP53 may serve as a rheostat to regulate autophagy in cancer [328]. Autophagy is a key survival process that is regulated in part by GSK-3 and mTORC1 [329–331].

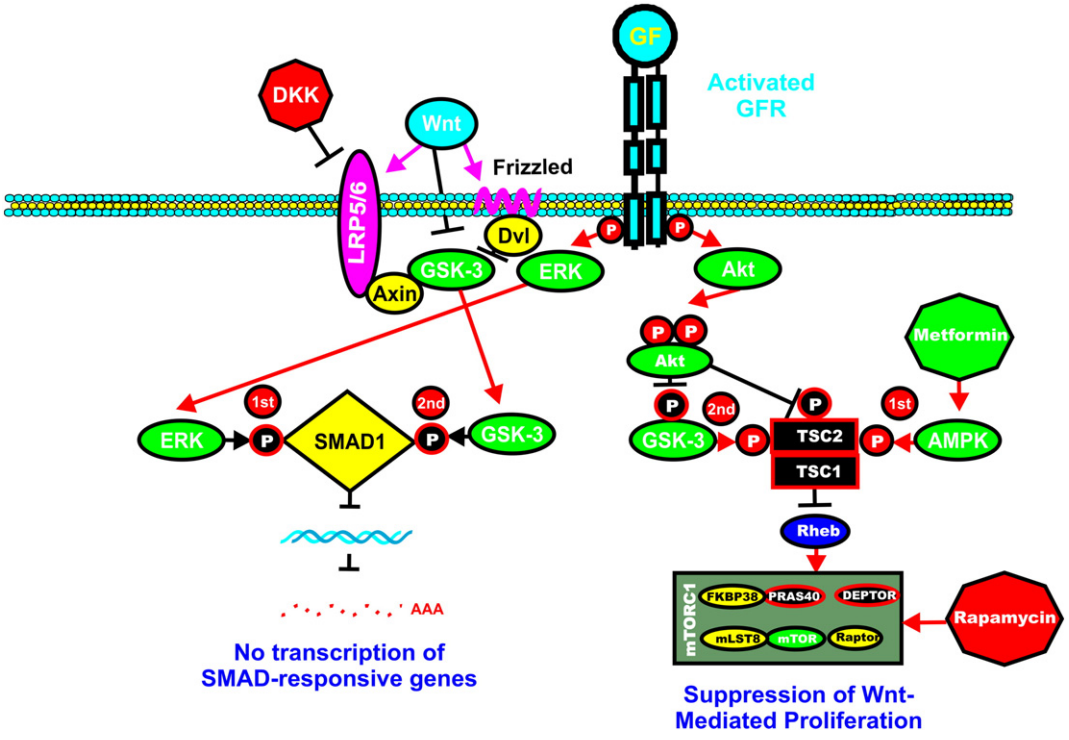

Fig. 10. Effects of Wnt and growth factor regulated GSK-3 activity. Both the Wnt/beta-catenin and growth factor mediated signaling pathways can have effects on GSK-3 activity which can alter cell growth. Wnt signaling may normally inhibit GSK-3 signaling via Dvl and/or LRP5/6 and Axin2. However, in the absence of Wnt, GSK-3 can be active and in combination with growth factor-mediated induction of ERK, stimulate the phosphorylation of SMAD1 to inhibit its effects on gene transcription. This diagram documents the priming phosphorylation by ERK (1st) and then the phosphorylation events by GSK-3 (2nd) which result in phosphorylation and suppression of SMAD1 transcriptional activities. On the right portion of the panel, the effects of growth factor and Wnt regulation of GSK3 and metformin stimulation of AMPK on the phosphorylation and activation of TSC2 and the effects of rapamycin on the suppression of the mTORC1 complex are shown. This can result in the suppression of Wnt-mediated proliferation. This diagram documents the priming phosphorylation by AMPK (1st) and then the phosphorylation events by GSK-3 (2nd) which result in phosphorylation and activation of TSC2 which is normally inhibited by Akt. This figure is presented to provide the reviewer some of mechanisms by which Wnt and growth factor mediated signaling pathways can interact to regulate GSK3 activity.

miRs have been shown to be important in many aspects of gene regulation. miR-100 can inhibit the expression of mammalian target of rapamycin or mechanistic target of rapamycin (mTOR) and insulin like growth factor receptor-1 (IGF-1R) and induce autophagy in HCC cells in vitro and inhibit the growth of HCC cells in xenograft studies. mTOR and IGF-1R were determined to be targets of miR-100 [332]. miRs are involved in the regulation of autophagy which is important for cell survival under hypoxic conditions. Hypoxia induced miR-96 in prostate cancer cells. This resulted in suppression of mTOR and stimulation of autophagy [333]. A diagram of the effects of miRs on autophagy is presented in Fig. 11.

Hypoxia has been shown to affect miR-155 expression that can induce autophagy in nasopharyngeal and cervical carcinoma cells [334]. Suppression of miR-155 inhibited hypoxia-induced autophagy. miR-155 targeted mRNAs involved in the PI3K/PTEN/Akt/mTORC1 pathway including: Ras homolog enriched in brain (RHEB), rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR),and ribosomal protein S6 kinase beta-2 (RPS6KB2). miR-155 had effects on cell proliferation [334].

In some cell types, miR-155 and miR-31 may inhibit PP2A activity which results in decreased GSK-3beta activity which has effects on autophagy. This also alters the Wnt signaling pathway [335]. A diagram of the effects of miRs on autophagy and Wnt signaling is presented in Fig. 12.

6. Abnormal activation of PI3K/PTEN/Akt/mTORC1 and Ras/Raf/MEK/ERK pathways can result in deregulated GSK-3 activity and drug resistance

These pathways are two of the most frequently activated signaling pathways due to mutations/amplification of upstream growth factor receptors in numerous cancers (Fig. 13). For example, components of the EGFR pathway are mutated/amplified in many different cancers [38,151]. Other growth factors and receptors also cause activation of these singling pathways [38,151]. In addition, other key signaling pathways are activated by these mutations and gene deregulations such as the Jak/STAT pathway [336]. Dysregulation of these pathways will alter GSK-3 activity by various kinases and phosphatases as well as miRs [3–6,337]. A diagram of how these mutations can alter gene expression is presented in Fig. 13.

The *PIK3CA* gene is one of the most frequently mutated genes in breast cancer [338]. Mutations at *PIK3CA* often serve to stimulate its activity [3–6,338]. Various effective small molecule inhibitors are being developed to suppress the activity of this pathway, as it is a key therapeutic target [338–345].

The PTEN phosphatase is also mutated frequently in human cancer [346,347]. PTEN is a tumor suppressor gene and is often silenced by mutations or epigenetic mechanisms in cancer. PTEN inhibitors are also being developed [348,349]. In particular, pharmacological inhibition of PTEN with the water-soluble vanadium-based complex (VO-OHpic), a potential antidiabetic drug [350], has recently been discovered to trigger induction of senescence in PTEN +/− prostate tumor cells, with no deleterious effect on PTEN WT cells [351]. This senescence response, referred to as PTEN-induced cellular senescence (PICS), was observed only in cells with partial PTEN activity (heterozygous). VO-OHpic treatment also induced cellular senescence in HCC cells with low endogenous PTEN levels [352]. Furthermore in HCC cells, treatment with VO-OHpic induced cell cycle arrest and expression of senescence-associated secretory phenotype (SASP) pro-inflammatory cytokine and protease mRNAs, IL-8 and MMP9 respectively. These findings indicated that VO-OHpic could be developed as a potential "pro-senescence" anti-cancer drug for the treatment of cancers with reduced PTEN expression. VO-OHpic has been

