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Review

In Vivo Models for Defining Molecular Subtypes of the Primitive Neuroectodermal Tumor Genome: Current Challenges and Solutions

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Abstract. *Primitive neuroectodermal tumors (PNET) of the brain include medulloblastoma (MB) and central nervous system primitive neuroectodermal tumor (CNS PNET) subtypes, which share histological features yet differ at the genomic level and in clinical outcome. Delineation of the genetic anomalies between PNET subtypes is a current challenge for establishing effective targeted therapeutic strategies against these aggressive tumors. Current efforts have demonstrated that specific molecular pathways drive a subset of MB and CNS PNET, but the genetic basis for the deadliest forms of these tumors remains poorly understood and anecdotal. This is in part due to an overall lack of biologically relevant in vivo and in vitro model systems capable of direct comparison and identification of the genetic origins among PNET subtypes. Forward genetic, random mutagenesis in mice is an effective phenotype-driven method to model the genetic origins of human disease including cancer. We have applied this method to PNET by developing a single Sleeping Beauty transposon insertional mutagenesis mouse model that recapitulates the morphological similarities and genetic heterogeneity of MB and CNS PNET capable of identifying genetic drivers important for genesis of PNET. Importantly, this model has allowed new PNET phenotypes to be observed and is designed to reveal biologically relevant candidate oncogenes and tumor suppressor genes for MB and CNS PNET molecular subgroups in mice and humans. The ultimate goal of the approach we have taken is to uncover new understanding of the genetic basis for MB and CNS PNET development, how they are distinguished from each other, and offer potential targets for therapeutic testing to improve patient clinical outcome.*

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Pediatric Brain Tumors

Clinical and histological presentation of MB and CNS PNET. Malignant brain tumors are the most frequent solid cancer in children and consist of several different subtypes that pose distinct clinical challenges (1). High-grade malignancies comprise a significant portion of these intracranial lesions including gliomas (15-20%), MB (20%) (2), and CNS PNET (2%) (2). This review focuses on MB and CNS PNET, which are PNET subtypes defined by the World Health Organization (WHO) with useful guidelines for diagnosing their heterogeneous histological characteristics and malignancy grade (3). MB and CNS PNET are WHO grade IV embryonal tumors that occur most frequently in children and share several histopathological features including poorly differentiated small round cells of dense cellularity, high nucleus-to-cytoplasm ratio with frequently observed Homer-Wright rosettes (4). MB and CNS PNETs have also been observed in rare adult patients (5, 6). Fundamental differences include physiological location, with MB always occurring in the cerebellum, while CNS PNET arise in the cerebrum. CNS PNET patients also suffer an overall poorer prognosis (7, 8). Furthermore, MB can be subdivided into five histology subtypes with varying frequencies: classic (~70%), desmoplastic (7%), anaplastic (2-4%), large cell (10-22%), and MB with extensive nodularity (3%) (3, 9). Classic MB is the most common subtype and shares the most histological overlap with CNS PNET. Although rare, CNS PNET also include several histological

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variants including CNS neuroblastoma, ganglioneuroblastoma and 'embryonal tumor with abundant neuropil and true rosettes' (ETANTR) (8, 10-12). The clinical significance of these histological subtypes indicates a more favorable prognosis for non-metastatic MB displaying desmoplastic or nodular features, while anaplastic tumors confer a worse outcome (8, 13). Overall, patients with CNS PNET experience a relatively low 5-year overall survival of 50% compared to approximately 80% for those with MB (4). CNS PNETs are large tumors that can present with distant spread throughout the CNS, and patients commonly suffer increased intracranial pressure, enlarged head circumference and seizures. MB also cause increased intracranial pressure and increased head circumference due to hydrocephalus stemming from tumor growth and compression in the cerebellar fourth ventricle (14). Subsequent symptoms for MB patients include headache, vomiting, irritability, and ataxia (14). The differences between MB and CNS PNET continue to be a focus of attention to delineate their clinical behavior.

Current MB and CNS PNET treatment strategies. Current risk stratification for patients with MB after initial surgical resection is based on several criteria: age at diagnosis, extent of residual tumor tissue, and presence of metastasis (14). Patients younger than 3 years of age or having residual tumor mass larger than 1.5 cm^2, or presenting with metastatic disease at the time of diagnosis are considered at high-risk, and patients that do not fit these criteria are considered at average-risk (15). Current average-risk MB patients over the age of 3 years have a 5-year disease-free survival rate of 80% after receiving low-dose (23.4 Gy) craniospinal and local boost radiotherapy (5.58 Gy) plus adjuvant chemotherapy in an effort to eradicate potential undetected microscopic disease and control disease recurrence (16). Chemotherapeutic drugs include cisplatin, *N*-(2-chloroethyl)-*N*-cyclo-hexyl-*N*-nitrosurea, vincristine, cyclophosphomide, and etoposide. High-risk MB patients over the age of 3 years undergo a similar treatment regimen, with the exception of a high-dose (3.6 Gy) craniospinal radiotherapy, and have approximately 60% 5-year disease-free survival (16). Children under the age of 3 years with MB pose a particularly difficult risk assessment as they do not undergo radiation therapy in order to avoid severe neurological morbidity due to the delicate developmental stage of the brain. The general strategy includes a chemotherapeutic approach to delay or preclude radiation if the patient reaches 3 years of age. Clinical management of this group is further complicated because up to 40% of these patients will present with metastatic disease at diagnosis (1). One benefit for this group is the high frequency of desmoplastic histotype MB that responds well to chemotherapy (1). Despite overall significant disease-free survival, current therapeutic strategies cause long-term morbidity among most MB survivors including neurological, neurocognitive and neuroendocrine problems. CNS PNET patients currently undergo treatment similar to high-risk MB patients. They encounter a 5-year disease-free survival of up to 60%, but suffer a high frequency of recurrence (17). Clear differences in the clinical behavior between MB and CNS PNET has spurred significant research to identify how these tumors differ on a genetic level, and possibly uncover molecular features predicating better risk stratification and potential target-specific therapeutic strategies to improve clinical response and reduce treatment-induced morbidity.

