locus was generally associated with chr19 loss or translocation (Fig. 4E, Suppl. Table 3), sequence analysis of the remaining *Sufu* allele did not reveal mutations and *Sufu* expression was detected in all tumors suggesting that a gene(s) other than Sufu was the important target on chr19. Further, *Sufu* haploinsufficiency doesn't appear to contribute to tumorigenesis as *Sufu*$^{+/-}$ mice are not tumor prone, and medulloblastoma in *Sufu*$^{+/-}$*p53*$^{-/-}$ mice results from inactivation of the WT copy of Sufu (29).

Secondly, we determined if Pten loss was associated with the HR-deficient tumors. We sequenced *Pten* cDNA from the tumors, but did not find any exonic mutations (data not shown). However, an immunohistochemical survey of Pten expression in ten different Brca2-deficient tumor samples showed many of the tumors had lost Pten immunoreactivity (5/8) and concomitantly had increased levels of phospho-Akt (Ser473), consistent with inactivation of Pten (Fig. 5). Some tumors retained Pten expression and did not show elevated levels of p-Akt suggesting heterogeneity of expression and that Pten inactivation only occurred in a subset of tumors.

Western blot analysis of 6 Brca2-deficient tumors revealed abundant Pten signaling as Akt and S6K activity were affected in many tumors (Fig 5B). While Pten-deficient cells *in vitro* showed strong P-Akt staining, we found elevated Akt phosphorylation in some *Brca2*-deficient tumors (3/6), suggesting that the *Pten* pathway is dysfunctional in many of the tumors. It is possible that PTEN haploinsufficiency may contribute to tumorigenesis, a hypothesis that has been supported by careful studies of the effects of Pten dosage in select animal models (41). Thus, while chr19 loss is a frequent event in medulloblastoma resulting from disabled HR, the specific gene activity that is affected remains unclear, although Pten inactivation or signaling abnormalities is a feature of many HR-deficient medulloblastomas.

Discussion.

To further characterize the tumor-suppressor role of DNA DSB repair pathways in defined tissues, we used conditional inactivation of *Lig4*, *Xrcc2* and *Brca2* throughout the nervous system, and found that in conjunction with *p53* mutations, rapid development of medulloblastoma occurred. A key finding from our data is the identification of *Ptch1* as a critical target in all DNAR-deficient medulloblastomas, indicating its unique importance for preventing transformation of GNPs.

Based on microarray analysis, medulloblastomas in our study showed a gene expression profile reflective of activated SHH signaling (14). This situation reflects medulloblastoma in *Ptch1*$^{+/-}$ mice in which *Ptch1* LOH occurs in pre-neoplastic lesions as incipient medulloblastoma develops in *Ptch1*$^{+/-}$ cerebella (25). Active SHH signaling is a potent growth promoting activity, and will contribute significantly to growth and expansion of GNP cells. Initial loss of a *Ptch1* allele may promote enhanced proliferation via mild mitigation of smoothened inhibition of Shh signaling, leading to an increase in replication stress such as premature termination, replication fork collapse and generation of DNA double strand breaks (42). Subsequently, defective DNA damage responses in the mutant mice will sustain increased DNA damage leading to oncogenic mutations (such as N-Myc/CyclinD2 amplification; see below) that overcome senescence and result in transformation (43-47).

Together with *Ptch1* inactivation, we also observed *N-Myc* or *CyclinD2* amplification in medulloblastomas, both of which are Gli targets. N-Myc is critical for GNP proliferation (48-50), while the D-cyclins influence cerebellar development and GNP proliferation (51-54). Thus, loss of Ptch1 function will lead to a Gli-dependent transcriptional upregulation of N-Myc and CyclinD2. Despite increased expression of N-Myc as a result of Ptch1 loss and activated SHH signaling we also found genomic amplification of *N-Myc* and *CyclinD2* in the medulloblastomas. N-Myc amplification was also found in medulloblastomas arising after

neural inactivation of *Xrcc4* (31). We also found a mutual exclusivity between N-Myc and Cyclin D2 genomic amplification suggesting, that in addition to increased Shh target expression, there is a significant advantage to a tumor cell for additional upregulation of the growth promoting factors. The tumorigenic properties of Myc have recently been linked to a function during DNA replication via interactions with minichromosome maintenance (MCM) subunits to participate in the control of DNA replication origin activity (55). Therefore, increased expression of N-Myc may lead to increased replication firing and associated DNA damage and checkpoint activation (55), generating a compounding scenario in DNAR-deficient GNPs that significantly promotes oncogenic mutation accumulation.