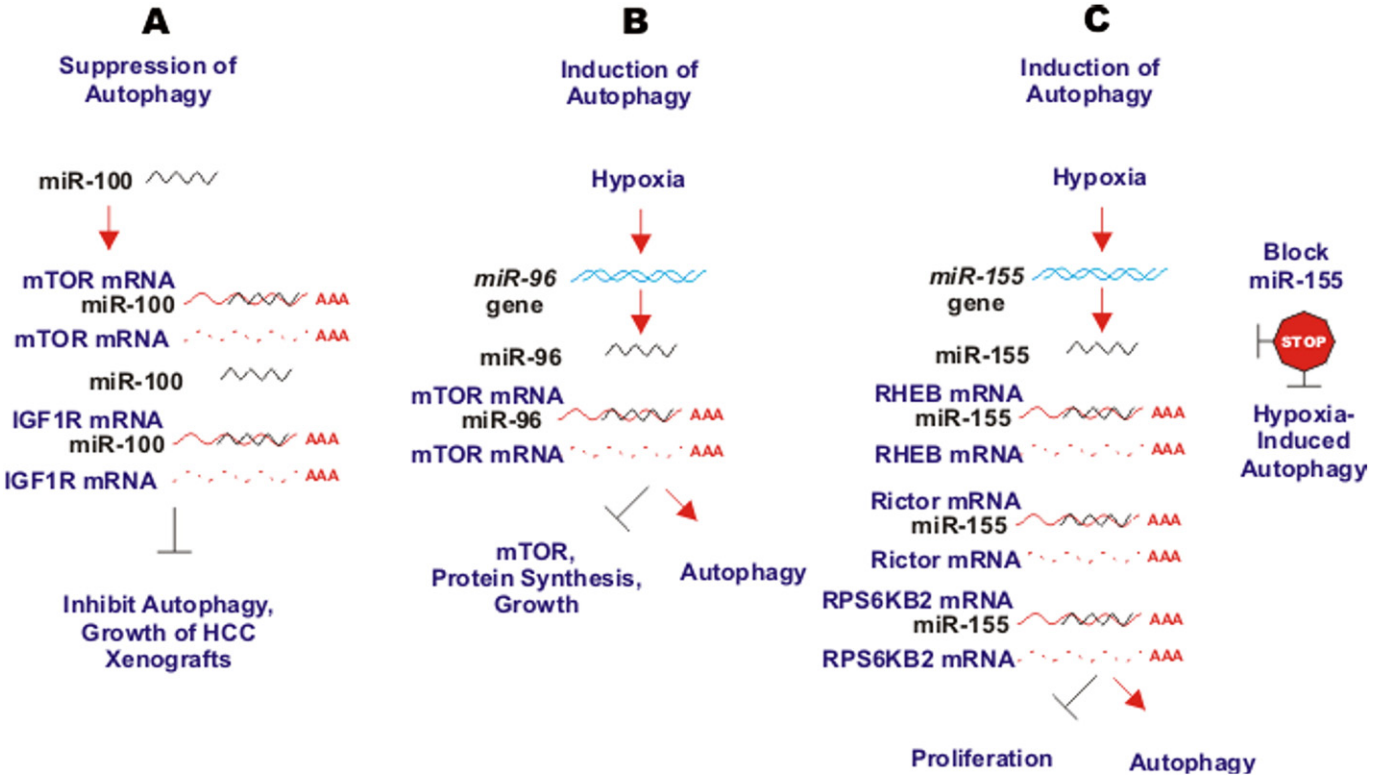

Fig. 11. Effects of miRs on autophagy and key components of the PI3K/PTEN/Akt/mTORC1 pathway. Panel A) miR-100 can suppress both mTOR and IGF-1R mRNA transcripts which can inhibit autophagy and the growth of HCC xenografts. Panel B) Hypoxia can induce miR-96 which can result in suppression of mTOR mRNA transcripts which will induce autophagy. Panel C) Hypoxia can induce miR-155 which can inhibit key genes involved in PI3K/PTEN/Akt/mTORC1 pathway including Rheb, Rictor and RPS6KB2. This can alter the induction of autophagy. Suppression of miR-155 will block hypoxia-induced autophagy. This figure is presented to provide the reader an idea of how autophagy can be regulated by miRs and hypoxia. Often components of the PI3K/PTEN/Akt/mTORC1 pathway are involved in the regulation of autophagy.

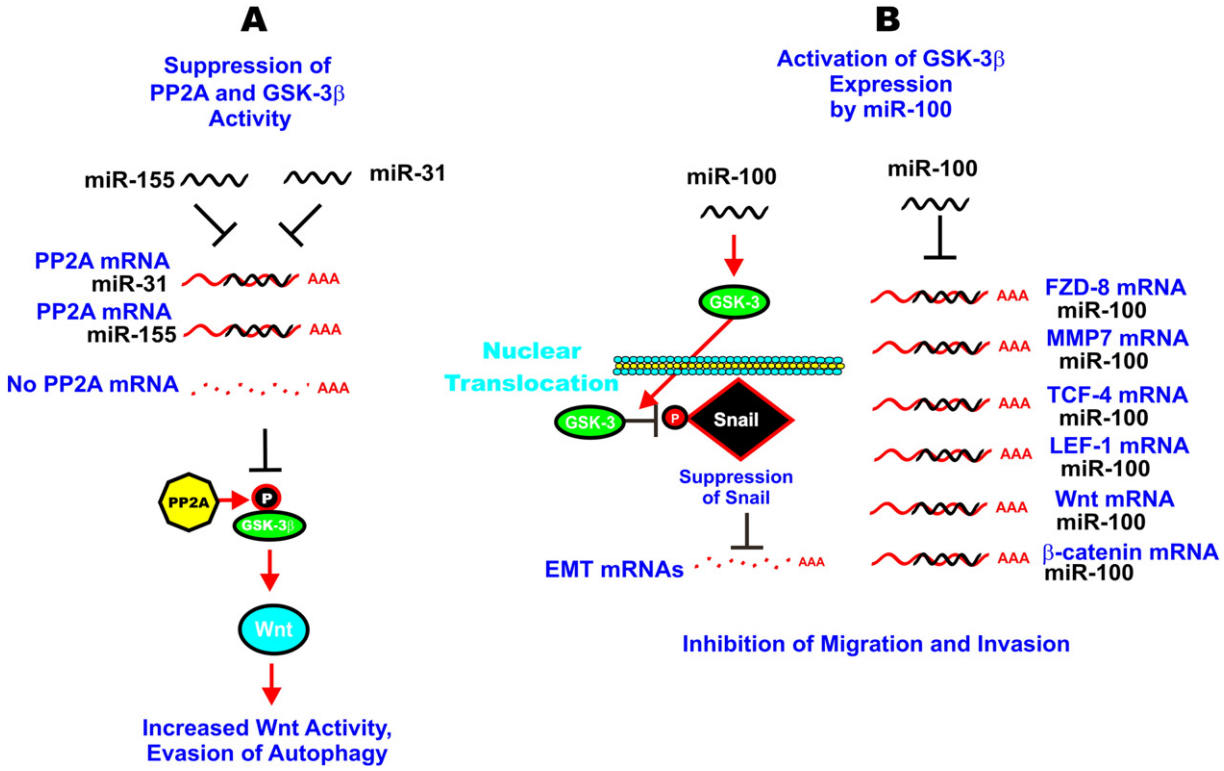

Fig. 12. Effects of miRs on GSK-3 activity which can result in modification of Wnt pathway and autophagy. Panel A) miR-155 and miR-31 can target PP2A mRNA which subsequently alters GSK-3 activity and the Wnt pathway as well as autophagy. Panel B) miR-100 can alter activity of GSK-3 which could affect Snail activity. miR-100 will affect activity of Wnt pathway members including: FZD-8, MMP7, TCF-4, Wnt and beta-catenin. miR-100 can have effects on migration and invasion. This figure is presented to provide the reader the concept of how miRs can regulate GSK-3 activity which can have effects on Wnt pathway activity and EMT.

also used in combination with 3-deazaneplanocin A (DZNep), a potent enhancer of zeste homolog 2 (EZH2) that is a histone-lysine N-methyltransferase enzyme inhibitor, to demonstrate the role of PTEN in polycomb repressive complex-2 (PRC2)-mediated apoptosis of colon cancer stem cells [353]. PTEN inhibition by VO-OHpic has a cardioprotective effect, in that VO-OHpic protects cardiomyocytes against cell death induced by ischemia and reperfusion [354]. In addition, it has been reported that myocardial infarct size was significantly reduced in VO-OHpic-pretreated mice [355]. Phospholipases, phosphatases and lipid kinases also play key roles in the regulatory circuits [356–361] and inhibitors to these enzymes are being developed.

Mutations at *KRAS* are detected in many different cancers. *KRAS* mutations are frequently observed in pancreatic cancer [115,116]. In addition, RAS-related genes are also important in human cancer and other disease [362,363]. One consequence of *KRAS* mutations is the activation of the Raf/MEK/ERK cascade which in turn can activate the transcription factor ETS which can induce GSK-3 transcription. GSK-3 can then induce the inhibitor kappa kinase (IKK) that results in phosphorylation of inhibitor kappa-B (I-kappaB) and activation of nuclear factor kappa B (NF-kappaB) that induces the transcription of many genes implicated in inflammation and cancer metastasis [364–369].

Various inhibitors have been developed to target molecules present in these pathways. They include both man-made monoclonal antibodies as well as small molecule membrane permeable inhibitors that often target kinases or phosphatases [5,338,348,364]. In summary, these pathways are frequently dysregulated in human cancer and their abnormal function often leads to increased proliferation and contributes to tumorigenicity.

6.1. GSK-3 and breast cancer drug resistance

In the following section we will focus on studies which involve GSK-3 and resistance to chemotherapeutic drugs and targeted therapy. Protein kinase D1 (PRKD1) has been shown to stimulate drug resistance and cancer stemness via GSK-3/beta-catenin signaling in breast cancer. PRKD1 is regulated by miR-34a. PRKD1 has been determined to be downregulated in certain invasive breast cancer cells. In drug resistant MCF-7-ADR breast cancer cells, PRKD1 was determined to be upregulated. miR-34a was determined to bind the 3′UTR of PRKD1 mRNA and negatively regulate its expression. PRKD1 expression was increased in MCF-7-Adr tumorspheres. Suppression of PRKD1 expression reduced self-renewal. The PRKD1 inhibitor CRT0066101 suppressed breast cancer stemness through GSK3/beta-catenin signaling [370].

GSK-3 has been determined to influence some of the effects that caveolin-1 has on the drug resistance in breast CSCs. Caveolin-1 is a membrane transport proteins which has been shown to be involved in drug resistance and CSC signaling. Caveolin-1 was detected at higher levels after chemotherapy which was associated with overexpression of beta-catenin and the ATP-binding cassette subfamily G member 2 (ABCG2) pathway which is associated with drug resistance. Suppression of beta-catenin eliminated the chemoresistance induced by caveolin-1 overexpression, indicating that beta-catenin was responsible for the effects of caveolin. Silencing caveolin downregulated beta-catenin/ABCG2 via inhibition of Akt and activation of GSK-3beta indicating the links between GSK-3beta, caveolin-1, ABCG2 and beta-catenin in the drug resistance of breast CSCs. These studies also demonstrated that the expression of caveolin-1 and beta-catenin/ABCG2 was associated in breast cancer specimens and caveolin-1 expression was upregulated in TNBC [371].

