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Epigenetic Pathways and Glioblastoma Treatment: Insights From Signaling Cascades

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ABSTRACT

There is an urgent need to identify novel therapies for glioblastoma (GBM) as most therapies are ineffective. A first step in this process is to identify and validate targets for therapeutic intervention. Epigenetic modulators have emerged as attractive drug targets in several cancers including GBM. These epigenetic regulators affect gene expression without changing the DNA sequence. Recent studies suggest that epigenetic regulators interact with drivers of GBM cell and stem-like cell proliferation. These drivers include components of the Notch, Hedgehog, and Wingless (WNT) pathways. We highlight recent studies connecting epigenetic and signaling pathways in GBM. We also review systems and big data approaches for identifying patient specific therapies in GBM. Collectively, these studies will identify drug combinations that may be effective in GBM and other cancers. J. Cell. Biochem. 116: 351–363, 2015.

KEY WORDS: EPIGENETICS; GLIOBLASTOMA; SIGNALING PATHWAYS; HEDGEHOG; NOTCH; WINGLESS

Glioblastoma (GBM) is the most common malignant brain tumor. Although novel GBM therapies are being tested, tumor recurrence after the current standard of care is nearly universal. Thus, there is an urgent need to identify novel targets for therapy in GBM. Epigenetic modulators have recently emerged as new drug targets in multiple cancers including GBM [Clarke et al., 2013]. Epigenetic enzymes modify histone and DNA, thus changing the transcriptional rates of multiple oncogenes and tumor suppressor proteins. One classical means of identifying therapeutic targets is to utilize sequencing information to pinpoint mutations identified in a specific tumor. Indeed, mutations in histone modifying enzymes have been detected in some GBM tumors. However, there is limited efficacy in choosing targets based on sequencing data alone as only a relatively small subset of tumors contain mutations in particular epigenetic modulators.

We reasoned that a possible means of identifying epigenetic targets in GBM is to concentrate on those regulators that affect expression of known cancer promoting pathways. For instance, the Notch, Wingless (WNT), and Sonic hedgehog (SHH) pathways have been implicated in GBM cell and stem-like cell proliferation. Thus, any epigenetic regulator that may positively regulate these pathways may be a particularly attractive therapeutic target in GBM. In this review we concentrate on the intersection of epigenetics and signaling pathways in GBM. We first discuss what is known regarding the role of epigenetic modulators in controlling Hedgehog, WNT, and Notch signaling in GBM. We then discuss methylation and microRNA networks that intersect with these signaling pathways, since both methyltransferases and microRNAs are emerging as interesting druggable targets in multiple cancers. Subsequently, we highlight our recent studies suggesting that patient specific

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assessment of epigenetic and signaling pathway dysregulation in GBM results in targeted therapies that increase patient survival and reduce tumor recurrence. Finally, we discuss our current efforts to use new Big Data resources to identify targets in GBM. We have used one such resource, the Library of Integrated Network-based Cellular Signatures (LINCS), to identify kinases implicated in GBM and that are part of epigenetic and signaling pathways. Collectively, our studies are aimed at utilizing these datasets to design effective combination therapies to combat GBM progression.

EPIGENETIC REGULATION OF HEDGEHOG SIGNALING IN GBM

Hedgehog (Hh) signaling is strongly implicated in many cancers, including GBM (Fig. 1). This signaling pathway is evolutionarily conserved among multicellular organisms and is essential for directing embryonic patterning by spatially and temporally controlling cell differentiation and proliferation. Aberrations in Hh signaling can result in developmental defects such as holoprosencephaly [Hayhurst and McConnell, 2003] when output is absent or decreased, or cancer predisposition, such as in Gorlin syndrome [Gorlin 1987; Caro and Low, 2010], when the pathway is unregulated or increased.

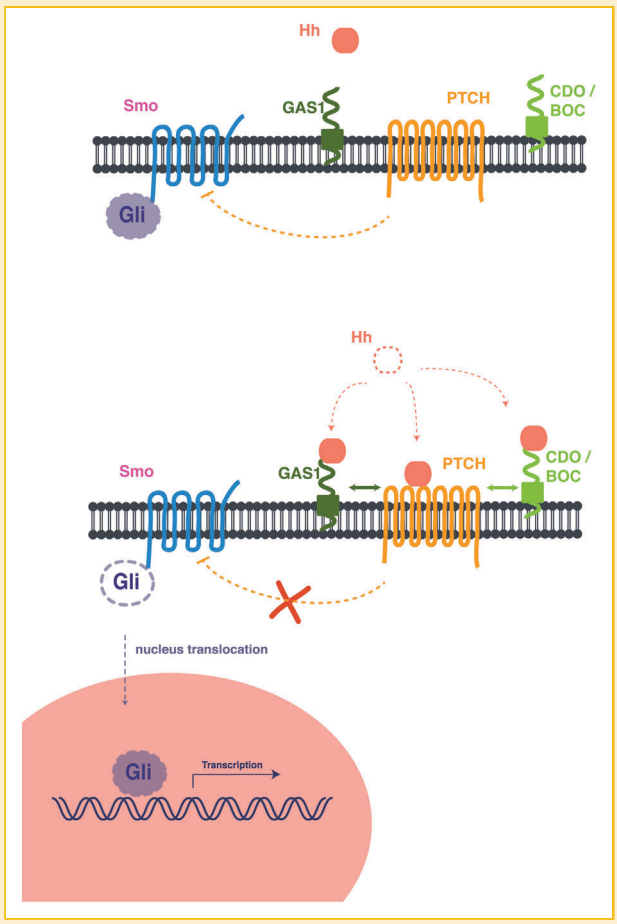

Fig. 1. Schematic showing SHH pathway interactions in the cell. Patched 1 (PTCH) inhibits smoothened from allowing GLI1 to translocate to the nucleus promoting the transcription of oncogenes that leads to cell proliferation. When Hh ligand binds to PTCH, disinhibition of SMO occurs allowing GLI1 to translocate to the nucleus.

Hh family proteins, which in humans include sonic hedgehog (SHH), Indian hedgehog (IHH) and desert hedgehog (DHH), are small, secreted, lipid-modified proteins that act as morphogens to direct the patterning of embryonic tissues in a concentration-dependent manner [Echelard et al., 1993; Ingham and McMahon, 2001]. The Hh proteins are ligands for the multipass cell-surface receptor, patched 1 (PTCH1), and its redundant paralog, patched 2 (PTCH2) (referred to here collectively as PTCH). PTCH binding to Hh ligands is promoted by the coreceptors CDO (cell adhesion molecule down-regulated by oncogenes), BOC (brother of CDO) and GAS1 (growth arrest-specific gene 1) [Allen et al., 2011; Robbins et al., 2012]. When not bound by an Hh ligand, PTCH inhibits the G-protein-coupled receptor, smoothened (SMO). However, when a Hh ligand binds PTCH, it no longer inhibits SMO, and the Hh signaling pathway is activated.