Finally, we observed genomic rearrangements of chromosome 19 in tumors resulting from HR disruption. The consistent loss of chromosome 19 among tumors suggests that this chromosome contains a tumor suppressor gene whose loss contributes to medulloblastoma formation. Two well-described tumor suppressors reside on this chromosome; *Sufu* and *Pten*. *Sufu* is mutated in a subset of human medulloblastomas (40) while *Pten* is responsible for Lhermitte-Duclos disease and is mutated in some gliomas (56, 57). PTEN has recently been found as a target for mutation in breast cancer when HR is disrupted, and Pten can modulate growth in animal models of medulloblastoma (39, 58). We surveyed Pten expression in Brca2-null tumors and found that a subset of the tumors had lost Pten immunoreactivity and showed a concomitant increase in phospho-Akt (Ser473). Despite this, some tumors retained PTEN expression although phospho-Akt was still increased. In these cases it is possible that PTEN haploinsufficiency may contribute to tumorigenesis, a hypothesis that has been supported by careful studies of the effects of Pten dosage in select animal models (41).

It is likely that defective DNA DSB repair generates a continuum of genomic alterations during cell proliferation that allow for uncoupling of the growth controls of the GNPs. The genomic changes reflect the specific minimal changes that are required to

transform granule neurons in vivo, providing the blueprint for GNP transformation. Generating other brain tumor types using the approaches described above will be particularly valuable in assessing important genetic changes that underpin other tumor types. For example, combined Brca2 loss and p53 haploinsufficiency can delay or bypass medulloblastoma resulting in gliomas (Frappart et al, unpublished observation). Given the relatively homogenous nature of these tumor models, they will provide important data regarding genomic changes that lead to tumorigenesis and will also be important tools for pre-clinical studies.

Acknowledgements

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Materials and methods.

Generation of Brca2$^{Nes\text{-}Cre}$, Xrcc2$^{Nes\text{-}Cre}$, Ligase4$^{Nes\text{-}Cre}$, p53-deficient mice

Brca2$^{Nes\text{-}Cre}$ and *Xrcc2*$^{Nes\text{-}Cre}$ were generated as described previously (9, 30, 33) and crossed with *p53*$^{+/-}$ mice in order to obtain the various groups used in this study. The conditional Lig4 mouse contains LoxP sites surrounding the single *Lig4* coding exon and was generated by standard methods. Tumor formation was monitored over a period of eight months.

SKY and array CGH analysis

Genomic DNA was prepared from brain tumors using the DNeasy® blood and tissue kit (QIAGEN), and metaphase spreads were from tumors after injection of colcemid (33). SKY and array CGH analysis were performed as described previously (24).

Microarrays analysis

Isolation of RNA, synthesis of cDNA, GeneChip Hybridization and Data analysis were performed as described previously (14, 16).

Real-Time RT-PCR

The *Ptch1*, *Ptch2*, *Gli1*, *Math1* and *Sufu* primer/probe sets for realtime PCR and the procedures have been described (24, 29). The primer/probe set for Titest was forward primer (5'GCGTGGTCAGGGACGAGTT), reverse primer (5' CCATCAGACAGCGGGATAAGA) and TaqMan probe (5' TCTCTGTCGGCCTTGACTCAGCCC).

Sequence analysis of candidate genes.