Previously we determined that kinase-dead (KD) GSK-3beta mutants would increase the resistance of MCF-7 breast cancer cells to doxorubicin and tamoxifen. Furthermore, constitutively-active GSK-3 mutants would decrease the resistance of the cells to doxorubicin. In contrast, the MCF-7 cells with the GSK-3beta KD mutant were more sensitive to the mTORC1 blocker rapamycin than the parental cells. Resistance to doxorubicin in the cells with the GSK-3beta KD mutant

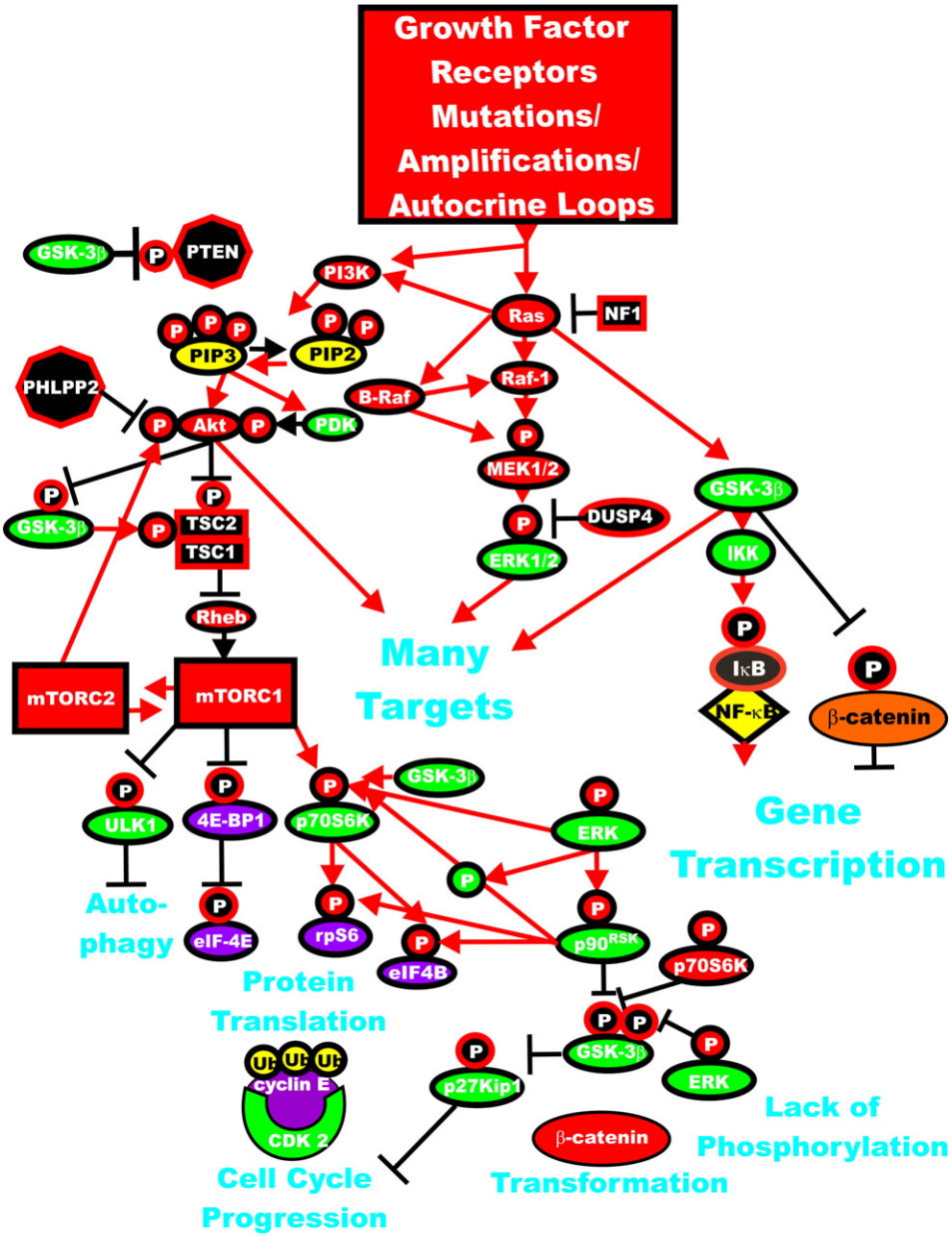

Fig. 13. Mutations which result in activation of the PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK pathways which alter GSK-3 activity. Sometimes dysregulated expression of growth factor receptors occurs by mutations, genomic amplifications or autocrine mechanisms which can lead to activation of the Ras/Raf/MEK/ERK, Ras/PI3K/PTEN/Akt/mTOR and other signaling pathways. Activating mutations occur in many genes in human cancer. These genes are indicated in red ovals and squares. Tumor suppressor genes inactivated in certain cancer are indicated in black squares, ovals or octagons. Other key genes are indicated in green ovals. Red arrows indicate activating events in pathways. Blocked black arrows indicate inactivating events in pathways. This figure is presented to provide the reader an idea of the diversity of the numerous molecules in which mutations have been detected which could alter the effects of GSK-3.

was alleviated upon treatment with a MEK inhibitor. Doxorubicin inhibited the detection of S9 phosphorylated GSK-3beta in parental MCF-7 cells but not in MCF-7 cells containing the kinase-dead GSK-3beta mutant, indicating that one of the effects of doxorubicin on MCF-7 cells is the induction of GSK-3beta activity. Suppression of GSK-3beta activity is associated with resistance to both doxorubicin and tamoxifen [6,372].

6.2. GSK-3 and CRC drug resistance

The GSK-3 pathway is hyper-activated in CRCs. In CRCs, hyperactivation of GSK-3beta results in drug resistance and survival. Protease-activated receptors (PAR1) and PAR2 exert key roles in activation of GSK-3 and the survival of CRC stem/progenitor cells. PAR2 activation was determined to decrease Caco-2 spheroids and proliferation. Thus PAR2 serves to suppress proliferation of cancerous Caco-2 cells. In contrast, PAR2 made normal cells more resistant to stress. GSK-3beta was dephosphorylated at S9 after PAR2 activation of both normal and tumor cells. Loss of PAR2 activity was associated with high levels of inactive GSK-3beta. These studies point to a novel pathway involving PAR2 and GSK-3beta in both normal and cancerous colon cells [373].

Regorafenib is a multikinase inhibitor which can target the Raf/MEK/ERK pathway. Part of the cytostatic effects of Regorafenib on CRC tumor

cells was determined to be via the induction of PUMA-mediated apoptosis through the NF-kappaB pathway, activation of GSK-3beta and suppression of ERK activity [374].

6.3. GSK-3 and gastric cancer drug resistance

The multi-drug resistance of a gastric cancer cell line can be inhibited by the proton pump inhibitor pantoprazole. The drug resistance in SGC7901/ADR cells was due to activated Akt/GSK-3beta/beta-catenin signaling which was reduced upon pantoprazole treatment [375].

6.4. GSK-3 and head and neck cancer drug resistance

Two different CSC phenotypes have been observed in head and neck squamous cell carcinoma (HNSCC). Both phenotypes express high levels of CD44, but they express different levels of epithelial-specific antigen (ESA). The CD44(high)/ESA(high) group of cells had epithelial features (Epi-CSC), while the CD44(high)/ESA(low) group is believed to have undergone EMT. GSK-3beta was determined to regulate the self-renewal and switching of both classes of cells. CD44(high)/ESA(low) cells were resistant to induction of apoptosis by 5-FU. This group of cells also had high expression of dihydropyrimidine dehydrogenase (DPD). Treatment with the DPD inhibitor 5-chloro-2,4-dihydroxypyridine (CDHP) increased 5FU-induced apoptosis in this group of cells. Suppression of GSK-3 caused these cells to undergo MET to CD44(high)/ESA(high) cells. Whereas treatment of regular CD44(high)/ESA(high) induced their differentiation. Importantly, co-treatment of the CD44(high)/ESA(low) cells with GSK-3beta and CDHP inhibitors resulted in increased 5FU mediated apoptosis. These studies indicate the roles of GSK-3 and CDHP in the chemoresistance of HNSCC CSCs [376].

6.5. GSK-3 and liver cancer drug resistance

Beta-escin is a mixture of saponins that are isolated from the horse chestnut (*Aesculus hippocastanum*). It has been determined that beta-escin reduced multidrug resistance in cholangiocarcinoma cells (CCA) by suppressing the GSK-3beta/beta-catenin pathway. The effectiveness of 5-FU, vincristine, and mitomycin on CCA could be enhanced by co-treatment with beta-escin. Beta-escin treatment resulted in decreased expression of P-gp but increased GSK-3beta phosphorylation at Y216 and dephosphorylation at S9 and degradation of beta-catenin [377].

6.6. GSK-3 and lung cancer drug resistance

miR-451 was determined to be detected at lower levels in docetaxel-resistant lung adenocarcinoma cells. miR-451 targets c-Myc in these cells. Overexpression of c-Myc induced ERK-dependent GSK-3 phosphorylation and inactivation and Snail phosphorylation and invasiveness [378].

6.7. GSK-3 and lymphoma drug resistance

The effects of the PI3K/mTOR inhibitor BEZ235 and pan-HDAC inhibitor panobinostat were examined on diffuse large B-cell lymphoma (DLBCL) cells. Synergy was observed between the two inhibitor treatments which resulted in Akt dephosphorylation, GSK-3 dephosphorylation and downregulation of Mcl-1 as well as other effects on key proteins involved in apoptosis. Activation of GSK-3 was suggested to be a key contributor to the synergy between the two inhibitor treatments in DLBCL cells [379].