Genetic Origins of Primitive Neuroectodermal Tumors

Current understanding of the genetic basis for MB and CNS PNET can be attributed to multiple studies of patients with heritable disease and genomic analyses of sporadic forms of these tumors.

Familial syndromes associated with MB and CNS PNET. Patients with nevoid basal cell carcinoma syndrome (NBCCS), or Gorlin syndrome, suffer an increased incidence of malignancy including basal cell carcinoma, meningioma and MB (18, 19). Gorlin syndrome is an autosomal dominant disorder manifested through mutations in the transmembrane receptor gene patched1 (*PTCH1*), a negative regulator of the Sonic Hedgehog (SHH) pathway. Interestingly, most MB driven by SHH have a desmoplastic histotype. Lack of active PTCH1 results in derepression of the serpentine receptor protein smoothened (SMO) and subsequent activation of Glioma-associated oncogene family zinc finger (GLI) transcription factors that help drive expression of SHH effector genes including v-myc myelocytomatosis viral-related oncogene (*N-MYC*), Atonal homolog 1 (*ATOH1/MATH1*) and cyclinD1 (*CCND1*) (20).

Patients with Turcot syndrome type I harbor mutations in deoxyribonucleic acid (DNA) mismatch repair genes mutL homolog 1 (*hMLH1*), mutS homolog 2 (*hMSH2*), postmeiotic segregation increased 1 (*hPMS1*) and postmeiotic segregation increased 2 (*hPMS2*) and develop colorectal adenocarcinoma, glial brain tumors, and have been linked with CNS PNET (21). Turcot syndrome type II is characterized by a germline mutation in the chromosome 5q21 adenomatous polyposis coli (*APC*) gene and these patients suffer an increased risk of colorectal carcinoma and MB (19). Most of these MB have a classic histotype. APC operates as part of a negative regulation complex of the Wingless and Int (WNT) signaling pathway. In the absence of WNT signaling, APC complexes with AXIN and glycogen synthase kinase-3-beta (GSK3β) to phosphorylate the WNT signaling effector molecule β-catenin (CTNNB1). Phosphorylated CTNNB1 is degraded *via* the ubiquitin-proteosome pathway (22). Mutant *APC* allows for uncontrolled

WNT signaling resulting in stabilized cytosolic CTNNB1 that can translocate into the nucleus, where it binds T-cell factor (*TCF*) transcription factors, facilitating transcription of multiple genes including *C-MYC* and *CCND1* (19).

Li-Fraumeni syndrome is caused by germline mutation of the tumor protein p53 (*TP53*) gene. These patients present with various types of cancer including sarcomas, acute leukemia, breast cancer, and brain tumors (23). Li-Fraumeni syndrome is also associated with MB (24) and CNS PNET (25). Sporadic forms of MB and CNS PNET can also harbor *TP53* mutations associated with increased P53 immunostaining (26, 27).

Genetic features of sporadic MB and CNS PNET. Pioneering research and retrospective genetic studies have identified additional significant genetic differences between MB and CNS PNET. Pomeroy and colleagues performed seminal experiments comparing the gene transcript expression profiles of several subtypes of pediatric brain tumors including MB and CNS PNET and confirmed these tumor subtypes indeed harbor distinct gene expression profiles (28). This study also demonstrated that MB histopathological subgroups could be distinguished by their gene expression. Namely, desmoplastic MB harbored differentially expressed genes among the SHH pathway including *PTCH1*, *GLI1* and *N-MYC*. Multiple subsequent studies using restriction length polymorphism (RFLP) and array comparative genomic hybridization (aCGH) demonstrated isochromosome 17 (i17q) being the most frequent genetic anomaly in MB, while rarely observed in CNS PNET (29-31). Differentially expressed candidate genes in this region are being identified (32). Work by Russo *et al*. also showed this discrepancy, as well as an association of 14q and 19q chromosomal loss with CNS PNET (33). Further studies linked focal deletion of 9p21.3, harboring the tumor suppressor gene (TSG) cyclin-dependent kinase inhibitor 2A (*CDKN2A*), and amplification of the 19q13.41 miroRNA cluster exclusively to CNS PNET (10, 31). In addition, Inda and colleagues identified a higher frequency of hypermethylation at the *CDKN2A* locus for *p14/ARF* in CNS PNET compared to MB (4). These studies provide evidence that despite morphological similarities, MB and CNS PNETs are genetically complex, with some similar and distinctive features. Current confounding issues for comparing and contrasting underlying genetic causes of MB and CNS PNET include the overall rarity of CNS PNET and that they harbor greater complexity of DNA copy number changes compared to MB (7).

As mentioned above, MB are subdivided into five histological subgroups: classic, desmoplastic, anaplastic, large cell, and MB with extensive nodularity (9), and MB also comprise distinct molecular subgroups (34-37). Notably, these studies identified gene expression signatures indicating aberrant SHH or activated WNT signaling in approximately 25 and 15% of all MB, respectively. The remaining majority of MB comprise molecular subgroups 3 and 4 that express high levels of the known MB oncogene orthodenticle homolog 2 (*OTX2*), and are not driven by SHH or WNT pathway activation (35, 37, 38). Hallmark characteristics of the SHH molecular subgroup include inactivating mutations in SHH pathway suppressors *PTCH1* or suppressor of fused (*SUFU*), loss of chromosome 9q (where *PTCH1* resides) or elevated of *N-MYC* expression. WNT MB can harbor activating mutations in *CTNNB1*, nuclear expression of CTNNB1 protein or monosomy for chromosome 6. Strikingly, patients presenting with non-SHH/WNT MB subgroups have an overall poorer prognosis, an increased frequency of metastatic disease, and the critical genetic driver pathways for these tumors are not well defined despite the fact they represent the majority of clinical MB. Molecular characteristics of these MB include i17q, *OTX2* and *MYC* amplification, TP53 immunopositivity, and are further subdivided by differential expression of genes involved in neuronal or photoreceptor differentiation (34-37).