The cDNA was synthesized as described (24, 29). The primers Ptch1-14 (5'-ACGCGCAATGTGGCAATGGAAGGC-3') and Ptch1-R1 (5'-GAAGCGGCCGCTTCAGATTTTAATTACCC-3') were used to amplify the full length Ptch1 in a first round. Then a second round of PCR was done to amplify five overlapping fragments covering the entire length. Fragment A : Ptch1-F (5'-ATGGCCTCGGCTGGTAACG-3') / Ptch1-1 (5'-AAGGCCGGTCCATGTACCCATGGC-3'); fragment B : Ptch1-2 (5'-GCTTAATCATTACACCTTTGGACTGC-3') / Ptch1-6 (5'-AAAGGAGCATAGTGCTTCTCTGC-3'); fragment C: Ptch1-5 (5'-TTGAGCCACAGGCCTACACAGAGC-3')/Ptch1-8 (5'-GTCTGAGGTGTCTCGTAGGCCG-3'); fragment D : Ptch1-7 (5'-TGGGAAACTGGGAGGATCATGC-3') / Ptch1-10 (5'-GCTCAGGCGAAGGAGTGGGCAGTCG-3'); fragment E : Ptch1-9 (5'-GTGGAGTTCACCGTCCACGTGGC-3')/ Ptch1-R2 (5'-GAAGCGGCCGCTCAGTTGGAGCTGCTCCCCCACGGC-3'). The PCR products were sequenced. Nevertheless, if several PCR products were found, they were cloned into pGEM-T easy vector (Promega) and sequenced. The primers used to amplify full length *Sufu* in a first round PCR were: SUFU-F (5'-CTCTACCCTCCCGGGTTCTCCGC -3') and SUFU-R (5'-CCCTGCAGGGCACAGCCCAGGC -3'). Sufu sequencing was done using the same primers, plus two additional internal primers SUFU3 (5'-GCGGGGAGAAACCATATTTGAGATCG-3') and SUFU4 (5'-CTCCGGCTATCCTCTTCATCCTCCG-3').

Immunohistochemistry and Western blots.

Tissue was disrupted in lysis solution (50mM Tris, 200mM Nacl, 1% Tween 20, 0.2% NP40, 2mM PMSF, 50mM B-glycerol phosphate, pH=7.5) for four hours. Proteins were then separated on 4-12% NuPage Bis-tris gels (Invitrogen) with MOPS buffer, and transferred to

nitrocellulose (Biorad). Antibodies used were; phospho ser473-AKT (1/500, Cell Signaling, #9271), phospho ser235/236-S6 ribosomal (1/500, Cell Signaling, #2211) and PTEN (1/1000, Cell Signaling, #9559).

Table 1. Medulloblastoma incidence in mice with defective DNA DSB repair.

Genotype	Affected/total animals	Incidence	Onset (weeks) ± SD
$Lig4^{Nes\text{-}Cre}$	0/42	0%	-
$Lig4^{Nes\text{-}Cre}$, $p53^{+/-}$	0/19	0%	-
$Lig4^{Nes\text{-}Cre}$, $p53^{-/-}$	17/18	94%	16.4 ± 3.67
$Xrcc2^{Nes\text{-}Cre}$	0/23	0%	-
$Xrcc2^{Nes\text{-}Cre}$, $p53^{+/-}$	0/10	0%	-
$Xrcc2^{Nes\text{-}Cre}$, $p53^{-/-}$	28/28	100%	15.6 ± 3.12
$Lig4^{Nes\text{-}Cre}$,$Xrcc2^{Nes\text{-}Cre}$	0/25	0%	-
$Lig4$,$Xrcc2^{Nes\text{-}Cre}$, $p53^{+/-}$	6/8	75%	22.5 ± 2.35
$Lig4$,$Xrcc2^{Nes\text{-}Cre}$, $p53^{-/-}$	3/3	100%	14 ± 1.41
$Brca2^{Nes\text{-}Cre}$	0/31	0%	-
$Brca2^{Nes\text{-}Cre}$, $p53^{+/-}$	34/47	72%	20.53 ± 5.64
$Brca2^{Nes\text{-}Cre}$, $p53^{-/-}$	19/22	87%	13.26 ± 2.86

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Figure Legends.