Importantly, primary cilia play a central role in this pathway in mammalian cells [Corbit et al., 2005]. Upon Hh binding to PTCH, SMO becomes enriched at the primary cilium membrane. This leads to the translocation of the GLI2 and GLI3 zinc finger transcription factors to the tip of the cilium, in a manner dependent on kinesin-related proteins [Liem et al., 2009; Humke et al., 2010]. The GLI2 and GLI3 proteins contain both transcriptional activation and repression domains, although GLI2 primarily acts as an activator and GLI3 as a repressor [von Mering and Basler, 1999; Aza-Blanc et al., 2000]. In the absence of Hh signaling, a highly regulated proteolytic process leads to specific cleavage of the activation domains, converting the GLI proteins to transcriptional repressors of Hh target genes. Hh pathway activation and GLI translocation blocks this proteolysis, preserving the full-length GLI proteins, which then translocate to the nucleus and activate Hh targets [Robbins et al., 2012]. These include an additional GLI family member, GLI1, which lacks a transcriptional repression domain and thus acts solely as an activator [Dai et al., 1999]. The GLI proteins act together to directly activate transcription of additional Hh target genes. Many of these genes are involved in cell proliferation, such as *MYCN* and *CCND1*.

When inappropriately activated, Hh signaling can lead to tumor formation. Germline mutations in *PTCH1* are the most frequent cause of Gorlin syndrome, an inherited disorder resulting from constitutive activation of Hedgehog signaling, and characterized by morphological abnormalities and the development of numerous basal cell carcinomas (BCCs) in adolescence, demonstrating the devastating effects of deregulations in this signaling pathway [Gorlin, 1987; Caro and Low, 2010]. Spontaneous mutations in Hh pathway components, including PTCH1, SMO and SUFU, are also commonly found in BCC, as well as in medulloblastoma, a form of malignant brain tumor commonly found in children [Pastorino et al., 2009; Kool et al., 2014].

Importantly, Hh signaling in cancer can be ligand-dependent. Among the Hh ligands, SHH has frequently been found to be upregulated in glioma, either in the tumor itself or in surrounding parenchymal cells [Bar et al., 2007; Ehtesham et al., 2007]. Further, consistent with its role in neural stem cell proliferation during

normal brain development [Palma and Ruiz i Altaba, 2004], the Hh pathway has been shown to be essential for the development and progression of glioma through the maintenance of cancer stem cells [Bar et al., 2007; Zbinden et al., 2010]. Consequently, this signaling pathway is an attractive target for GBM therapy.

It has recently been proposed that epigenetic modulators may play a role in promoting cancer via dysregulating the Hh pathway [Malatesta et al., 2013]. The main focus of research efforts has been on epigenetic reader proteins, the BET bromodomains and the histone acetyltransferase PCAF, which directly interact with this signaling pathway to coordinate cell proliferation and direct tumor initiation and progression. The implications of these epigenetic modulators will be described in the context of hedgehog signaling to encourage interest in epigenetic cancer therapies.

BET BROMODOMAINS

Bromodomains recognize and bind to ε-*N*-lysine acetylation motifs on open chromatin, such as those found on K27 residues of H3 histone *N*-terminal tails [Filippakopoulos et al., 2010]. They interact with the positive transcription elongation factor (P-TEFb) and phosphorylate Ser2 of RNA polymerase II (PolII), facilitating gene transcription at enhancer sites across the genome (Fig. 2). BRD-containing complexes often localize to promoter regions of oncogenes such as MYC and their inhibition has led to a decrease in cell proliferation among many cancers including GBM. We have shown that BET inhibitors arrest cells at the G1/S transition [Pastori et al., 2014]. Recently, we and others have shown that bromodomain-containing protein 4 (BRD4) is a critical regulator of GLI1 and GLI2 transcription through direct occupancy of GLI1 and GLI2 promoters [Robbins et al., 2012], [Jun et al., in revision; Allen et al., in preparation]. Therefore, transcriptional activation at cancer-specific GLI promoter-binding sites is markedly inhibited by the BET inhibitors I-BET151 and JQ1. Both I-BET151 and JQ1 inhibit multiple BET proteins and act as acetylated histone mimics, occupying the binding site of the bromodomain-containing protein and inhibiting its ability to modify chromatin and activate transcription [Delmore et al., 2011; Cheng et al. 2013]. It may seem counterintuitive to target BET proteins to modulate a cancer-signaling pathway such as Hedgehog when the pathway itself can be targeted pharmacologically; however, a hallmark of cancer signaling pathways is their ability to develop resistance to a single inhibitor. Synthetic analogs of cyclopamine have allowed the development of potent inhibitors of SMO, which have shown clinical efficacy against medulloblastoma and glioblastoma [Tang et al., 2014]. Nevertheless, resistance is almost always encountered. Therefore, using BET inhibitors in combination therapy with Hedgehog inhibitors may lead to higher rates of patient survival and less tumor recurrence.