Similarly, CNS PNET subcategories have been identified based on their histological and genetic characteristics (8, 10). *CDKN2A* deletion is more common in CNS neuroblastoma, and 19q13.42 amplification frequent in ETANTR, both with a dismal prognosis (8). Recently, an immunohistochemistry screen was performed to determine WNT pathway activation by nuclear CTNNB1 staining in a series of MB and CNS PNET. Rogers *et al*. found 36% of CNS PNETs analyzed were immunopositive for nuclear CTNNB1, similar to 27% of MB, and that patients with these characteristics tended to have better prognosis (39).

The age and sex of patients with MB and CNS PNET also contribute to overall tumor burden and have been linked with particular genetic anomalies. MB occurs more often in males overall, but SHH and WNT pathway subgroups predominantly affect females (36, 37). Additionally, these studies revealed SHH-driven MB arise most often in infants younger than 3 years old or adults over 16 years old. Furthermore, CNS PNETs have also been observed in adult patients and these tumors have a higher incidence (5/11, 45%) of *TP53* mutations compared to pediatric CNS PNET (6). Isocitrate dehydrogenase 1 (*IDH1*) mutations that generate the $IDH1^{R132H}$ mismatch observed in secondary glioblastoma also occur more often in adult CNS PNET (2/11, 18%) (6), (2/6, 33%) (40). Collectively, these studies further demonstrate the demographic and genetic complexity of MB and CNS PNET, providing a challenge for clinicians to establish appropriate treatment strategies.

Current *In Vivo* Mouse Models for MB and CNS PNET

Molecules and pathways that are affected in human PNET tumors have also been manipulated in mice to model PNET development, and have provided insight into potential cells of origin for MB and CNS PNET.

In humans, normal cerebellar development begins during gestational week 6 and stems from two distinct germinal zones, the ventricular zone and rhombic lip (9). The ventricular zone is the neural stem cell primary germinal zone located at the dorsal aspect of the fourth ventricle and gives rise to gamma-aminobutyric acid (GABA)ergic cells, including glia and Purkinje cells that take final residence in the cerebellar cortex. The rhombic lip is a secondary germinal zone comprised of granule neuron precursor cells (GNPC) that migrate rostrally to form the external granule cell layer (EGL) that creates a perimeter around cells derived from the ventricular zone. These precursor cells undergo rapid proliferation and then proceed to migrate inward along Bergmann glial fibers, past the Purkinje cell layer, to form mature neurons of the internal granule cell layer (IGL). This process is completed within the second year after birth (9). Mouse cerebellar development occurs in a similar fashion, beginning at embryonic day 10, with GNPC migration persisting through two weeks after birth. This process can be tracked during mouse brain development using specific molecular markers for cells derived from their respective germinal zones (41, 42). At embryonic day 14, GNPC derived from the secondary germinal zone have migrated to form the EGL and express the transcription factor *Math1*. Simultaneously, developing cells of the fourth ventricle can be detected by glial fribrillary acidic protein (GFAP) expression. At postnatal day 7, GNPCs in the EGL achieve peak expansion and begin migrating to form the IGL, and cells derived from the fourth ventricle are observed in the cerebellar cortex, near the EGL. By postnatal day 21, the cerebellum is fully developed. GNPC have completed their migration from the EGL to become mature granule neurons of the IGL, cease to express *Math1* and are marked by the neuronal differentiation marker *NeuN*. Cells derived from the fourth ventricle take final residence in the cortex, including the Purkinje and molecular cell layer. In addition, a third germinal zone was identified within the white matter of the postnatal cerebellum that can generate astrocytes, oligodendrocytes and neurons other than granule neurons (43). Perturbations in the organization and movement of these germinal cells circumvent normal cerebella development and lead to pathological conditions, including MB, as exemplified in $Ptch1^{+/-}$ mice.

Goodrich *et al*. created the first MB mouse model through use of a germline inactivating mutation of *Ptch1* by omitting its first transmembrane domain to genocopy Gorlin syndrome and induce Shh signaling (44). These $Ptch1^{+/-}$ mice suffered a range of phenotypes seen in Gorlin syndrome including extra digits, soft tissue tumors and MB (15%). Although Tp53 mutant mice designed to mimic Li-Fraumeni syndrome do not form MB, 95% of $Ptch1^{+/-}Tp53^{-/-}$ mutant mice develop MB within four months (45). Reviewed in Wu *et al*., many other Shh-activated MB mouse models combine *Ptch1* heterozygosity with cyclin-dependent kinase inhibitor 2C (*Ink4c*), cyclin-dependent kinase inhibitor 1B (*Kip1*), or hypermethylated in cancer 1 (*Hic1*) mutations. Other Shh-mediated MB mouse models harbor constitutively active *Smo* or combine mutated *Sufu* with *Tp53* deficiency (46). Seminal research by Wechsler-Reya and Scott demonstrated critical regulation of the Shh pathway for proper development of the cerebellum and how altered Shh signaling affects granule cell precursors as a MB cell of origin (47).