6.8. GSK-3 and melanoma drug resistance

Suppression of GSK-3 activity by the LY2090314 GSK-3 (alpha and beta) inhibitor has been shown to be effective in the treatment of certain melanoma cells, even those which have *BRAF* and *NRAS* mutations and were resistant to the BRAF inhibitor vemurafenib. Loss of beta-catenin occurs during melanoma progression. It has been observed that cellular proliferation is inversely associated with nuclear beta-catenin levels. Inhibition of GSK-3 activity by administration of nanomolar doses of LY2090314 to the melanoma cell lines resulted in stabilized beta-catenin and increased expression of Axin2. In contrast, other tumor cells were not as sensitive to LY2090314. Beta-catenin stabilization was determined to be required for apoptosis induction after LY2090314 treatment of the melanoma cell lines. Thus certain Wnt activators such as LY2090314, which suppresses GSK-3, may be effective in the treatment of certain cancers such as melanoma [380].

6.9. GSK-3 and NSCLC drug resistance

Treatment of NSCLC patients with inhibitors targeting either EGFR or c-Met can result in the development of resistance to these inhibitors. The H1975 and H2170 NSCLC cell lines were used to investigated some of the mechanisms which resulted in the development of resistance to inhibitors which target EGFR or c-Met. Inhibitor-resistant cells were isolated and some had mutations in either *EGFR* or *MET* genes. In the inhibitor-resistant cells, the Wnt and mTOR proteins underwent changes in expression. beta-catenin and a transcriptional activator of Wnt, GATA-6 were upregulated in inhibitor-resistant H2170 cells. Inactive GSK-3beta (P-GSK-3beta) was also increased in inhibitor-resistant H2170 cells, suggesting that the suppression that active GSK-3 normally exerts on the Wnt and mTOR pathways was absent in the resistant cells. In contrast in H1975 cells, beta-catenin, GATA-6 and P-GSK-3beta were not detected at elevated levels and treatment with the Wnt inhibitor XAV939 did not significantly suppress proliferation. However combined treatment with the mTORC1 blocker everolimus and the EGFR inhibitor erlotinb resulted in synergistic suppression of growth. These studies point to roles of GSK-3beta, Wnt, beta-catenin, EGFR and mTORC1 in the growth of drug sensitive and resistant NSCLC and at least in some cells that combined Wnt and mTORC1 inhibition may be an approach to overcome resistance to EGFR and c-Met inhibitors [381].

6.10. GSK-3 and ovarian cancer drug resistance

Dishevelled-1 (DVL1) has been shown to be involved in the paclitaxel-resistance of human ovarian cancer cells by its interaction with Akt/GSK-3beta/beta-catenin signaling. DVL1 has been previously linked with cancer progression and in the following studies it has been associated with paclitaxel-resistance of the A2780/Taxol ovarian cancer line. Decreased expression of DVL1 was demonstrated to decrease paclitaxel-resistance in the cells. Increased expression of DVL1 in 2780 was determined to increase the levels of targets of beta-catenin such as: Bcl-2, BCRP and P-gp. In contrast, silencing DVL1 decreased their expression and led to beta-catenin accumulation. DVL1 activated Akt/GSK-3beta/beta-catenin signaling. Suppression of Akt prevented the DVL1-induced inhibition of GSK-3beta in both drug sensitive and drug resistant A2780 cells. Thus the authors suggested that DVL1 is important in the regulation of Akt/GSK-3beta/beta-catenin signaling and that suppressing DVL1 could result in paclitaxel sensitivity in ovarian cancer cells [382].

6.11. GSK-3 and pancreatic cancer drug resistance

Pancreatic cancer patients are often treated with the nucleoside analogue gemcitabine. Often after treatment of pancreatic cancer patients with gemcitabine, drug resistance occurs. Thus it is important to develop alternative approaches to treat gemcitabine-resistant patients. Zidovudine [azidothymidine (AZT)] is an anti-viral drug used to treat HIV/AIDS. Zidovudine was determined to sensitize gemcitabine-resistant pancreatic cancer cells to gemcitabine. Zidovudine treatment

was determined to decrease activation of Akt-GSK-3beta/Snail1 pathway, reverse EMT and increase human equilibrative nucleoside transporter 1 (hENT1) in gemcitabine-resistant pancreatic cells. Thus suppression of active GSK-3beta, which occurs in drug resistant pancreatic cells, may be eliminated after Zidovudine treatment and result in a therapeutic response [383]

TNFα-related apoptosis-inducing factor (TRAIL) has been evaluated as an anti-cancer agent for various types of cancer. Unfortunately cells often become resistant to TRAIL therapy. Pancreatic cancers are often resistant to treatment with TRAIL. Interactions between GSK-3 inhibition and TRAIL have been observed in pancreatic cancer cells [384].

7. Overview of the hedgehog (Hh) signaling pathway

GSK-3 also can regulate the Hh signaling pathway. Suppression of GSK-3 activity decreases the Hh pathway. The Hh pathway has essential roles in development and segmental pattern formation and is also involved in neoplasia and other developmental diseases. Hh ligands include: desert hedgehog (DHH), Indian hedgehog (IHH) and sonic hedgehog (SHH). The Hh ligands interact with the 12-pass transmembrane receptor patched (PTCH1) to regulate developmental signaling [385]. Depending on the circumstances, Hhs can act as mitogens, or in other conditions, they may promote differentiation. Fig. 14 presents a brief overview of the Hh pathway and indicates where some of the key mutations in this pathway occur and have effects on certain cancers and developmental disorders.

When a Hh binds PTCH1, PTCH1 is internalized and degraded. This results in the release of Smoothened (SMO), a G protein coupled receptor (GPCR). SMO then stimulates the dissociation of a suppressor of fused (SUFU)-glioma-associated oncogene homologue (GLI) complex. Upon stimulation of active SMO and dissociation of the SUFU/GLI complex, the transcription factors GLI1 and GLI2 can translocate to the nucleus and stimulate the transcription of certain genes including: *BCL2*, *CCND1*, *GLI1*, *GLI2*, *IGF2*, *MYC*, and *PTCH1* [386]. Thus there is yet another regulatory loop between GSK-3 and the Hh pathway. SUFU normally serves to inhibit the activity of GLI-1 by preventing the nuclear translocation of GLI-1. GSK-3 elevates Hh activity by phosphorylating SUFU that causes disassociation of SUFU from GLI3.

PKA can prime GLI3 for subsequent phosphorylation by CK1 and GSK-3. These phosphorylation events convert GLI3 into a transcriptional repressor. GLI3 is subsequently degraded. GLI1 and GLI2 proteins stimulate the transcription of Hh target genes as mentioned above. Many of these gene products are involved in the stimulation of cell growth and the prevention of apoptosis.