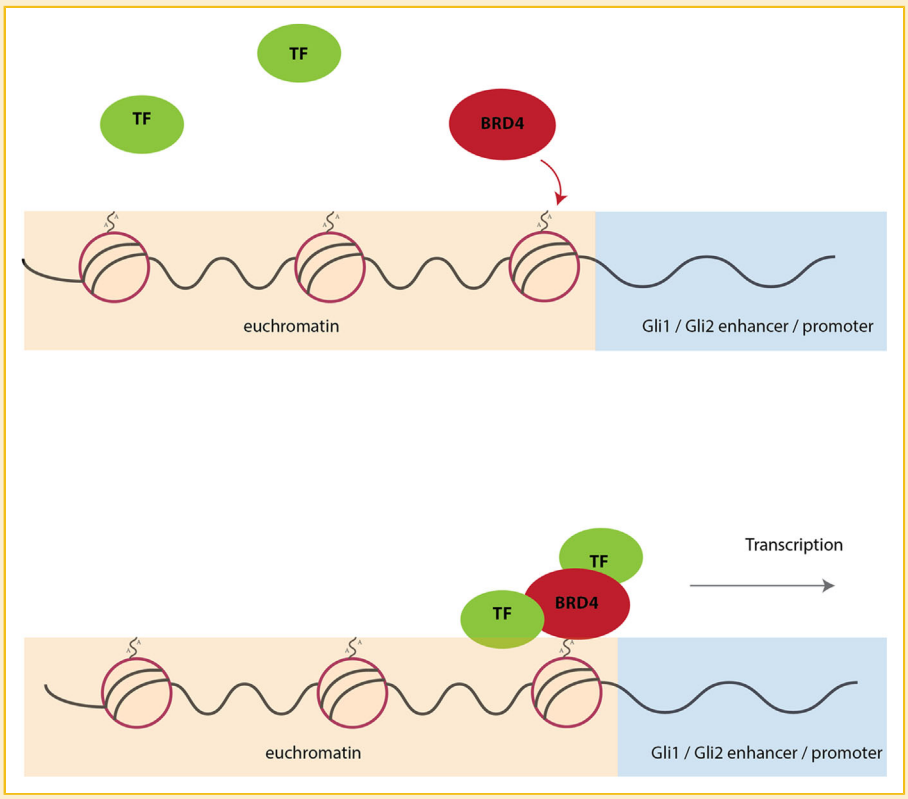

Fig. 2. BRD4 interaction with SHH pathway. Bromodomain containing protein 4 (BRD4), one of the best studied BET proteins, is a critical regulator of GLI1 and GLI2 transcription through direct occupancy of GLI1 and GLI2 promoters. TF (Transcription Factor).

PCAF/KAT2B

Another epigenetic modulator important for Hedgehog signaling in GBM is p300/CBP-associated factor, also known as K(lysine) acetyltransferase 2B (PCAF/KAT2B) [Malatesta et al., 2013]. *PCAF* is a human gene and transcriptional coactivator known mostly for its acetylation of the carboxy-terminal end of p53, leading to its activation and tumor suppressive function (Fig. 3). It contains acetyltransferase and E3 ubiquitin ligase domains, as well as a bromodomain, for interaction with other proteins [Ghizzoni et al., 2010]. Previous studies have shown that the protein encoded by the *PCAF* gene associates with p300 and CBP, which are large nuclear proteins that bind sequence specific factors involved in cell growth and/or differentiation, such as c-jun and the adenoviral oncoprotein E1A [Schiltz et al., 1999]. PCAF has been shown to compete with E1A for binding sites in p300/CBP, indicating its direct interaction with oncoproteins. However, PCAF is known mostly for its histone acetyl transferase activity with core histones, indicating its direct role in transcriptional regulation. The acetyltransferase activity and cellular location of PCAF are regulated through acetylation of PCAF itself, either through autoacetylation or acetylation by p300. When acetylated, PCAF migrates to the nucleus where its acetyltrasferase activity is enhanced, and induction of transcription occurs. HDAC3 negatively regulates PCAF by deacetylating it, leading to its localization in the cytoplasm. PCAF has a number of targets due to its acetyltransferase activity, although recently it has been shown that PCAF is a positive cofactor in the Hh–Gli signaling pathway [Malatesta et al., 2013]. Experiments have shown that PCAF depletion impairs Hh activity and reduces expression of Hh target genes [Malatesta et al., 2013]. It was also shown that PCAF down regulation in medulloblastoma and glioblastoma cells by siRNA knock down decreased cell proliferation and increased apoptosis. Further experiments probed whether PCAF interacts with GLI1 and if PCAF or GLI1 loss reduced levels of H3K9 acetylation on Hh target gene promoters. Their findings suggested that PCAF binds to GLI1, indicating that both proteins are required for increased H3K9Ac levels on Hh target gene promoters in response to Hh–Gli activation. This evidence showed that the association of PCAF with GLI-regulated promoters is dependent on GLI1, triggering the authors to conclude that activation of the Hh–Gli signaling pathway leads to GLI1-dependent recruitment of PCAF, which in turn leads to H3K9acetylation of Hh target gene promoters and their activation. With this conclusion in place, therapeutic strategies can be designed to inhibit PCAF interaction with GLI1 and/or its acetyltransferase activity. Treatment of Hh–Gli-dependent tumors with histone acetyl

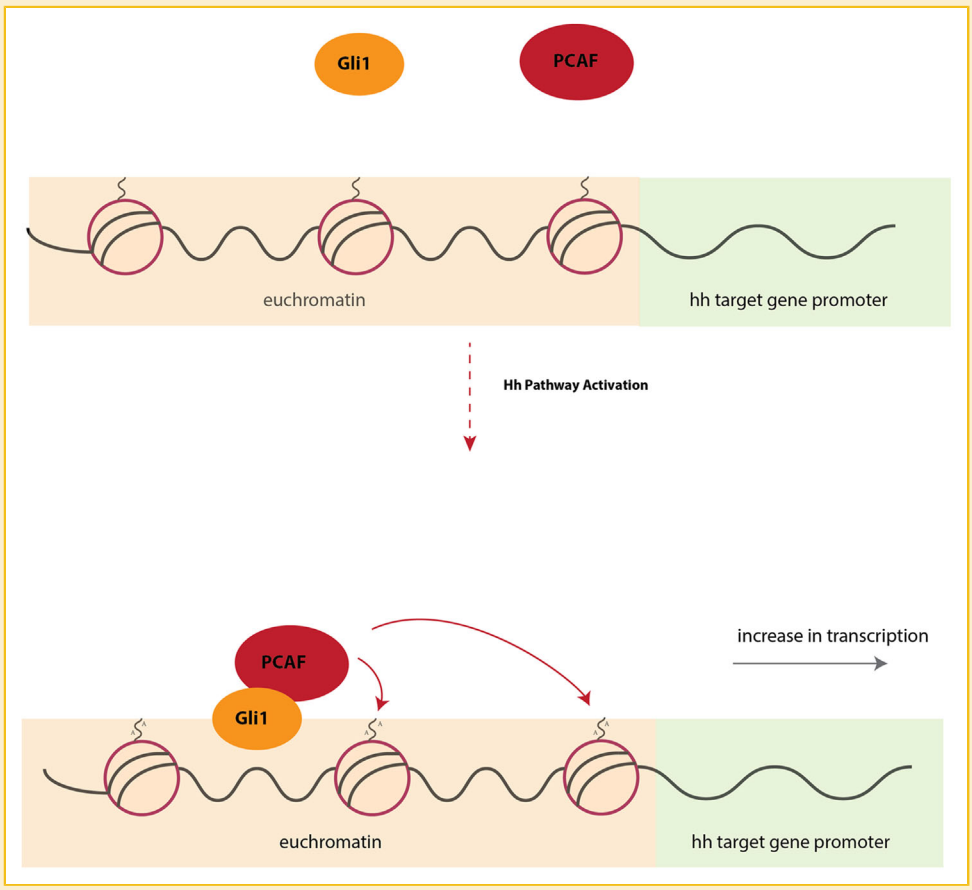

Fig. 3. Model of PCAF function. Upon Hh pathway activation, PCAF is recruited to Hh target gene promoters through an interaction with GLI1. The association between PCAF and GLI1 leads to the H3K9 Acetylation of the Hh target gene promoters, resulting in an increase of the Hh pathway.

transferase (HAT) inhibitors known to inhibit the activities of PCAF, such as Anacardic Acid, led to reduced expression of PTCH, a Gli-target gene, and a clear increase in apoptosis in DAOY-medulloblastoma cells. As described earlier, combination therapies are more effective at combating tumor resistance and evolution, therefore PCAF inhibitors may be combined with SMO inhibitors, anti-Gli agents, and anti-Shh antibodies to completely deplete cell proliferation capacity, induce apoptosis, and eradicate tumorigenesis.