Wechsler-Reya and Scott used $Ptch1^{+/-}$ mice and showed that Shh is produced and secreted by Purkinje cells and normally received by GNPC *via* the *Ptch1* receptor for proper regulation of proliferation in the EGL (47). Subsequent studies revealed that although only a fraction of $Ptch1^{+/-}$ mice develop MB, all of these animals possess ectopic, pre-neoplastic lesions located in the EGL, further suggesting these cells as a cell of origin for MB and their need for proper Shh signaling (48). Furthermore, the ideas of potential MB cells of origin were expanded through Cre-lox conditional knockout of *Ptch1* using *Math1*-Cre (expressed in GNPC) or *GFAP*-Cre (expressed in neural stem cells that give rise to neuronal and glial cells including GNPC) (42). Both experiments yielded MB at a complete penetrance and reduced latency, suggesting proper Shh pathway regulation is required both in early stem cell populations after they commit to the granule cell lineage and the later GNPC of the EGL. Sporadic mouse models of MB have been developed using *in vivo* postnatal gene transfer with replication-competent avian leukosis virus-subgroup A (ALV-A) splice acceptor (RCAS) vectors to deliver different combinations of oncogenes in a tissue-specific manner (49). The RCAS method works by physically injecting one or more RCAS vector-producing cell lines designed to express exogenous oncogenes to the brain of a transgenic mouse engineered to express the ALV-A receptor, TV-A, in neural stem cells under control of the *Nestin* promoter. When the RCAS retrovirus infects the respective TV-A-expressing cell, virus replication does not occur, but retroviral RNA is reverse-transcribed to generate a proviral DNA (49). This DNA then integrates into the host genome and the exogenous gene is expressed as a spliced message driven by the constitutive retroviral promoter long terminal repeat (LTR). RCAS-mediated delivery of *c-Myc*, insulin-like growth factor 1 (*Igf1*), *N-myc* or hepatocyte growth factor (*Hgf*), in combination with Shh overexpression to *Nestin*-TV-A cells demonstrated tumor-promoting interaction between multiple molecular pathways (50-53). Other mouse models of MB have been generated by combining $Tp53^{-/-}$ with various defective DNA damage repair genes including ligase IV (*Lig4*), X-ray repair complementing defective repair in Chinese hamster cells 4 (*Xrcc4*), breast cancer 2 (*Brca2*) and poly (ADP-ribose) polymerase 1 (*Parp1*) in *Nestin*-Cre

expressing neural stem cells (46). Interestingly, subsequent expression profiling and chromosomal analysis of these tumor models showed significant defects including loss of *Ptch1* and *Gli1* overexpression, further implicating the Shh pathway in MB development.

One of the first non-Shh MB mouse models developed combined a conditional Cre-lox activation mutant *Ctnnb1* with conditional *Tp53* deficiency (54). The brain lipid-binding protein (BLBP)-Cre transgene promoted recombination in cells throughout the hindbrain including the ventricular zone, GNPCs of the EGL, and progenitor cells of the lower rhombic lip. Gibson *et al*. found MB located in the fourth ventricle with expression profiles comparative to human WNT subgroup tumors, including elevated levels of *Dickkopf1-3*, targets of the WNT pathway (54). Notably, they went on to show that these tumors do not derive from GNPCs, but from cells derived from the lower rhombic lip that develop and populate the brainstem. Moreover, these tumors present a classic morphology similar to their human counterpart and distinct from Shh-derived tumors that often have a desmoplastic morphology. Together, these results indicate WNT subgroup MB harbor distinct molecular profiles and are derived from a specific cell of origin compared to Shh subgroup MB. More recently, mice engineered with the Tet system to overexpress *N-MYC* in cerebellum cells expressing glutamate transporter 1 developed several classic and large cell anaplastic MB histotypes with characteristics of non-Shh-mediated tumors including elevated *Otx2* and a lack of *Math1* and *Gli1* expression (55). New mouse models of Group 3 MB were recently developed by engineered expression of *c-Myc* coupled with *Tp53* suppression in isolated prominin-positive cerebellar stem cells (56) or GNPCs (57). Importantly, these models molecularly mimic human forms of Group 3 MB and are unresponsive to therapies targeting the Shh pathway, and demonstrate a cerebellar stem cell as a cell of origin for this MB molecular subtype.

Mouse models for CNS PNET that mimic human tumors are scarce. Mice that lack *Tp53* and different combinations of cyclin-dependent kinase inhibitor 2D (*Cdkn2d*) and cyclin-dependent kinase inhibitor 2C (*Cdkn2c*) deficiency often suffer vascular tumors, and also develop MB and CNS PNET at a low frequency implicating proper cell cycle control to suppress these tumors (58). *CDKN2A* promoter methylation has been weakly observed in several studies of MB and CNS PNET (59, 60), but *CDKN2A* deletion is found to be significantly more common in CNS PNET (31). Recently, Jacques and colleagues conditionally inactivated retinoblastoma 1 (*Rb1*) and Tp53 in prospective subventricular zone stem cells of adult mice and observed CNS PNET at a frequency of 19.8% and approximately nine-month latency (61). When combined with conditional phosphatase and tensin homolog (*Pten*) loss, this model develops CNS PNET faster (approximately 100 days) and more frequently (51%), further implicating proper cell cycle control, genome stability and phosphoinositide-3-kinase signaling in CNS PNET development. Interestingly, the phenotype is shifted to glioma when only *Tp53* and *Pten* are deleted in these cells. Furthermore, this study importantly demonstrates subventricular zone stem cells as being a potential spatial and temporal CNS PNET cell of origin requiring the correct genetic insult, especially loss of cell cycle control by the *Rb1* pathway. Momota *et al*. utilized the RCAS system and delivered *c-Myc* overexpression to *GFAP*-expressing subventricular neural stem cells in $Tp53^{-/-}$ neonatal mice to generate CNS PNET within five months (11/32, 34%) (62). Similar tumor incidence was observed when this model was coupled with stabilized *β-cateninS37A* (10/21, 32%), however with a reduced two-month latency, further implicating subventricular neural stem cells as a potential CNS PNET cell of origin. Although these studies are informative, robust CNS PNET mouse models are needed to decipher the genome-wide characteristics of CNS PNET biology.

Overall, these studies provide valuable insight into the heterogeneous molecular basis and histogenesis for MB and CNS PNET. Nevertheless, CNS PNET and MB subgroups associated with poor clinical outcome still require further molecular characterization to distinguish driver from passenger mutations in these tumors. Therefore, appropriate mouse models for these molecular subgroups are urgently needed to assist this characterization and provide a true biological context for direct comparison to human disease, and ultimately pinpoint clinically relevant genetic targets for better patient stratification and therapeutic applications.