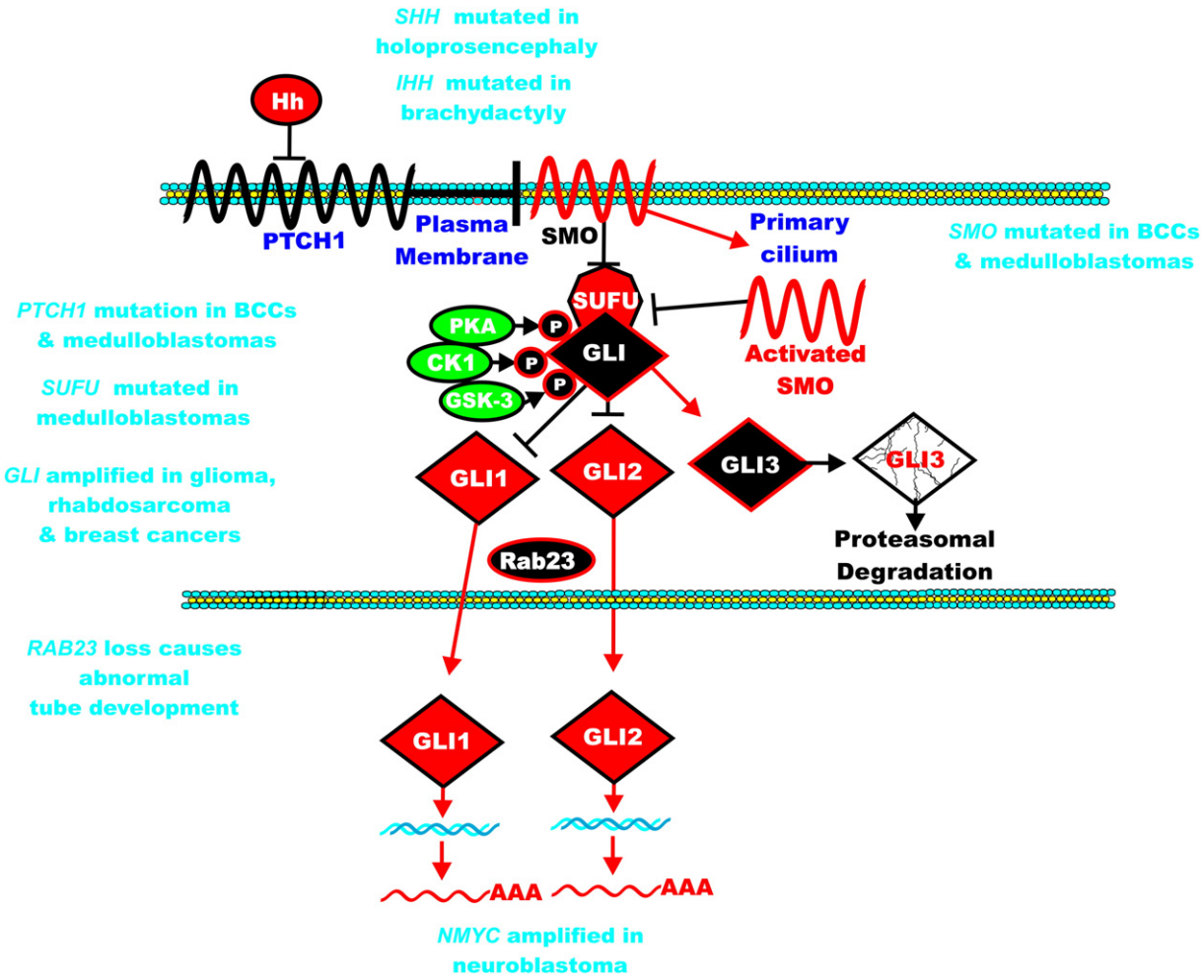

Fig. 14. Overview of hedgehog signaling pathway and sites where mutations alter activity of Hh signaling pathway. The Hh pathway normally inhibits the membrane spanning PTCH1 GPCR receptor which is indicated by a squiggly purple line crossing the membrane 14 times. PTCH1 normally inhibits SMO which is indicated by a red squiggly line which crosses the membrane 7 times. SMO is then activated in the primary cilium and serves to inhibit SUFU which is indicated by a red octagon. SUFU normally inhibits the GLI1 and GLI2 transcriptions factors which are indicated in red diamonds. The Rab23 GTPase may aid in the nuclear translocation of the transcription factors and is indicated by a black oval. In contrast, GLI3 is activated by SUFU and may serve as a transcriptional repressor and is regulated by GSK-3. It is indicated in a black diamond. Activating mutations are indicated in red. Brief descriptions of mutations are indicated in blue text to the sides of mutations. This figure is presented to provide the reader an idea of the diversity of mutations in Hh pathway components and the types of cancers they have been detected in.

7.1. Mutations in the Hh pathway

Mutations that result in activation of constituents of the Hh pathway occur in some sporadic and familiar skin (basal cell carcinoma, BCC) and brain (medulloblastoma) cancers. The Hh pathway is also involved with rhabdosarcoma, CRC and likely other cancers which have an embryonal nature as the Hh pathway plays key roles in development. The Hh pathway may be important in regulation of CSCs present in these cancers [385,386]. Patients with Gorlin syndrome harbor germline mutations in *PTCH1*. *PTCH1* mutations result in hyperactivation of the Hh pathway which lead to the development of multiple BCCs during the lifetime of the patients *PTCH* is thus a tumor suppressor gene. Gorlin syndrome patients are predisposed to other types of cancer, such as medulloblastoma. Medulloblastoma is the most common brain cancer in young children. Approximately one-third of the medulloblastomas examined in children in one study displayed hyper-activation of the Hh pathway due to mutations in critical pathway genes [387].

Mutations at components of the Hh pathway are found in various human diseases including cancer and other disorders. Targeting this pathway has become a key goal [385,386]. Mutations in *SHH* result in holoprosencephaly (HPE) which is a developmental disorder affecting the midline of the face and nervous system [387]. HPE is the most common malformation of the human forebrain. There are also mutations in the *IHH* gene that have been linked with Brachydactyly type A1 (BDA1) [388].

Activating mutations in the *SMO* gene lead to constitutive activation of the Shh pathway. These mutations have been identified in human medulloblastoma. These mutations are oncogenic and thus *SMO* can in some cases be considered an oncogene [389].

Dysregulation of *PTCH* is the underlying cause of nevoid basal cell carcinoma syndrome (NBCCS). *PTCH* mutations are also found in sporadic cancers where it is thought that *PTCH* serves as a tumor suppressor [390,391]. *PTCH* and suppressor of fused (*SUFU*) are mutated in medulloblastomas [392–400]. Activating mutations in *PTCH* and *SMO* have been detected in BCC [401–403]. *SUFU* mutations have been detected in NBCCS and certain forms of medulloblastomas [404]. Mutations have been detected in the putative PTCH1 coreceptor, *CDON* in HPE [405].

BCC frequently displays constitutive Hh pathway activity. Approximately 90% have loss of *PTCH1* and 10% have activating mutations in *SMO* [406]. Medulloblastomas are not as uniform in Hh pathway activation as BCC. Approximately 30% of medulloblastomas have a gene expression pathway consistent with Hh pathway activation. In these medulloblastomas, approximately 50% have mutations in a component of Hh pathway such as loss of *PTCH1* or *SUFU* or activated *SMO* [385]. Therefore in some medulloblastomas (i.e., the remaining 50%), there was Hh pathway activation but no mutations were detected in the Hh pathway genes examined (*PTCH1*, *SUFU*, or *SMO*). Some medulloblastoma harbor genetic mutations in both the Hh and Wnt pathways [407].

The *GLI* gene is an abbreviation for glioma associated oncogene where it was detected [408]. It is also mutated in breast cancer [409]. Mutations of components of the Hh pathway have also been detected in rhabdomyosarcomas (RMS) as well as amplification of the *GLI1* gene; however, the roles of these genetic mutations are not clear. Interestingly, in a PTCH+/− mouse model that develops RMS, the tumor cells were not dependent on SMO activity as the SMO-blocker cyclopamine did not prevent tumor development. It was therefore suggested that loss of PTCH activity may contribute to tumor initiation but was not required for tumor maintenance [410]. However, upwards of 25% of human cancers apparently lack mutations in key Hh pathway genes, but they demonstrate sensitivity to SMO inhibitors [411].

The Rab23 GDP/GTP exchange protein is an essential negative regulator of the Shh signaling pathway. Loss of function mutations of the *RAB23* gene results in abnormal neural tube development in mice. It has also been postulated to have roles in various cancers such as lung and HCC. Inhibition of Rab23 decreases the expression and nuclear localization of GLI1 [412].

Hh pathway activity is also elevated in neuroblastomas. Neuroblastoma is the most common and deadly extracranial tumor of children. *MYCN* is amplified in approximately 30–40% of high risk neuroblastomas. *MYCN* is considered an oncogenic driver gene in neuroblastoma [413]. MYCN is expressed in the developing neural crest where it is critical for the proliferation and differentiation of neuroblasts [414]. Hh pathway activation induces MYCN expression.

7.2. Hh pathway inhibitors and development of inhibitor resistance

Small molecules such as cyclopamine that block SMO function have been investigated for their effects on various cancers. SMO inhibitors have been tested in approximately fifty clinical phase I and phase II trials with various cancer patients and patients with certain developmental disorders. Substantial results have been observed in patients with BCC and medulloblastoma. These cancers often have mutations in components of the Hh pathway which results in its activation. Other types of cancer that rely on Hh signaling may also be responsive to SMO inhibitors. Combining SMO inhibitors and additional targeted, chemo-, or endocrine therapy may prove to be effective approaches for the treatment of certain cancers.

Resistance to some of the SMO antagonists (GDC-0449, vismodegib) was observed in one of the treated medulloblastoma patients who initially showed a significant response [415]. Thus there is a rationale for development of more effective inhibitors or appropriate combination therapies. Analysis of the medulloblastoma patient who developed resistance to vismodegib revealed that prior to treatment; the patient had a *PTCH1* (*W844C*) mutation and also had elevated Hh pathway signaling. After relapse, loss of heterozygosity for *PTCH1* was observed as well as a *SMO* mutation that was not detected prior to treatment with vismodegib [415]. The *SMO* mutation (*D473H*) prevented the binding of vismodegib. This *SMO-D473H* mutation was not inherently oncogenic as it was suppressed by functional PTCH1; however, the mutation blocked drug binding in cells with activated Hh signaling. Second generation SMO antagonists are being isolated which inhibit the proteins encoded by *SMO-D473H* mutations. In addition, other inhibitors are being developed which act mechanisms different from cyclopamine [416].

Inhibitors such as GANT61, which block GLI function, could suppress the resistance to SMO inhibitors [417]. Upregulation of GLI-2 expression by gene amplification is another known mechanism of resistance to vismodegib [418]. The effects of targeting the Hh pathway were examined in neuroblastoma by determining the ability of SMO inhibitors (cyclopamine and SANT1) or GLI1/2 inhibitors (GANT61) to inhibit neuroblastoma growth [419].

GANT61 was identified as a small molecule GLI inhibitor by a cell-based screen [417]. Interestingly, GANT61 reduced the transcriptional activity of GLI1 and GLI2 and interfered with GLI1 binding in the nucleus. Inhibiting the Hh pathway at the level of GLI was determined to be an effective means of suppressing the Hh pathway. GANT61 downregulated GLI1, c-Myc, MYCN and cyclin D1 expression, induced cell cycle arrest in early S phase, as well as promoted caspase 3 activation, PARP cleavage and apoptosis. Interestingly, while the effects of GLI1 inhibition by GANT61 and GLI knock-down were similar, they were not similar to the effects elicited by SMO knockdown. GANT61 also enhanced the effects of the chemotherapeutic drugs examined on neuroblastoma. Namely, GANT61 synergized with doxorubicin in all neuroblastoma cell lines examined. GANT61 synergized with vincristine in two out of three neuroblastoma cell lines examined [419].