EPIGENETIC REGULATION OF WNT PATHWAY IN GBM

The WNT signaling pathway plays a crucial role in cell fate determination and dysregulations in this pathway are closely associated with oncogenesis, often through the modification of key components that lead to loss of β-catenin regulation [Clevers 2006; Klaus and Birchmeier, 2008]. Secreted glycoproteins from the WNT pathway bind to frizzled (Fz), a transmembrane receptor that activates both canonical and noncanonical WNT pathways [Widelitz 2005]. Activation of the canonical WNT pathway leads to inhibition of glycogen synthetase-3 (GSK-3) through the stabilization and accumulation of cytoplasmic β-catenin (Fig. 4). GSK-3 is a critical component of the activated deconstruction complex that functions to phosphorylate β-catenin leading to its rapid degradation. β-catenin is translocated to the nucleus when cytoplasmic levels increase, where it binds to T cell factor/lymphocyte enhancer factor (TCF/LEF) transcription factors. Activation of TCF/LEF allows for the transcription of multiple Wnt target genes which promote cell proliferation and differentiation, including c-myc, c-jun, and cyclin D1. Inhibition of WNT signaling is observed when extracellular secreted antagonists block binding of Wnt glycoproteins to Fz. Two mechanisms have been discovered to complete this objective. Wnt inhibitory factor-1 (WIF1), the secreted frizzled-related proteins, and Cerberus block WNT signaling by directly binding Wnt glycoproteins, disrupting their ability to bind the Fz receptor. Dickkopf-1 (DKK1) and other members of the DKK family inhibit WNT signaling by sequestering low-density lipoprotein (LDL) receptor-related protein 5/6 (LRP5/6). LRP5/6 is a coreceptor required by the Fz receptor to activate the canonical signaling pathway. Many studies reveal that WNT pathway inhibitors function as tumor suppressor genes, due to their inhibition of oncogenic WNT pathway signaling [Chen et al., 2008; Bouteille et al., 2009] Noncanonical WNT pathways function independent of β-catenin. Unfortunately, the role of noncanonical Wnt signaling in cancer is not well defined.

Large-scale whole-genome approaches have been used to identify epigenetically silenced genes that may function as tumor suppressors in GBM [Foltz et al., 2006]. These studies identified three genes that are known inhibitors of canonical WNT signaling, *DKK1*, *SFRP1*, and *WIF1* [Clevers 2006; Klaus and Birchmeier, 2008]. The authors show that these tumor suppressors are epigenetically silenced by DNA methylation and histone modification in the promoter regions of DKK1, SFRP1, and WIF1 in relation to transcriptional repression in GBM. Tumor samples show decreased expression of these proteins as compared to nontumor brain tissue. They also show that treatment of T98 cells with the HDAC inhibitor TSA restores expression of all three genes and decreases cell proliferation and sensitizes cells to apoptosis. However, when treated with the demethylating agent 5-azacytidine (5-Aza), only DKK1 expression is restored, indicating the presence of promoter hypermethylation. However, other studies showed that SFRP1–5 is hypermethylated in four GBM cell lines (U87, U138, LN18, and A172), and when treated with 5-Aza, demethylation was observed. Demethylation of the SFRP promoter regions and increased expression of corresponding proteins should therefore lead to inhibition of canonical Wnt signaling, attenuating tumorigenesis [Schiefer et al., 2014].

EPIGENETIC REGULATION OF THE NOTCH SIGNALING PATHWAY IN GLIOBLASTOMA

The Notch-signaling pathway is an evolutionarily conserved cell signaling pathway, which functions in the development of organisms from multiple lineages. It regulates cell fate determination, and when dysregulated, becomes oncogeneic. Abnormal Notch signaling inhibits apoptosis and promotes cell survival, and is found in many cancers such as breast, cervix, colon, pancreas, skin, and brain. Inhibition of constitutively active Notch signaling leads to growth arrest and differentiation of cancer cells, providing a therapeutic means for targeting cancer.

Notch signaling in mammals is triggered by direct interaction of receptors with ligands expressed on neighboring cells. There are four different Notch receptors and five ligands, which are Notch1, Notch 2, Notch 3, Notch 4, and Delta-like 1, Delta-like 3, Delta-like 4, Jagged 1, and Jagged 2, respectively. When Notch intracellular domain (NICD) is released from the membrane after cleavage by gamma-secretase, it translocates into the nucleus and associates with transcription factors, which leads to the expression of Notch target genes. Dysregulated Notch-signaling pathway causes a variety of disorders such as glioblastoma, where maintenance of GBM stem cells has been shown. Elevated Notch1 expression in glioblastoma has also been reported and is possibly influenced by hypoxia in the tumor microenvironment [Fan et al., 2004]. Notch targeted therapies such as GSI, depleted stem like cancer cell proliferation and increased apoptosis in GBM [Chen et al., 2010]. Glioma cell lines also showed a decrease in cell growth, enhanced cell cycle arrest, and apoptosis by knockdown of Notch1 gene [Zhao et al., 2010]. Recent studies have shown that epigenetic events play a pivotal role in the development and progression of cancer, with DNA methylation being one of the most common events. Methylation occurs at cytosine nucleotides usually adjacent to a guanine nucleotide (CpG islands) by DNA methyltransferase (DNMT) enzyme, forming 5-methylcytosine in the upstream region of promoter sequences. Both Notch hypermethyulation and hypomethylation have been reported in some human cancers and epigenetic silencing of DLL1, HEY1, DTX1, HDAC1, Notch2, and Jag1 has been detected in a variety of cancers but has not been shown specifically in glioblastoma [Aktas et al., 2010]. However, due to a lack of sufficient evidence relating DNA methylation to its effect on Notch signaling and