SB Transposon Mutagenesis *In Vivo* – Forward Genetics to Model PNET Molecular Subgroups and Define Genetic Drivers of Tumor Development

Applications and limitations of genome-wide association studies. In this era of cancer genomics, an ongoing challenge for identifying genetic anomalies in cancer pathogenesis on a genome-wide scale is distinguishing the genetic changes that directly contribute to disease progression from those that do not play a role in tumor growth. Recent efforts have proven beneficial for this purpose in several human cancer including colorectal and breast (63), lung (64), glioblastoma (65, 66) and MB (67, 68) tumors. These studies combined multiple technologies including gene exon re-sequencing, high-resolution single nucleotide polymorphism (SNP) genotyping, aCGH, and microarray gene expression analysis on a genome-wide scale. Importantly, these studies confirm previously known somatically altered oncogenes and TSG, as well as genes not previously implicated in tumor development. Northcott and colleagues sampled 201 primary MB using

high-resolution SNP arrays and reidentified the most common MB chromosomal aberration i17q, characterized by a net loss of 17p and a net gain of one copy of 17q, in 28% of samples (67). Known MB oncogenes (*MYC*, *GLI1* and *OTX2*) were also amplified, while multiple oncogenes and TSG involved in histone 3 lysine 9 (H3K9) methylation were identified as novel candidate MB genes. Non-coding RNAs such as the microRNA cluster *miR-17/92* have also been identified in multiple studies to be recurrently amplified in SHH-driven MB (69). In addition to structural genetic anomalies, altered gene expression profiles due to epigenetic forces also significantly contribute to tumor phenotype. Genome-wide expression and methylation profiling studies have also uncovered novel oncogene and TSG candidates in MB (34, 35, 70). Overall, such studies exemplify the importance of identifying significant changes at the genetic, epigenetic and expression levels of gene regulation for better understanding the cancer genome in order to ultimately uncover potential therapeutic drug targets. While informative, such studies are not without caveats including the significant costs and resources required to complete them. In addition, specific genetic alterations that possibly create tumorigenic functional portions of genes, transcripts or proteins may be overlooked by mutation, expression or chromosomal structure analyses. Therefore it is important to compliment this research with basic research animal models capable of mimicking the genetic and biological context of human cancer to correctly identify relevant candidate cancer genes (CCG).

Sleeping beauty transposon insertional mutagenesis in mice: mobilizing cancer genetics. Since many types of cancers are derived from a series of somatic genetic mutations and anomalies, forward genetic somatic mutagenesis screens in mice have become an extremely useful approach to mimic this behavior. Mice are well suited for comparative functional genomics studies that model human disease because of their genetic and physiological similarities and both mouse and human genome sequence are publicly available for comparative analysis (71). Insertional mutagenesis in mice is an efficient method for inducing tumorigenesis by altering the nascent genome, and subsequent identification of the precise genes and gene loci affected (72). Retroviruses have been developed as *in vivo* copy-and-paste insertional mutagens and shown to be effective for identifying CCG in a variety of tumors including lymphoma (73), glioma (74) and mammary tumors (75). Areas of the genome that recurrently harbor integrated provirus among multiple tumors are referred to as common insertion sites (CIS) and represent gene loci that were important for tumor development. These studies demonstrated biological relevance of insertional mutagenesis because genes that are altered in human cancer were identified, as well as previously unknown CCG. Although amenable to genome-wide, high-throughput identification of CCG in mice, retroviruses hold significant limitations for this work, including activity restricted to few cell types and a propensity for proviral insertion at the 5' end of coding genes.

The Sleeping Beauty (SB) DNA transposon has emerged as a pioneering tool for somatic genome manipulation including insertional mutagenesis in mice. SB transposon and transposase sequences were originally reconstructed based on dormant *Tc1/Mariner* fish transposon genes harboring inactivating mutations (76). SB operates as a 'cut-and-paste' DNA transposon and was engineered to function as a two-component system consisting of the SB transposon and transposase enzyme shown to be active in mammalian cells (76). SB transposase recognizes inverted repeat/direct repeat (IR/DR) sequences flanking the DNA cargo, binds as a homodimer to these IR/DRs and excises the transposon from its genomic location, leaving behind a three base pair footprint (CA/TG, Figure 1A and B) (76). The transposon is then subsequently reintegrated into a new genomic location at a TA dinucleotide that becomes a flanking duplication (Figure 1C). SB transposons provide an enhancement to the use of retroviruses as they do not discriminate towards coding genes or their 5' ends. An ultimate benefit to *in vivo* mobilization of SB transposons in the mouse genome is that the IR/DRs serve as marker sequences to identify the exact location of the reintegration site using ligation-mediated PCR (77). Several iterations of the SB transposase and transposon IR/DR sequences have been engineered to increase mobilization activity (78). This SB system has been used successfully for multiple experiments in mice including germline transgenesis (79), forward genetic phenotype screening (80, 81), and somatic cell gene delivery for *in vivo* cancer induction (82, 83) or suppression (84). These studies have also shed light onto limitations of SB-mediated transposition including the tendency for local hopping of the transposon from its donor chromosomal concatomer and incompletely random nature of TA dinucleotide site integration (80, 81). Nevertheless, recent studies have used SB transposon insertional mutagenesis as a successful genome-wide method for generating cancer in mice and subsequently identifying CIS as candidate genetic tumor drivers (85). The transposons T2/Onc and T2/Onc2 work as insertional mutagens through their murine stem-cell virus long terminal repeat/splice donor sequence (MSCV LTR/SD) and bidirectional splice acceptor/polyadenylation sequences (SA/pA) (Figure 2A). These mutagenic transposons are provided as a multi-copy chromosomal concatomer mobilized by SB to other chromosomal loci (Figure 2B), and designed to promote proto-oncogene (Figure 2C) or halt TSG (Figure 2D) expression, respectively. Both vectors are effective when mobilized in all somatic cells and combined with the correct predisposing mutant background to induce neoplastic prostate lesions, lymphomas and sarcomas (86-89). Brain tumors, including MB, CNS PNET and high-grade glioma, were also observed at low frequency using this strategy (88, 89) with