Interestingly, neuroblastomas with amplified *MYCN*, which expressed high levels of MYCN and low levels of GLI, were not sensitive to GANT61. A negative correlation was observed between MYCN and sensitivity to GANT61, as the cells which expressed higher levels of MYCN were less sensitive to GANT61. In addition, neuroblastoma cells which expressed low levels of MYCN and high levels of GLI1 were the most sensitive to GANT61. The authors of this interesting study have suggested that this may represent a transition from Hh signaling dependency to MYCN-

driven proliferation and differentiation of neuroblasts which occurs during neural crest development and was Hh pathway-independent [419].

Hh pathway signaling is also important in CRC. GANT61 was also shown to be more effective than SMO inhibitors in suppressing the growth of CRCs [420,421]. This group observed activation of the ATM/CHK2 axis and co-localization of gamma-H2AX and CHK2 in nuclear foci proceeding inhibition of GLI1/GLI2. CHK2 is a S/T kinase that is activated in response to DNA damage and is involved in cell cycle arrest. ATM is a S/T kinase and is activated by double strand DNA breaks. Upon DNA double strand breaks, histone H2AX (one of many histone H2A genes) becomes phosphorylated on S139 and is then called gamma-H2AX. gamma-H2AX is frequently used as a marker for DNA double strand breaks.

This group also observed by cDNA microarray analysis that down-regulation of genes involved in DNA damage response and DNA repair occurred. Aberrant activation of the Hh pathway was associated with genomic instability, inactivation of DNA repair mechanisms, defects in checkpoint activations and cancer development.

Increased expression of the PI3K/PTEN/Akt/mTOR pathway has been observed in certain mouse models of Hh-driven medulloblastoma that have acquired resistance to the SMO inhibitor NVP-LDE225 [422]. The investigators determined that there were some genetic alterations in Hh pathway genes (*SMO* mutations and *GLI2* amplification) in cells which acquired resistance to SMO antagonists as well as upregulation of the PI3K/PTEN/Akt/mTOR pathway. Treatment of the medulloblastoma mouse model with the SMO inhibitor NVP-LDE225 and either the PI3K inhibitor NVP-BKM120 or the dual PI3K-mTOR inhibitor NVP-BEZ235 delayed the development of resistance. Thus in the Hh pathway, there are both tumor suppressors such as *PTCH1* and oncogenes such as *GLI2* and *SMO*. Certain medulloblastomas exhibit the phenomenon of oncogene-addicted growth that is dependent on GLI activation. Mutations at *SMO* can occur at multiple sites and they prevent binding of SMO antagonists. In addition, amplification of cyclin-D1 (*CCND1*) has been observed in a vismodegib-resistant line which has been shown previously to be important in medulloblastoma formation [423]. A diagram illustrating where mutations can occur in the Hh pathway that leads to resistance to small molecule inhibitors is presented in Fig. 15.

At least 90% of BCCs have mutations in the PTCH1 or SMO genes. If the BCC metastasize, they may be sensitive to inhibitors which target Hh and PI3K/PTEN/Akt/mTORC pathways [424].

SMO mutations may be responsible for BCC resistance to SMO inhibitors. In one study, 50% (22 out of 44) of BCC that were resistant to SMO inhibitors had SMO mutations. These mutations resulted in Hh signaling in the presence of the SMO inhibitors. The mutations occurred in the ligand binding pockets. The cells with the SMO mutations outcompeted cells with the WT SMO in the presence of the SMO inhibitor. Importantly, the cells with the SMO mutations did respond to inhibitors of downstream targets such as GLI2 and some PKC isoforms [425].

Vismodegib is a SMO inhibitor that is now approved for the treatment of locally advanced and metastatic BCC. Resistance occurs due to Hh pathway reactivation. This can result from SMO mutation or less frequently from changes in copy numbers of SUFU and GLI2. These studies have also indicated that targeting the Hh pathway at multiple levels may be necessary to overcome resistance [426].

Many inhibitors to SMO have been developed. These include: vismodegib, BMS-833923, saridegib (IPI-926), sonidegib/erismodegib (LDE225), PF-04449913, LY2940680, LEQ 506, and TAK-441. These inhibitors have shown promising effects as monotherapy in patients with BCC and medulloblastomas but not in other cancer types [427].

NL-103 is an inhibitor that targets histone deacetylases and the Hh pathway. It can also suppress BCC which have become resistant to the SMO inhibitor vismodegib that have *SMO* mutations. NL-103 is a novel inhibitor that is a chimeric compound. One component of NL-103 is similar to vismodegib while the other component has structural elements of the HDAC inhibitor vorinostat. NL-103 treatment suppressed the vismodegib-resistance that resulted from SMO point mutations. In contrast to vismodegib, NL-103 decreased GLI2 expression [428].

Different mutations in *SMO* have been observed in BCCs. One *SMO* mutation was observed to inhibit drug binding to *SMO* (*SMO* G497W). This was responsible for the primary resistance of this patient. Another patient initially had a *PTCH1* nonsense mutation and had a complete clinical response on vismodegib treatment for five months but then had progression after eleven months on vismodegib. This patient had acquired a *SMO* D473Y mutation. The *SMO D473Y* mutation had an effect on the binding site geometry which resulted in loss of the hydrogen bond network. Inactivating *PTCH1* mutations have been observed in BCCs which result in constitutive SMO activity as PTCH1 normally suppresses SMO [429].

The ERIVANCE BCC phase II trial evaluated the efficacy and safety of vismodegib in patients with locally advanced or metastatic BCC. This drug has also been examined in patients with Gorlin syndrome [430]. The effects of vismodegib on NSCLC cells resistant to the EGFR inhibitor erlotinib have been examined. It was determined that treatment of NSCLCs with vismodegib suppressed the resistance of the cells to erlotinib and cisplatin. Treatment of the cells with vismodegib resulted in sensitivity to erlotinib. Vismodegib treatment resulted in decreased CSC gene expression and upregulation of miR-200b and let-7c [226].

Additional signaling pathways that are often deregulated in colon carcinomas can also drive GLI1 transcriptional activity. Oncogenic mutations at *KRAS* and *AKT* or loss of *PTEN* or *TP53* will influence GLI1 activity. These effects were observed in both non-metastatic as well as CD133 + CRCs. GLI1 activity can also be enhanced by upregulation of beta-catenin by *KRAS* and *TP53* mutations [430,431].

7.3. Cross talk between Hh and other signaling pathways

As with other signaling pathway, the Hh pathway is cross-regulated by many other signaling pathways, including Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTORC1. Mutations in *KRAS* and *BRAF* occur frequently in CRC that will result in abnormal MEK activity [432–434]. MEK inhibitors suppress GLI1 activity in CRC cells [420,421]. Cancer cells may become resistant to SMO antagonists due to acquired mutations in *SMO* or due to non-canonical activation of Hh pathway downstream of SMO. This latter point reinforces the concept of targeting the *GLI1* gene products may be a more effective therapeutic approach than targeting SMO. Bcl-2 is a target gene of GLI1, thus GLI1 inhibitors suppress Bcl-2 expression. Inhibition of MEK may be an approach to suppress GLI1 activity. Inhibiting GLI as opposed to SMO may also a more effective approach in suppressing rhabdosarcoma growth [435].

8. The Notch signaling pathway and interactions with GSK-3

The Notch signaling pathway is another critical pathway regulated by GSK-3 [436–438]. Briefly, Notch signaling is critical in many cellular processes including cancer and is a therapeutic target [439–445]. Activation of the Notch pathway occurs after cell-to-cell interactions. A gamma secretase induces the activity of Notch protein stimulating the cleavage of Notch into the Notch intracellular domain (NICD). NICD can then translocate into the nucleus to regulate gene expression through its interactions with other transcription factors, HATs and other chromosomal proteins [441–445].

GSK-3 and Akt can have opposite effects on the Notch pathway [446,447]. The transcriptional activity of NICD is increased upon GSK-3 phosphorylation. This phosphorylation event also increases the stability of NICD by preventing its proteasomal degradation [446]. GSK-3 phosphorylates NICD on the domain involved in nuclear localization. This increases the transcriptional activity of Notch [448]. On the other hand, the transcriptional activity of NICD is regulated negatively by Akt phosphorylation [447]. Some other studies have shown that phosphorylation of Notch1 by GSK-3 resulted in decreased levels of Notch1