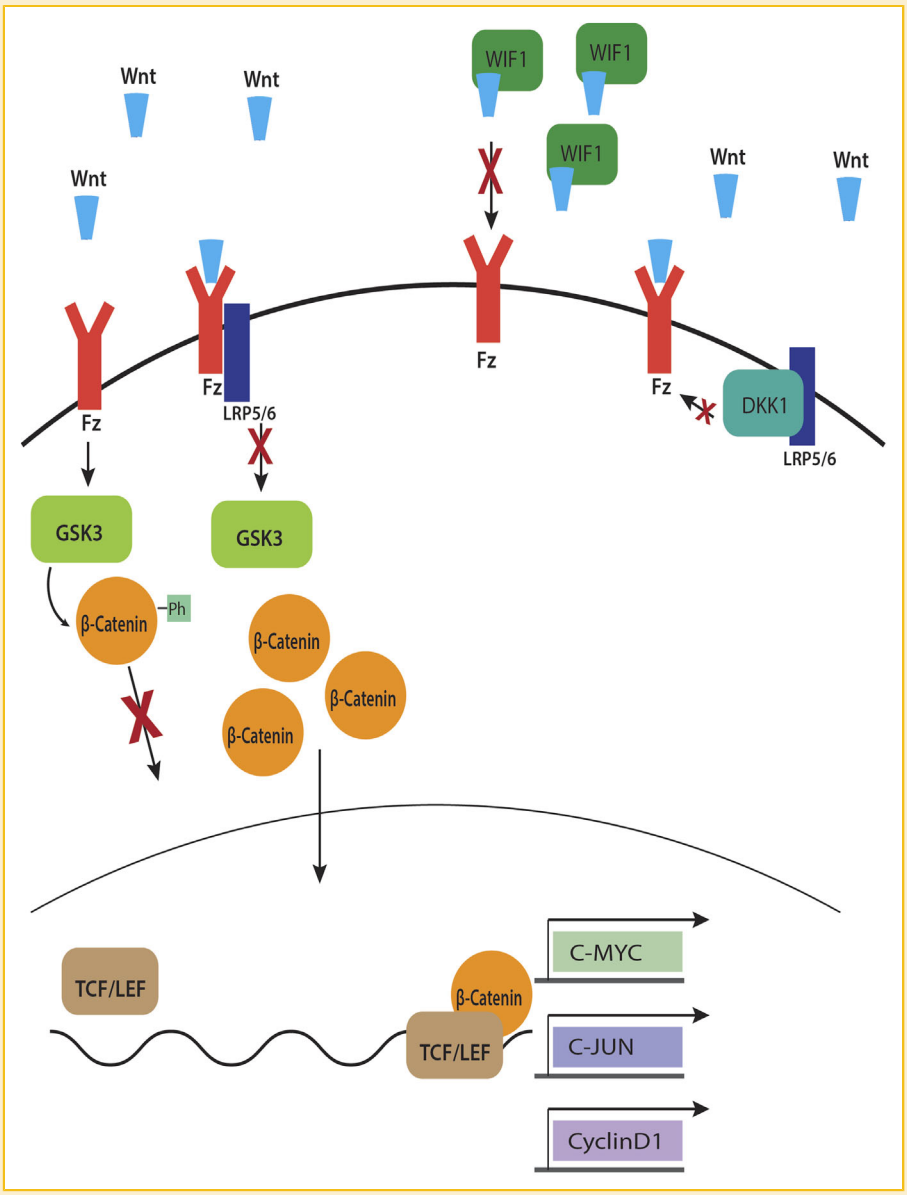

Fig. 4. Canonical WNT pathway and cancer. In the canonical WNT pathway, WNT ligands bind to receptor Fz on target cells. Fz with co-receptor LRP5/6 blocks GSK3 phosphorylation of β-catenin, allowing nuclear entry. β-catenin binding to TCF/LEF transcription factors and leads to the expression of Wnt downstream proteins c-myc, c-jun, and cyclin D1. Wnt inhibitors prevent expression of these genes in two ways. WIF1 directly binds WNT and prevents receptor activation. DKK1 sequesters LRP5/6, blocking its interaction with Fz and preventing GSK3 inhibition.

gene expression, presently there are no biomarkers developed using methylated Notch gene promoter for cancer diagnosis.

ABERRANT METHYLATION AND GBM

As highlighted above, alterations in WNT inhibitor methylation status affect tumor formation. By examining methylation profiles of DNA and of chromatin, one can distinguish GBM tumors from normal tissue and other brain tumor subtypes. Changes to promoter and histone methylation status impact the major proto-oncogenic signaling pathways implicated in GBM including WNT, Notch and to some degree Hh, as well as the inhibitor pathway, BMP.

Analysis with CpG Island Methylator Phenotype (CIMP) reveals global hypermethylation in GBM tumors versus normal brain tissue, and the degree of methylation can impact patient survival rates [Piperi et al., 2010; Shinawi et al., 2013]. Brain tumor subtypes possess distinct global CpG island methylation signatures that can be used to distinguish GBM tumors [Campos et al., 2012; Shinawi et al., 2013]. For instance, methylation of the promoter for DNA repair gene O^6-methylguanine-DNA-methyltransferase (MGMT) is increased in GBM tumors specifically and can be used as a GBM

marker [El Hindy et al., 2013; Shinawi et al., 2013]. MGMT methylation is correlated with tumor invasion severity and can be a prognostic tool for chemotherapy efficacy [Piperi et al., 2010; Wick et al., 2014].

Methylation induced gene silencing for several Wnt inhibitors is apparent in GBM as well. WIF1 mRNA expression is reduced in a subset of GBM tumors and in many GBM cell lines, and this reduction appears to derive from methylation at its promoter [Lambiv et al., 2011]. SFRP1 promoter hypermethylation occurs in two-thirds of primary GBM tumors, and like MGMT, its expression level is correlated with tumor prognosis [Shahi et al., 2011, Shinawi et al., 2013; Delic et al., 2014]. miR-328 appears to partially regulate the expression of SFRP1. In secondary GBM tumors, miR-328 overexpression occurs, and while this rise does not impact tumor proliferation, it does appear to increase the amount of tumor invading cells [Delic et al., 2014]. Furthermore, promoters for SFRP1–5 are hypermethylated in several GBM cell lines. Usage of methyltransferase inhibitors on these cell lines not only increased expression levels of SFRP1–5, but also resulted in reduced cell viability for all GBM lines tested [Schiefer et al., 2014]. This evidence suggests that aberrant methylation of WNT inhibitory gene promoters greatly impacts tumor formation and virulence.

There is limited evidence on promoter methylation for Hh signaling genes with GBM. Partial to complete promoter methylation for SMO occurs in half of GBM tumors examined [Shahi et al., 2008], but the significance of this methylation remains to be elucidated.