novel candidate glioma genes successfully identified (90). In a seminal paper, Wu *et al*. applied this system for identifying new CCG important in MB metastasis by driving SB expression from the endogenous mouse *Math1* promoter facilitating mutagenic transposition in granule neuron precursors, a cell of origin for MB, in the context of deficient *Ptch1* or *Tp53* expression (91). Furthermore, T2/Onc mutagenesis has been used to induce hepatocellular carcinoma and gastrointestinal tract cancer when applied in a tissue specific manner utilizing a Cre-lox regulated SB transposase transgene, *Rosa26-LoxSTOPLox-SB11* (92, 93). Importantly, subsequent transposon insertion site analysis identified gene targets also altered in human disease, as well as candidate oncogenes and TSG not previously associated with their respective tumors. Significantly, SB-insertional mutagenesis was also able to reveal oncologically functional portions of gene products including amino-terminal truncated v-raf murine sarcoma viral oncogene homolog B1 (Braf) (Figure 2C) (87) and carboxy-terminal truncated epidermal growth factor receptor (Egfr) (Figure 2C) (92). Therefore, these studies provide proof-of-concept that SB-mediated insertional mutagenesis can successfully model relevant stages of cancer in mice, can be applied to specific tissues for particular tumor development, and is capable of identifying known and novel candidate cancer genes including onco-functional portions of genes for accurate delineation of the genetic basis of different cancers in proper biological context. Precise location of T2/Onc insertion within a candidate cancer gene is an important consideration for interpreting induced gene expression or suppression as this can influence the abundance or absence of protein domains critical for binding, activation, stabilization or subcellular localization. Data from SB-insertional mutagenesis mouse models can also reveal multiple genetic alterations occurring in the same tumor simultaneously contributing to tumor progression (94). Together, these results have important influence on subsequent analysis of cancer candidate genetic drivers especially their biological relevance, possible function as tumor-associated antigens and potential development of targeted therapeutic strategies.

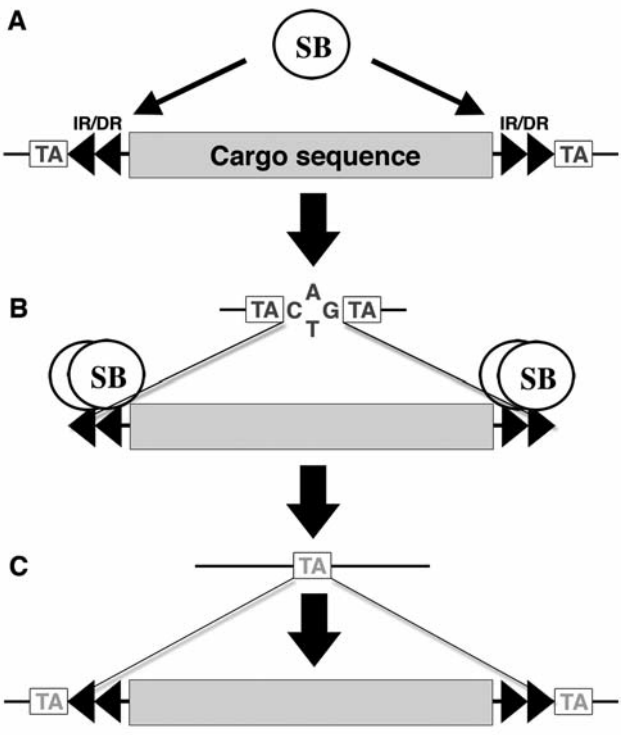

Figure 1. *SB Transposon Mechanism Of Mobilization. A: SB transposase recognizes and binds as a homodimer to SB transposon IR/DR sequences. B: SB transposon is excised from its genomic location. C: Reintegration of the SB transposon into a new TA dinucleotide genomic location.*

A novel mouse model to reveal the genetic basis for molecular subgroups of MB and CNS PNET. In an effort to focus the SB system on brain tumors, we activated mutagenic transposition in early mouse neural precursor cells by combining a tissue-specific *Nestin*-Cre transgene (95) with the conditional SB11 and multiple lines of T2/Onc transgenic mice. We observed Cre-mediated SB expression throughout cells of the developing cerebellum and anterior brain especially prospective cells of origin for MB (granule cells, white matter and fourth ventricle; Figure 3A) and CNS PNET (subependymal midbrain and subventricular zone; Figure 3B). These experiments generated aggressive and infiltrative tumors in the cerebellum or anterior (Figure 3C and D). Histological analysis revealed the presence of small round tumor cells, rosette formation and mitotic figures confirming MB and CNS PNET, respectively (Figure 3E and F). We observed a modest frequency of these tumors when associated with an otherwise normal or conditional $Pten^{flox/+}$ (96) genetic background. MB and CNS PNET occurrence was significantly enhanced with the conditional dominant negative $p53^{lsl\text{-}R270H}$ transgene that mimics Li-Fraumeni syndrome (97). In particular, analysis of T2/Onc transposon insertion sites from MB and CNS PNETs revealed several distinguishing candidate oncogenes and TSG and pathways that also represent distinct tumor molecular subtypes reflective of human tumors (Larson *et al*., in preparation). Furthermore, expression of multiple CCG we identified not previously associated with MB were similarly altered in specific human MB molecular subgroups especially SHH, and Groups 3 and 4, and a majority of CNS PNET harbor transposon insertion profiles affecting regulators of rat sarcoma virus oncogene (Ras) signaling corresponding with pathway activation. Therefore, *Nestin*-Cre combined with Cre-lox regulated SB transposon insertional mutagenesis is amenable to generating MB and CNS PNET on a variety of