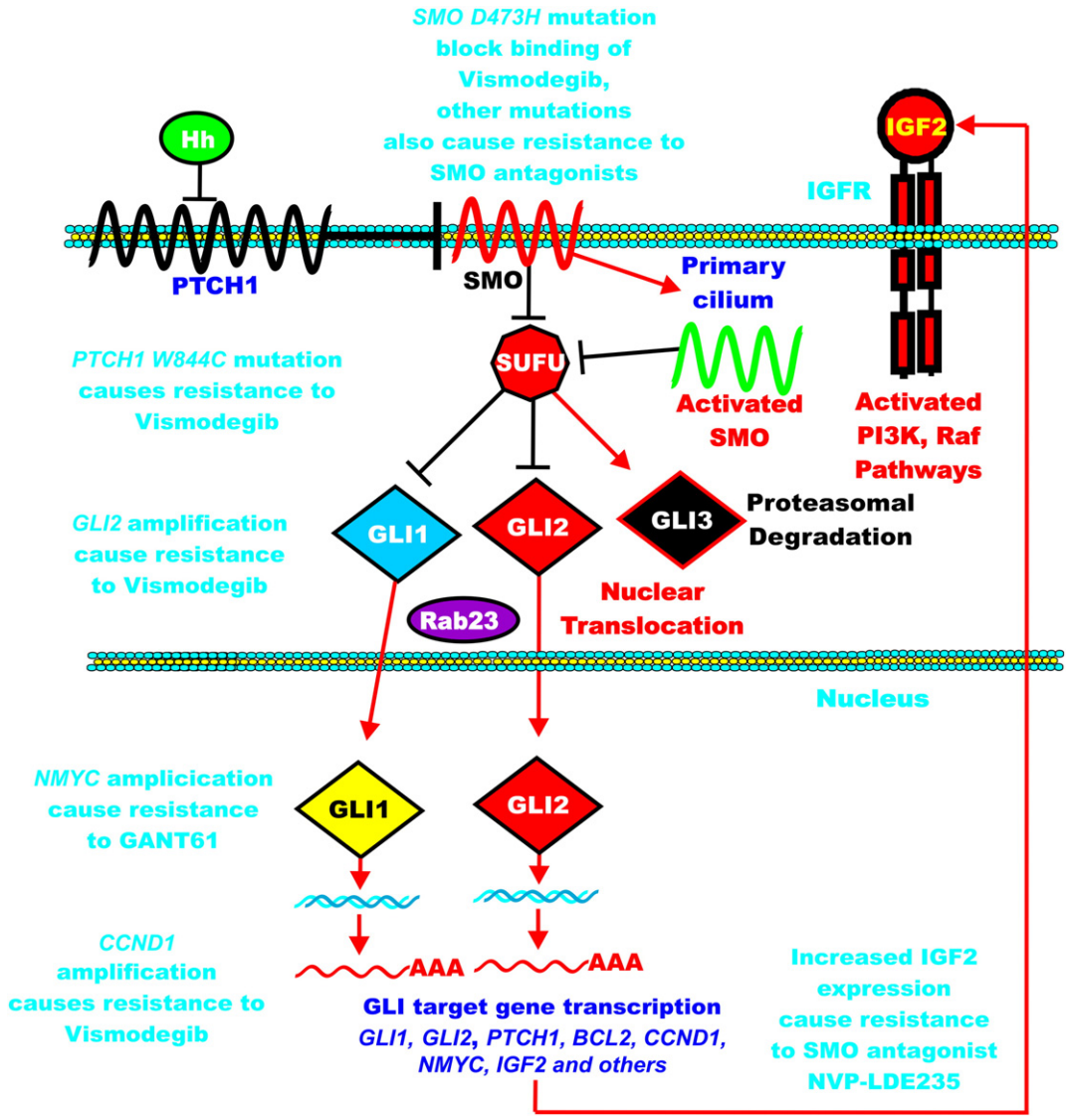

Fig. 15. Sites where mutations confer resistance to Hh pathway inhibitors. In this figure, sites which when mutated confer resistance to Hh pathway inhibitors are indicated in blue text. Activating mutations are indicated in red. This figure is presented to provide the reader an idea of how mutations in key Hh pathway genes can confer resistance to Hh pathway directed targeted therapies.

as well as transcriptional activity [449–451]. Beta-catenin can also influence the transcriptional activity of Notch1/NICD [450].

Notch1 mutations are detected in approximately 50% of T-ALL patient samples. These T-ALL patients can be treated with γ-secretase inhibitors (GSIs). Mutant Notch1 will activate various signaling and apoptotic pathways, including: PI3K/PTEN/Akt/mTORC1/GSK-3 and c-Myc [452].

Additional targets of the miR-200 family include the Notch signaling pathway. The Notch pathway is also involved in EMT and metastasis. miR-200 can suppress Jagged 1, mastermind-like protein 2 (Maml2) and Maml3 which have effects on ZEB1/2 expression, that can in turn regulate miR-200 levels. Notch signaling (Jagged2) can regulate GATAs which will suppress miR-200. These pathways have multiple autoregulatory loops [209]. miR-200 prevented the metastasis of human prostate cancer cells by suppressing the Notch ligand Jagged2, Maml2 and Maml3 [453,454]. Thus the PI3K/PTEN/Akt/mTORC1, Wnt/beta-catenin, GSK-3 pathways and miRs can interact and regulate Notch signaling.

9. Brief overview of GSK-3 inhibitors

We will briefly summarize some of the key aspects about GSK-3 inhibitors. Additional details of GSK-3 inhibitors in clinical trials have been recently reviewed [5]. GSK-3 is a complicated target as it is involved in many biological processes from neurology, diabetes to cancer. In some circumstances, GSK-3 is considered a tumor suppressor protein while in other cases it is considered a tumor promoter [3]. Many GSK-3 inhibitors have been developed.

Long-standing clinical studies have indicated the effects that lithium has on hematopoietic stem cells (HSCs) and other hematopoietic cells [4,5,455–459]. Treatment of patients with lithium resulted in augmentation of the number of circulating HSCs as well as peripheral blood cells [459]. In 1998, it was suggested that lithium treatment might be useful in bone marrow transplantation as it could mobilize HSCs [458]. A target of lithium has been shown to be GSK-3 [460]. The effects of lithium and GSK-3 on hematopoiesis, leukemia and leukemia stem cells have been reviewed recently [4].

Lithium has also been shown to affect other cell types besides hematopoietic cells. Treatment of glioma cells with lithium was determined to suppress cell migration in spheroid cultures by inhibiting GSK-3 activity. In contrast, treatment of these glioma cells with lithium did not extinguish cell viability but did induce alterations in cellular morphologies [461]. The lithium-treated glioma cells displayed retraction of the normally long extensions at their leading edges [290]. Some of the effects of GSK-3 inhibition by lithium and other GSK-3 inhibitors are stimulation of the Wnt/beta-catenin and PI3K/PTEN/Akt/mTORC1 pathways. Suppression of GSK-3 will affect many integral components present in the PI3K/PTEN/Akt/mTORC1 pathway as well as interacting components including: PTEN, mTORC1, TSC1, Rictor, Akt and p70S6K. However, as pointed out in this review, GSK-3 interacts with many other signaling pathways [462,463]. Thus the effects of inhibiting GSK-3 are predicted to be diverse.

Numerous GSK-3 inhibitors have been discovered by basic scientists and pharmaceutical companies [464]. Some studies have suggested that GSK-3 inhibitors may be appropriate for the treatment of certain cancers [465–470]. The GSK-3 inhibitor SB-415286 induced: cell growth inhibition, cell cycle arrest at G_2/M, cyclin B downregulation, beta-catenin stabilization, GSK-3beta S9 phosphorylation and apoptosis in certain leukemia cell lines examined [470]. SB-415286 as well as arsenic trioxide and LiCl inactivated GSK-3beta in certain AML cells by inducing the phosphorylation GSK-3beta at S9 that is associated with its inactivation [471]. In the above-mentioned scenarios, GSK-3 is acting as a tumor promoter and suppression of GSK-3 activity inhibited cell growth.

The situation with GSK-3 is complicated as in some cases GSK-3 can act as a tumor suppressor. In this scenario, suppression of GSK-3 would be predicted to promote growth or as we observed induce the chemotherapeutic- and hormonal-based drug resistance of breast cancer cells [6]. Likewise in other studies with breast cancer and medulloblastoma, increased GSK-3 expression was associated with the induction of apoptosis, documenting the tumor suppressor functions of GSK-3 [472, 473]. These tumor suppressor properties of GSK-3 have resulted in caution in terms of the use of GSK-3 inhibitors.

As discussed previously, GSK-3 can interact with the TP53 pathway. In some cells, inhibition of TP53 altered the effects of the GSK-3 inhibitors. In contrast, activation of Wnt signaling led to decreased viability through a TP53-independent pathway in these cells [474].

As predicted from the effects that GSK-3 has on Wnt/beta-catenin signaling, suppression of GSK-3 activity by GSK-3 inhibitors can result in activation of beta-catenin. Interestingly, the activation of beta-catenin mediated by GSK-3 inhibitors can augment the cytotoxicity of cisplatin in HEI-OCI cells [475].

One important aspect about GSK-3 inhibitors is their effect on pluripotency of cells [4]. Many GSK-3 inhibitors are ATP-competitive and suppress both GSK-3alpha and GSK-3beta [476]. BIO was shown to suppress GSK-3 activity and promote Wnt/beta-catenin signaling. This combination of events preserved the pluripotency of human and mouse embryonic stem cells (ESCs) [477]. However, prolonged inhibition or activation of GSK-3 and Wnt signaling respectively resulted in differentiation of ESCs into multipotent mesendodermal progenitors or their differentiated progenitors [478–480]. The Hh, Notch and Wnt pathways can regulate cell fate determination and maintenance of stem cells. All of these pathways are regulated by GSK-3. GLI2 is a part of the Hh pathway that can be phosphorylated by GSK-3 [481]. Suppression of GSK-3 activity by BIO in canine melanoma cells enhanced beta-catenin activity and reduced migration and proliferation [482].

Various diseases including: neurological, diabetes, obesity, ischemia, sepsis, colitis and cancer have been postulated to be targets for GSK-3 inhibitors. However, caution has been suggested before treatment of cancers with GSK-3 inhibitors as GSK-3 can phosphorylate such pro-oncogenic factors as beta-catenin, c-Jun and c-Myc that target them for degradation [476]. However, as stated above, in certain circumstances, enhanced beta-catenin activity observed after GSK-3 inhibitor treatment was associated with reduced migration and proliferation. Indeed, suppressed proliferation was observed after either GSK-3 inhibitor treatment or GSK-3 knock-down in many cancers examined, including: brain, CRC, hematopoietic, melanoma, ovarian, pancreatic, paraganglioma, pheochromocytoma, prostate and thyroid cancers [483]. Patients with bi-polar disorder have been treated with the GSK-3 inhibitor lithium for decades and there does not appear to be any evidence of increased cancer incidence in these patients [484].