While GBM cells show global and gene promoter specific increases in methylation, characteristic histone hypomethylation also occurs in these tumor cells. Polycomb repressive complex 2 (PRC2) facilitates chromatin remodeling through its catalytic component, the histone lysine *N*-methyltransferase, enhancer of zest homolog 2 (EZH2) [Lewis et al., 2013; Natsume et al., 2013; Venneti et al., 2013]. EZH2 is responsible for histone3 lysine 27 trimethylation (H3L27me3), resulting in developmental gene silencing [Venneti et al., 2013], Figure 5. Most GBM tumors have a mutation at this lysine residue (H3L27M) that prevents EZH2 mediated gene silencing and leads to EZH2 overabundance [Venneti et al., 2013]. Additional mutations to other H3 putative lysine residue

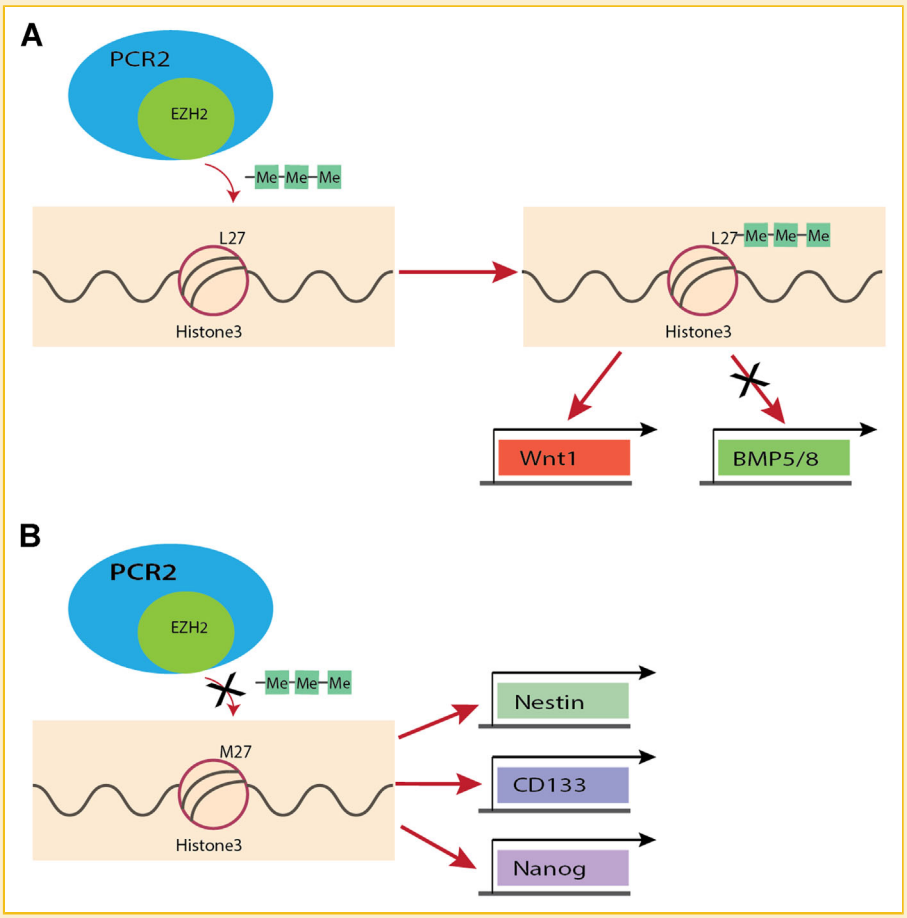

Fig. 5. Perturbations in H3K27 methylation promote GBM proliferation. (A) PCR2 catalyst EZH2 mediates methylation of lysine 27 on histone 3. When H3 is located near promoter regions L27me3 can regulate gene expression, by up-regulating WNT and downregulating BMP. These changes to gene expression reduce proliferation and promote differentiation. (B) Many GBM tumors have a K27M mutation, thereby preventing EZH2 mediated methylation. As a result, cells express high levels of stem cell genes Nestin, Nanog, and CD133, thereby maintaining proliferation and preventing differentiation.

methylation sites do not appear to be as detrimental [Bender et al., 2013; Lewis et al., 2013; Natsume et al., 2013]. H3L27M appears restricted to GBM tumors and does not occur in surrounding healthy tissue [Venneti and Thompson, 2013].

Low levels of H3L27me3 in GBM tumors promote proliferation while inhibiting cell differentiation. Hypomethylation due to H3L27M increases expression of the stem cell genes, nestin, nanog, and CD133. Downregulation of EZH2 in non-tumor cells yields consistent expression data for these genes. Many sites of H3L27me3 are located at or near promoter regions for these and other genes. Loss of H3L27me3 at the promoter for Wnt1 reduces its expression and promotes GBM growth. Similar tumor growth occurs in WNT1 downregulation experiments [Natsume et al., 2013]. Paradoxically, hypermethylation of Histone3 Lysine 9 near WNT antagonists, DKK1, SFRP1, and WIF1 increases GBM proliferation as well [Foltz et al., 2010; Shinawi et al., 2013]. As stated above, SFRP1 and WIF1 promoters are hypermethylated in GBM tumors as well, so methylation impacts expression of these inhibitors directly and indirectly. Tumor proliferation is most pronounced with concurrent DKK1 reduction, and it is hypothesized that DKK1 inhibits tumor growth by suppressing the non-canonical Wnt pathway [Foltz et al., 2010]. Thus, it is possible that multiple WNT signaling pathways are differentially active in GBM tumor cells.

While loss of PRC2 mediated chromatin methylation sites contributes to GBM proliferation, increased methylation in others can also produce tumor growth. As discussed above, MGMT methylation is higher in GBM cells. Concomitant to MGMT silencing, Notch and its downstream pathway gene expression levels increase. In GBM, Notch is implicated in tumor proliferation and angiogenesis [El Hindy et al., 2013]. CIMP profiling of glioblastoma cell cultures reveal BMP8A/B and BMP5 hypermethylation [Natsume et al., 2013; Shinawi et al, 2013]. BMP, WNT, and Notch signaling pathways establish expression profiles for different cell types during development. In the case of GBM epigenetic modulation of chromatin methylation appears to cause tumor proliferation via aberrant silencing of BMP expression in conjunction with increasing WNT and notch expression.

MicroRNAs AND GBM

miRNAs are small non-coding RNAs that can regulate gene expression by either binding to a complimentary mRNA and inhibiting its translation or promoting its destabilization and degradation. By contributing to the regulation of various signaling pathways, miRNAs have been shown to affect the proliferation, differentiation and survival of cancer cells. More specifically, many studies have highlighted the importance of miRNAs in Glioblastoma development and their potential use as biomarkers and therapeutic targets. Below are some of the most important miRNAs that directly modulate signaling pathways.