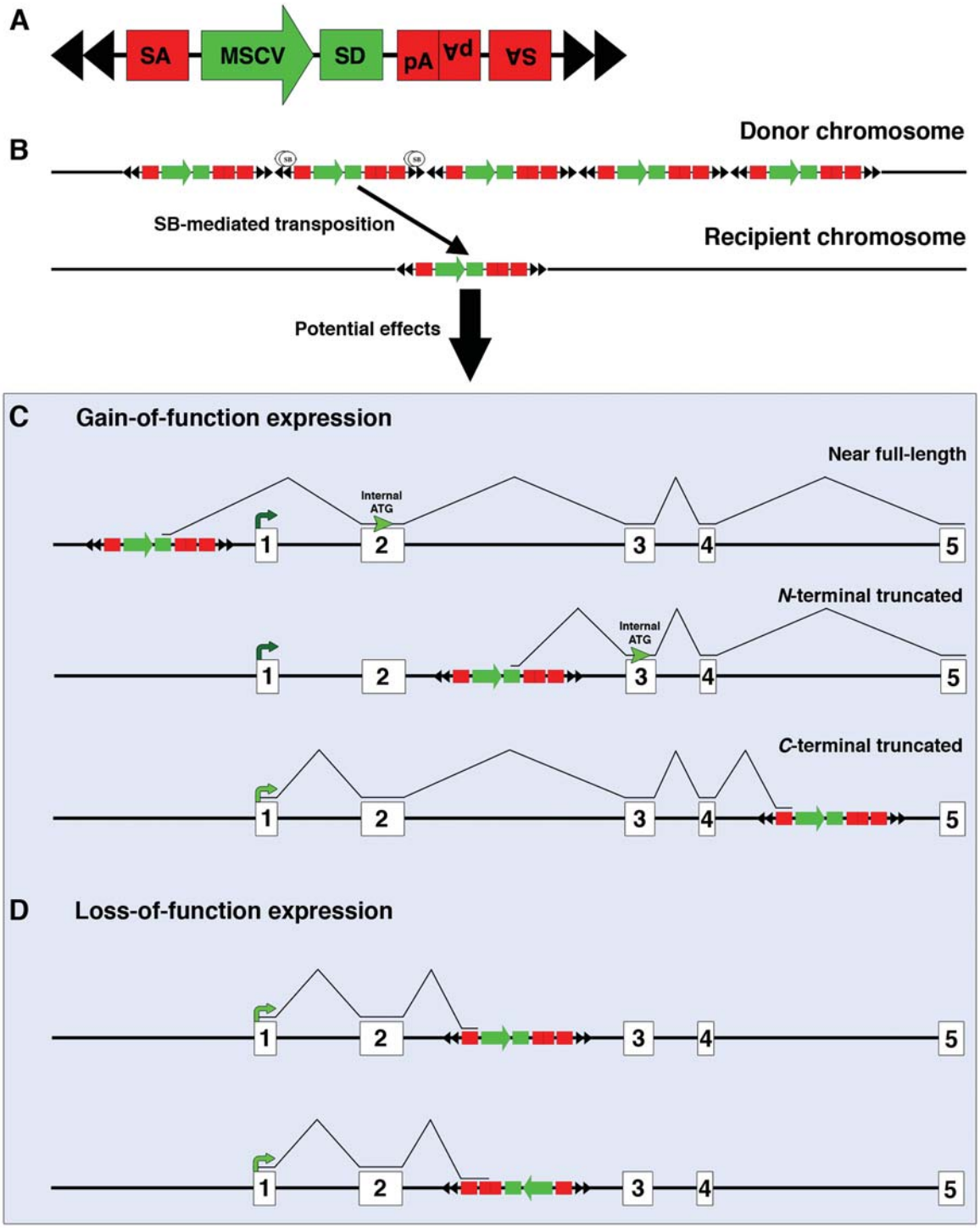

Figure 2. *Structure And Function Of Mutagenic Transposons. A: Schematic of T2/Onc/T2/Onc2. Murine stem cell virus 5' long terminal repeat with promoter and enhancer elements (MSCV) and splice donor (SD) sequences promote proto-oncogene expression. Bi-directional splice acceptor (SA) and polyadenylation (pA) sequences disrupt expression of tumor suppressor genes. B: T2/Onc transgenic mice harbor transposon concatomers located on the donor chromosome. SB-mediated transposition of T2/Onc achieves reintegration at essentially random TA dinucleotide loci of the recipient chromosome. T2/Onc elicits different biological effects upon candidate genes depending on insertion orientation and location. C: Gain-of-function expression: T2/Onc MSCV-SD sequences in the sense orientation relative to a proto-oncogene full-length or near full-length (upstream insertion), or N-terminal-truncated proteins (intronic insertion) via an internal in-frame ATG start codon. Alternatively, C-terminal-truncated proteins can be generated by SA-pA sequences regardless of insertion orientation. D: Loss-of-function expression: independent of insertion orientation, the T2/Onc SA-pA sequences inhibit expression of a tumor suppressor gene.*