It is also possible to increase the effects of GSK-3 inhibitors by combining them with inhibitors that target key components of other signaling pathways. Combining the GSK-3 inhibitor AR-A014418 with the PKC-beta inhibitor enzastaurin was shown to have enhanced cytotoxicity against cutaneous T-cell lymphomas (CTCL). This combined treatment resulted in increased beta-catenin protein and transcriptional activity and decreased the level of CD44 [485]. Not surprisingly, a common aspect of many GSK-3 inhibitors is that they increase beta-catenin protein levels and transcriptional activity which is also necessary for their cytotoxic effects of the GSK-3 inhibitors.

10. Brief overview of Wnt/beta-catenin inhibitors

It is not surprising that Wnt-beta-catenin has been a key therapeutic target for over 20 years due to the observations that elevated Wnt/beta-catenin signaling is detected in colorectal and other cancers. This has resulted in the discovery of inhibitors that can suppress this pathway [486–491]. Recently an Achilles' heel has been observed in beta-catenin, its activity is iron-dependent. This iron-dependency has been exploited in the discovery and characterization of beta-catenin inhibitors that suppress beta-catenin stabilization due to their iron chelation abilities. Some of the inhibitors have been examined in clinical trials [492–494]. In certain cancers, if iron is elevated so is Wnt/beta-catenin signaling. This can result in enhanced tumorigenesis [494].

Another approach to suppress Wnt/beta-catenin is with common drugs such as non-steroidal anti-inflammatory drug (NSAIDs) and celecoxib. Aspirin, ibuprofen and naproxen are common NSAIDS which inhibit both COX1 and COX2, while celocoxib inhibits COX2. These drugs have been shown to inhibit beta-catenin-dependent transcription in CRCs and other cell types [495,496]. Moreover the more specific COX2-inhibitor celecoxib can reduce polyp formation in patients with FAP [497,498].

The beta-catenin/TCF complex is an important target in the Wnt/beta-catenin pathway. This complex has proven challenging to target. However, natural inhibitors of this complex have been identified. One group of inhibitors is called the inhibitors of Wnt response (IWR-1/2). IWR-1/2 targets Axin and stabilizes it [488,499]. XAV939 is another inhibitor that also targets and stabilizes Axin [489]. These inhibitors, IWR-1/2 and XAV939, act by suppressing the enzyme tankyrase that results in beta-catenin degradation. Tankyrase is a poly-ADP ribosylase and normally poly-ADP ribosylates Axin1/2 that results in its turnover. Axin becomes stabilized when tankyrase is inhibited. This results in the destabilization of beta-catenin and inhibition of Wnt signaling. Axin is poly-ADP ribosylated that is recognized by the ring finger protein 146 (RNF146). This results in the ubiquitination and subsequent degradation of Axin. CK1 also exerts effects on the stability of beta-catenin. CK1 activators, such as the FDA-approved drug Pyrvinium, will also modulate the stability of beta-catenin [491].

The enzyme Porc (short for porcupine) is a transmembrane O-acyltransferase in the endoplasmic reticulum. Porc is essential for Wnt palmitoylation and maturation [500,501]. Lipid-free Wnts are not secreted. Suppression of Porc activity with the IWP2 compound inhibited Wnt palmitoylation which also suppressed Wnt/beta-catenin signaling, and Wnt-C59 is a Wnt/Porc inhibitor which modifies Wnt activity. Wnt-C59 was determined to suppress the growth and stemness properties of nasopharyngeal (SUNE1 and HNE1) carcinoma cell lines [502].

10.1. Combining chemotherapy with Wnt/beta-catenin pathway inhibitors

Certain Wnt/beta-catenin natural pathway inhibitors have minimal effects on cancer cells. A WIF1 expression construct was transfected into PC3 cells and reduced levels of activated Akt were detected in the WIF1-transfected cells compared to control cells. Ectopic expression of WIF1 in the PC3 cells increased the sensitivity of the cells to the chemotherapeutic drugs paclitaxel and etoposide. The PC3 cell line lacks PTEN and normally expresses high levels of activated Akt levels [503]. These results indicated a cross talk between the Wnt/beta-catenin pathway and Akt activation, at least in the context of *PTEN*-mutant cells.

Likewise, DDK1 can increase the sensitivity of U87MG glioblastomas to various chemotherapeutic drugs [504]. These authors have previously shown that DKK-1 was induced by TP53 [505]. The authors demonstrated that DKK-1 was an important link between TP53 and Wnt signaling pathways. It should be pointed out that U87MG glioblastoma cell line also lacks functional PTEN and has high levels of activated Akt.

10.2. Wnt pathway expression increases sensitivity to therapy

The expression of certain Wnt molecules will increase the sensitivity of certain cancers to chemotherapy as well as targeted therapy. Wnt5A is expressed at high levels in ovarian cancers as compared with benign tumors and normal ovaries [506]. The levels of Wnt5a were modulated by either transfection with a Wnt5a expression construct or a construct encoding a miR specific for Wnt5a. Increased Wnt5A expression increased the sensitivity of certain ovarian cancer cells to multiple chemotherapeutic drugs (epirubicin, etoposide, 5FU, oxaliplatin and paclitaxel) [507]. The authors have suggested that Wnt5a levels may be a useful prognostic indicator in patients with ovarian cancer and that Wnt5a expression may play an important role in controlling chemosensitivity to chemotherapeutic drugs in ovarian cancer.

Increased Wnt/beta-catenin activity can influence the sensitivity of certain cancer cells to targeted therapy, as activation of beta-catenin increased the responses of melanoma cells, which often have mutations at *BRAF*, to the BRAF inhibitor PLX4720 [507]. It was determined that endogenous beta-catenin was essential for PLX4720-induced apoptosis. Activation of Wnt/beta-catenin signaling in the *BRAF*-mutant melanoma cells synergized with PLX4720 to increase apoptosis in vitro as well as decrease tumor growth in vivo. Axin1 levels were also reduced in the cells. Axin1 is a negative regulator of beta-catenin. Knockdown of Axin1 converted BRAF-inhibitor-resistant cells to BRAF-inhibitor-sensitive cells indicating that Axin1 was essential in controlling the response to BRAF inhibitors in these cells. These important results indicate that Wnt/beta-catenin/Axin1 levels and activity may have modulated the sensitivity of certain melanomas to BRAF inhibitors. This same research group subsequently demonstrated that treatment of *BRAF*-mutant and *NRAS*-mutant melanomas with Wnt3A and the MEK inhibitor AZD6244 resulted in the induction of apoptosis. Susceptibility to combination treatment in the melanomas correlated with negative regulation of Wnt/beta-catenin pathway activity by the Raf/MEK/ERK pathway in those particular melanomas. The authors observed decreases in the levels of Axin1 in the cells. As in their previous studies, they documented that apoptosis-resistant *NRAS*-mutant lines can be sensitized to the MEK inhibitor by lowering the levels of Axin1 specifically by pretreatment with Axin1 siRNA. These studies indicate the importance of Wnt3A and Axin1 in controlling the sensitivity of *NRAS*-mutant melanoma to MEK inhibitors [508]. These and many other studies in different model systems document the complex interactions between signaling pathways and the critical roles that genetic mutations play in determining sensitivity/resistance.

11. Summary

The roles of GSK-3 in biological processes have evolved significantly since it was first identified as an enzyme that phosphorylated and inactivated GS, a key metabolic enzyme. GSK-3 is clearly a key moonlighting enzyme that has critical roles in many biological processes and diseases. As expected, GSK-3 was shown to be important in metabolic diseases such as diabetes and cardiovascular diseases. GSK-3 was demonstrated to be the target of lithium that is a key remedy in various neurological disorders. GSK-3 was identified as playing key roles in Wnt/beta-catenin signaling which is frequently aberrantly regulated in CRC due to mutations in the pathway at *APC* genes as well as mutations at the sites of phosphorylation of beta-catenin by GSK-3 and CK-1. GSK-3 is regulated by many kinases, including: Akt, PKA, Src, ERK, $p38^{MAPK}$, Src, Fyn, PYK2 and others. In addition, GSK-3 activity is regulated by phosphatases such as PP1 and PP2A. Thus there are many biochemical mechanisms to regulate GSK-3 activity. Due to the high frequency of mutations of *PIK3CA* (PI3Kalpha) and PTEN in human cancers, as well as aberrant expression of upstream growth factor receptors, Akt is frequently activated in human cancer that can result in abnormal Akt activity that in turn will normally extinguish GSK-3 activity. These effects on GSK-3 activity can influence the activity of other pathways such as: Wnt/beta-catenin, Hh and Notch. Moreover, GSK-3 can also serve to prevent Akt activation by phosphorylation of Rictor which results its inability to catalyze the "second" phosphorylation event involved in Akt activation. Thus, there are complicated regulatory loops between Akt-GSK-3-Rictor-Akt that serves to control this critical cascade important in cellular growth and malignant transformation. So we can begin to see how mutations in pathways associated with cellular proliferation can have effects on EMT and developmental pathways. Some of the normal activities of these pathways are "normally coordinated or fine-tuned" by GSK-3. However, in cancer these activities normally controlled by GSK-3 may disappear and have deleterious consequences.

miRs and epigenetic events have been shown to be important in the regulation of various signaling pathways. Thus these pathways can be fine-tuned by many different genetic and epigenetic mechanisms to control their activity. Abnormal activity of these pathways can contribute to cancer as well as other developmental disorders and aging.

Conflicts of interest

The authors declare that they have no conflicts of interest with publication of this manuscript.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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