Mir-21

mir-21 is upregulated in many cancers, including gliomas. Mir-21 can affect the proliferative capacity of glioma cells by modulating the expression of EGFR through the direct targeting of the 3′-UTRs of VHL and PPARα. This repression of VHL and PPARα leads to the activation of β-catenin and AP-1 and thus to the increase in EGFR. Moreover, inhibition of mir-21 can have an advantageous effect on survival when combined with nimotuzumab, the EGFR humanized monoclonal antibody.

Mir-34a

The Notch pathway is also regulated by miRNAs. Mir-34a is a transcriptional target of p53 and belongs to the mir34 family, which has been extensively associated with anti-proliferative and anti-apoptotic functions. Mir-34a is down-regulated in gliomas and potentially plays an important role in regulating the Notch and c-Met pathways. More specifically mir-34a was shown to inhibit Notch-1, Notch-2, and c-Met and therefore act as a tumor suppressor. Thus, mir-34a may be an ideal therapeutic target [Li et al., 2009].

Mir-34c-34p

Another member of the mir-34 family has also been shown to interact with the Notch pathway. Mir-34c-3p is the first of the two isoforms of mir-34c miRNA and has been established as a tumor suppressor in numerous cancers. Unlike mir-34a, which induces G0/G1 cell cycle arrest, mir34c-3p causes S phase arrest, indicating that members of the same family can be implicated in different mechanisms. Finally, mir-34c-3p targets only Notch-2 while mir-34a has a wider variety of targets [Wu et al., 2013].

Mir302–367

Glioma-initiating cells (GICs) are important targets in GBM treatment as these pluripotent cells may be the main contributors to tumor resistance observed after Temozolomide treatment. The miRNA cluster mir302–367 may be a good candidate for disrupting GIC's stemness and tumorigenicity through the indirect disruption of the SHH pathway. This miRNA cluster drastically inhibits the CXRC4 receptor, which then leads to the repression of the SHH signaling pathway and to the suppression of GIC stemness [Fareh et al., 2012].

Mir-181-d

Finally, the WNT pathway can also be regulated by miRNAs. One reported means of miRNA dependent control of the WNT pathway is the direct targeting of *CTNNB1* and *CREBBP*, which are the genes that encode GPB protein (part of the WNT transcriptional complex, together with β-catenin, TCF4 and Lef-1).

IDENTIFYING PATIENT SPECIFIC EPIGENETIC AND SIGNALING CASCADES TO TARGET THERAPEUTICALLY

We have attempted to highlight some of the epigenetic enzymes and microRNAs that may be interesting therapeutic targets in GBM. However, it is rare that a large number of GBM tumors have dysregulation of any one modulator. This is partly due to the heterogenous nature of GBM. GBM is often characterized by vast heterogeneity at the genomic level. Many approaches have been utilized in order to elucidate this heterogeneity, with The Cancer

Genome Atlas (TCGA) as one of the most successful. By using gene expression data from more than 200 Glioblastoma patients, TCGA managed to segregate the patients into four subtypes (proneural, neural, classical, and mesenchymal) showing that the efficacy of aggressive treatment differs significantly depending on the subtype [Verhaak et al., 2010]. This difference makes oncogenomics all the more relevant in a clinical setting. Patient specific therapies will not only improve the effectiveness of the therapeutic scheme by inhibiting specific signaling pathway deregulations but will also help alleviate the drug resistance obstacle. By aiming at multiple targets at once, treatment will limit the tumor's ability to overcome the changes in their microenvironment and therefore limit their proliferative capacity.

Apart from gene expression abnormalities in signaling pathways, TCGA also highlighted the role of epigenetics in glioblastoma with the most well known being the methylation status of MGMT (Cancer Genome Atlas Research, 2008; Fouse et al., 2014]. As an increasing number of epigenetic enzymes are starting to be linked with cancer signaling pathways (NOTCH, WNT, and SHH) [Foltz et al., 2010; Tang et al., 2014], more global approaches should be used in order to uncover the full spectrum of these interactions. Furthermore, the dual inhibition of these targets may prove to be advantageous in the therapy of glioblastoma. The investigation of epigenetic-signaling pathway interactions is therefore of great importance. By utilizing the large number of publicly available interaction and expression databases, scientists can investigate and identify critical epigenetic-signaling relationships. Most of the software tools analyzed below have the ability to uncover potential interactions between genes or proteins by utilizing preexisting databases (see Fig. 6). In the context of personalized medicine, lists containing differentially expressed/mutated epigenetic and signaling pathway genes directly derived from a patient's tumor can be used as input in the following software.

One of the most important modeling environments for elucidating interactions is Cytoscape [Demchak et al., 2014]. Cytoscape is an open-source network visualization and analysis software platform with the ability to modify its output depending on the plug-in that is being used. Most network analysis tools and databases have also a corresponding Cytoscape plug-in making it easier to integrate information from different databases into the same network. Data processed through the Cytoscape plug-ins may include pathway data, different types of physical interaction data, experimental data and/or post translation data.

Some of the most popular and most useful plug-ins include:

Pathway Commons: A tool for providing pathway and interaction information on a given gene list using the following databases: BioGRID, Cancer Cell Map, HPRD, HumanCyc, IMID, IntAct, MINT, NCI/Nature PID, Reactome, All Intergated.

GeneMania: A gene function prediction tool utilizing information from GEO, BioGRID, Interpro, Pfam, Ensembl, MGI, InParanoid8, Pathway Commons.

Genoscape: A bioinformatics tool that retrieves gene expression data from GenoScript and enriches them with pathway data from KEGG pathways.

MiMI: Retrieves interactions from the MiMI Database, which merges and uses data from well-known protein interaction databases such as BIND, DIP, HPRD, SwissPROT, and IPI.

STRING: A database including direct (physical) and indirect (functional) protein interactions. The curated data that STRING extracts come from the following databases: Biocarta, BioCyc, GO, KEGG, and Reactome.