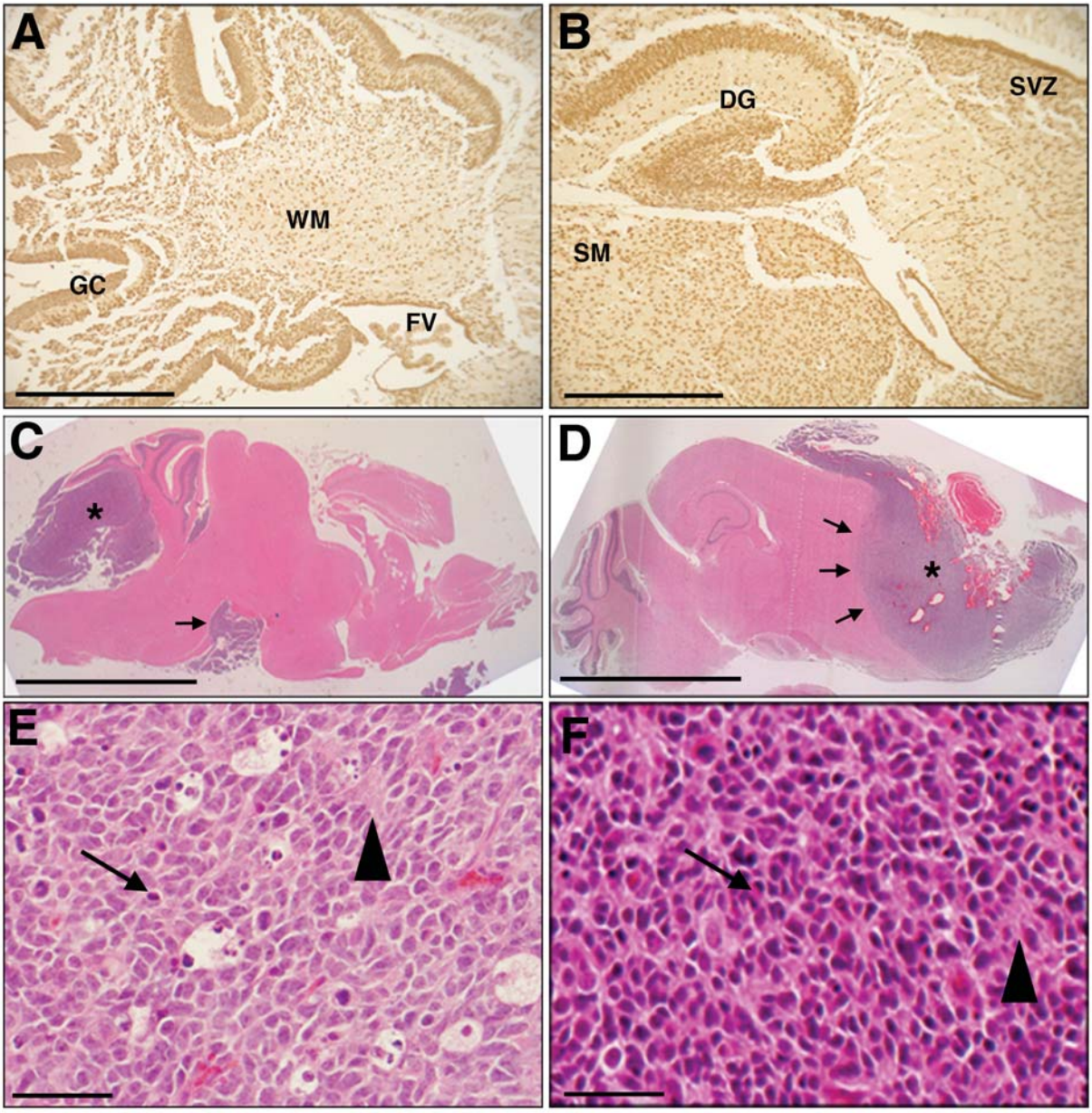

Figure 3. *Nestin-Cre-Mediated Mutagenic Transposition Generates MB and CNS PNET. A and B: Immunohistochemistry for SB transposase protein throughout the brain of a Nestin-Cre, Rosa26-lsl-SB11 double transgenic mouse. Sagittal section at p1 reveals SB expression in neurons and glial cells throughout the brain including the cerebellum (A) and anterior brain (B). Scale bars=350 μm. C-F: Hematoxylin and Eosin staining of tumors in mice undergoing mutagenic transposition. C: MB form in the cerebellum (asterisk) and typically exhibit leptomeningial spread (arrow). D: CNS PNET form in the anterior brain, consume the cereberal cortex and olfactory bulb (asterisk) and invade the parenchyma (arrows). Scale bars=5 cm. E and F: High-power magnification of an area from bulk tumor depicting small, round tumor cells, rosette formations (arrowheads) and mitotic nuclei (arrows) in MB (E) and CNS PNET (F). Scale bars=50 μm. GC, granule cells; WM, white matter; FV, fourth ventricle; SM, subependymal midbrain; DG, dentate gyrus; SVZ, subventricular zone.*

genetic backgrounds. These experiments contribute the first mouse model to simultaneously pheno/geno-copy PNET subtypes MB and CNS PNET, recapitulate multiple molecular subgroups, and moreover provide a single *in vivo* method to better define the genetic causes of these tumors.

Conclusion and Future Perspectives

The genetic distinctions between MB, CNS PNET and their molecular subgroups have become more defined in the recent past, but remains a challenge and critical factor for proper

patient stratification and predicting clinical outcome for those harboring deadly forms of these tumors. Identification and characterization of as yet unknown specific genetic defects and molecular mechanisms is warranted to better delineate the causes of these poorly understood tumor groups. Forward genetic SB transposon insertional mutagenesis in mice has evolved into a high-throughput method for genome-wide interrogation of various tumor types, including MB and CNS PNET. These studies revealed high fidelity candidate gene targets for cancer development and progression, and help pinpoint MB and CNS PNET subgroup-specific genetic drivers.

Further experiments can be implemented to accentuate and improve upon this original system for MB and CNS PNET. For example, additional transposon mutagenesis *in vivo* tissue-specific mouse models have been developed. T2/Onc3 is an SB mutagenic transposon similar to T2/Onc and T2/Onc2 except that a cytomegalovirus (CMV) enhancer/chicken β-actin promoter replaces the MSCV sequence for driving oncogene expression, with activity in a wider range of cell types especially epithelial cells (98). *PiggyBac* (PB) is a similar cut-and-paste transposon system derived from the cabbage looper moth (99) that has been used for insertional mutagenesis in mice (100). PB transposase functions differently than SB by recognition of and binding to the specific PB transposon inverted terminal repeat sequences to facilitate mobilization. PB transposons target TTAA nucleotide sequences for genome integration, predominantly insert at transcription start sites and can carry larger cargo compared to SB (101). Such a system could be fashioned for targeted activation in neural stem cells *in vivo*. Alternative predisposing transgenic backgrounds can be employed to render particular molecular interaction with unbiased transposon mutagenesis. Moreover, gene targets found in the current PNET model system can be transgenically manipulated for functional validation of their participation in aberrant development and tumor progression.

Ultimately, the present model serves as an important *in vivo* method for advancing the knowledge of PNET genetic origins. The key for this and future experimental approaches is a focus on accurate representation of biologically relevant genetic origins that discriminate between MB and CNS PNET subtypes and their corresponding molecular subgroups. This attention will continue to provide valuable information in the pursuit of effective therapeutic strategies to improve clinical outcome of patients with PNET, especially those who suffer from poorly understood forms of their disease.

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