IDENTIFYING GBM SPECIFIC SIGNALING PATHWAYS USING A NOVEL BIG DATA RESOURCE—THE LIBRARY OF INTEGRATED NETWORK CELLULAR SIGNATURES (LINCS)

We have recently begun utilizing some of the software tools mentioned above to identify gene–gene interactions in GBM samples. We are then coupling this information to perturbation data derived from small molecule treatments of GBM cell lines. The main reason for performing these studies is to identify possible drug combinations. To achieve this goal, we utilize information from the

	Link	Types of information	Databases used
Pathway Commons	www.pathwaycommons.org	Pathway and Interaction Information	BioGRID, Cancer Cell Map, HPRD, HumanCyc, IMID, IntAct, MINT, NCI/Nature PID, Reactome, All Intergated.
GeneMania	www.genemania.org	Prediction of Gene Function	GEO, BioGRID, Interpro, Pfam, Ensembl, MGI, InParanoid8, Pathway Commons.
Genoscape	www.pasteur.fr/recherche/unites/Gim/genoscape/	Gene Expression Data	KEGG Pathways
MiMI	www.mimiplugin.ncibi.org	Interactions from MiMI Database	BIND, DIP, HPRD, SwissPROT, IPI.
STRING	www.string-db.org	direct and indirect protein inderactions	Biocarta, BioCyc, GO, KEGG, and Reactome

Fig. 6. Some common tools for detecting gene–gene or protein–protein interactions.

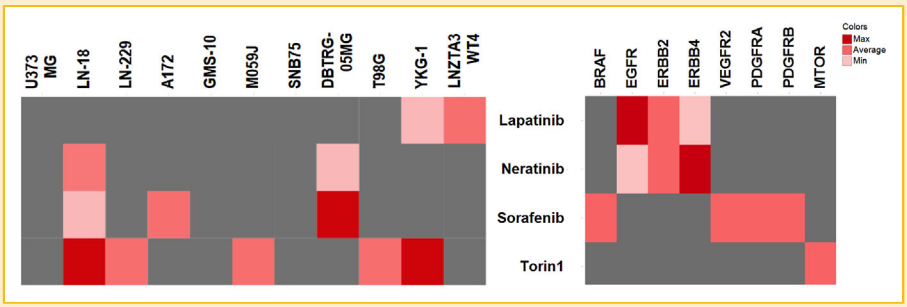

Fig. 7. LINCS data visualization. Shown on the left, cell viability profile of 4 LINCS over-expressed kinase inhibitors in 11 LINCS glioblastoma cell lines, and on the right kinase activity profile for 4 LINCS compounds (shown only KINOMEscan kinases inhibited).

LINCS (Library of Integrated Cell Signatures) project. The LINCS project is a large-scale coordinated effort to build a network-based understanding of cell biology. The goals of the project include generating and cataloging cellular signatures (such as genome-wide transcriptional signatures, biochemical protein binding profiles, cellular phenotypic response profiles, and many others) for a wide range of cell model systems and molecular and genetic perturbations, as well as developing novel informatics and computational tools to integrate, analyze, and make the data readily accessible.

Based on the various LINCS datasets, we identified a few drugs that are active in a set of glioblastoma cell lines (HMS Cell viability assay and are also kinase inhibitors) based on the KINOMEscan assay for a set of kinases over-expressed in TCGA. The set of over-expressed kinases contains EGFR kinase, a well-known cancer target, and also MELK, WEE1, CHEK1, TTK, AURKB, and CDK2. Among the inhibitors of these kinases we identified Sorafenib, Lapatinib, Neratinib, Torin1, BI-2536, HG-6-64-01, and NVP-TAE684; these also exhibit cell viability below 20% for the 11 glioblastoma cell lines (Table S1 in the Supplementary Material). In Figure 7 we show a heat map with the experimental activities of these compounds for cell lines and a subset of 277 kinases that at least one of the four compounds inhibit.

Additionally, we applied Laplacian-corrected naive Bayesian classification models [Schurer and Muskal, 2013] to predict activity of these compounds as well as all LINCS compounds across the modeled kinome.

We identified kinases in the pathways that have been implicated in glioblastoma: Notch, WNT, and Sonic hedgehog pathways. For this purpose, we used the NCI curated pathway information and identified eight pathways of interest (Table S2 in the Supplementary Material) with 31 kinases (Table S3 in the Supplementary Material) involved in at least one of them. The LINCS KINOMEscan dataset shows 42 of the LINCS compounds (Table S4 in the Supplementary Material) being active against these kinases and our computational predictions suggest that a few hundred LINCS compounds could be active across these eight pathways. Furthermore, we identified eight epigenetic targets in these pathways, including CBP, KDM1A, EP300, SETB1, HDAC2, HDAC1, NCOA1, and CHD7. Although these epigenetic targets were not tested in the biochemical LINCS assays, the expression level of *HDAC2* gene was reported in the LINCS L1000 transcriptional assay [Peck et al., 2006] and six of them (CBP, KDM1A, EP300, SETB1, HDAC2, HDAC1) were knocked down in the L1000 genomic perturbation studies (by corresponding shRNAs). Among 31 kinases identified in the eight pathways, the expression levels of 7 kinases were also measured in the L1000 assay (AKT1, CSNK1A1, CSNK1E, MAPK9, PIK3CA, PRKACA, and PRKCD) and all were knocked down (by shRNA) in the L1000 genomic perturbation studies. Although no glioblastoma cell lines were tested in this assay, there is an ongoing effort to expand the assay to many more cell lines as part of a large-scale systems-biology approach. Data used here and additional information can also be obtained via the LINCS Information FramEWork (LIFE) search system, the LINCS website, and the LIFE project website (http://lifekb.org/wp/).

As data generation capabilities continue to increase, several large-scale (Big Data) resources have been developed or are being developed. Examples of such resources that are focused on cellular model systems include the Cancer Cell Line Encyclopedia [Barretina et al., 2012], Genomics of Drug Sensitivity in Cancer [Yang et al., 2013], and LINCS. In order to enable data integration and analysis across different sources, we have developed metadata standards that cover many of the assays, molecular entities, model systems, data types, and screening results generated in the LINCS project and we have obtained rich annotations for LINCS cell lines, small molecules, and proteins [Vempati et al., 2014]. Based on these standards we have recently demonstrated global relationships among transcriptional signatures, kinome-wide affinity profiles, and cell viability profiles to characterize drug action at the systems level [Vidovic et al., 2014].

CONCLUSIONS

Epigenetic modulators have emerged as promising therapeutic targets in multiple cancers including GBM. Mutations and deletions of genomic regions containing epigenetic enzymes and microRNAs have suggested specific therapeutic targets. However, these patient specific alterations are usually rare, thus making the identification of targets difficult. In this review we suggest that another means of choosing epigenetic targets for drug discovery is to concentrate on those modulators, which intersect with cancer promoting pathways. As examples, we have discussed the role that epigenetics plays in the

Notch, Hh, and WNT pathways as these signaling networks are closely linked to GBM cell and stem-like cell expansion. To help identify the connections between signaling and epigenetic pathways we are utilizing Big Data resources such as the LINCS resource. Collectively, these studies will identify drug combinations that will limit tumor recurrence in GBM and other cancers.

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