Figure 1: *p53* deficiency induces medulloblastomas formation in *Lig4*$^{Nes\text{-}Cre}$ and *Xrcc2*$^{Nes\text{-}Cre}$ mice. (A) Survival curves of *Lig4/p53* deficient mice. *Lig4*$^{Nes\text{-}Cre}$ (n=42), *Lig4*$^{Nes\text{-}Cre}$, *p53*$^{+/-}$ (n=19) and *Lig4*$^{Nes\text{-}Cre}$, *p53*$^{-/-}$ (n=23) mice, were monitored over a period of 32 weeks for survival and medulloblastoma development. The life span of *Lig4*$^{Nes\text{-}Cre}$,*p53*$^{-/-}$ mice was significantly shorter compared with the *Lig4*$^{Nes\text{-}Cre}$, and *Lig4*$^{Nes\text{-}Cre}$, *p53*$^{+/-}$ cohort ($p<0.0001$). (B) Survival curves of *Xrcc2/p53* deficient mice. *Xrcc2*$^{Nes\text{-}Cre}$ (n=23), *Xrcc2*$^{Nes\text{-}Cre}$, *p53*$^{+/-}$ (n=11), *Xrcc2*$^{Nes\text{-}Cre}$, *p53*$^{-/-}$ (n=33). The life span of *Xrcc2*$^{Nes\text{-}Cre}$, *p53*$^{-/-}$ mice was significantly shorter compared with the *Xrcc2*$^{Nes\text{-}Cre}$, *p53*$^{+/-}$ and *Xrcc2*$^{Nes\text{-}Cre}$ ($p<0.0001$). (C) Survival curves of *Ligase4/Xrcc2/p53* deficient mice. *Lig4,Xrcc2*$^{Nes\text{-}Cre}$ (n=20), *Lig4,Xrcc2*$^{Nes\text{-}Cre}$, p53$^{+/-}$ (n=8), *Lig4,Xrcc2*$^{Nes\text{-}Cre}$, p53$^{-/-}$ (n=3). The survival curves are statistically significantly different ($p<0.0001$). (D) Representative aCGH analysis of chr 11 of *Brca2* and *Lig4,Xrcc2* deficient medulloblastoma associated with *p53* heterozygosity. (E) Summary of genomic rearrangements of chr11 in medulloblastomas of *Brca2*$^{Nes\text{-}Cre}$ and *Lig4/Xrcc2*$^{Nes\text{-}Cre}$. Each column represents a single tumor. Red color indicates that the chromosome exhibit genomic loss.

Figure 2: *Ptch1* is lost in *Brca2*, *Ligase4*, *Xrcc2; p53* deficient medulloblastomas.
(A) Representative aCGH analysis of chr13 of *Brca2*, *Lig4*, *Xrcc2* deficient medulloblastomas in *p53* mutant backgrounds. (B) Summary of the genomic rearrangements of chr13 in the medulloblastomas. Each column represents a single tumor, each line represent one chromosome. Each red square indicates that the chromosome exhibit genomic loss. (C) Representative FISH analysis of chr13 showing either deletion or translocation. (D) Sequence analysis of *Ptch1* mRNA from *Lig4*, *Xrcc2*, and *Brca2* deficient medulloblastoma.

Figure 3 : Global gene expression analysis of medulloblastoma. (A) Hierarchical cluster analysis of a selective cohort of genes (from total 319 genes differentially expressed, 46 genes up-regulated/273 genes down-regulated) differentially expressed in the medulloblastomas compared to wildtype (WT) and $p53^{-/-}$ postnatal day 5 and adult (3 month old) cerebellum. The first column shows the address on the MOE 430 A and B gene chips, and the second column shows the GenBank accession numbers. Asterisks indicate multiple hits of the same gene. High (red) to low (blue) expression changes ranging from 0 to 2.5 of normalized values. (B) Quantitative real-time RT-PCR measures the expression level of several signature genes expressed in medulloblastoma to confirm the array analysis.

Figure 4: Genomic analysis of chr 6, 12 and 19 in *Brca2*, *Lig4*, *Xrcc2*, *p53* deficient medulloblastomas. (A) Representative aCGH of chr 6, 12 and 19 in medulloblastomas examined. (B) Representative complete SKY analysis of $Brca2^{Nes\text{-}Cre}$, $p53^{-/-}$. (C) FISH analysis for N-Myc amplification in medulloblastoma. (D) Representative SKY of chr19 showing either deletion or translocation. (E) Summary of the genomic rearrangements of *N-Myc*, *cylin D2*, *Sufu* and *Pten* in the medulloblastomas. Each column represents a single tumor, and each line represents one chromosome. Each red square indicates that the chromosome exhibit genomic loss, each green square indicates that the chromosome presents genomic amplification.

Figure 5: Pten pathway disruption in Brca2/p53 deficient medulloblastoma. (A) Representative medulloblastoma sections showing loss of expression of Pten and up-regulation of levels of phosporylated Akt (P-Akt) and S6 (P-S6) in Brca2/p53 deficient medulloblastoma (Magn.x10 and Magn.x20). (B) Western-blot analysis of Pten, P-S6 and P-

Akt in Brca2/p53 deficient medulloblastoma. (1-3,6: Tumor presenting loss of Pten locus in CGH analysis, 4-5: absence of Pten locus loss in CGH).

Figure 1

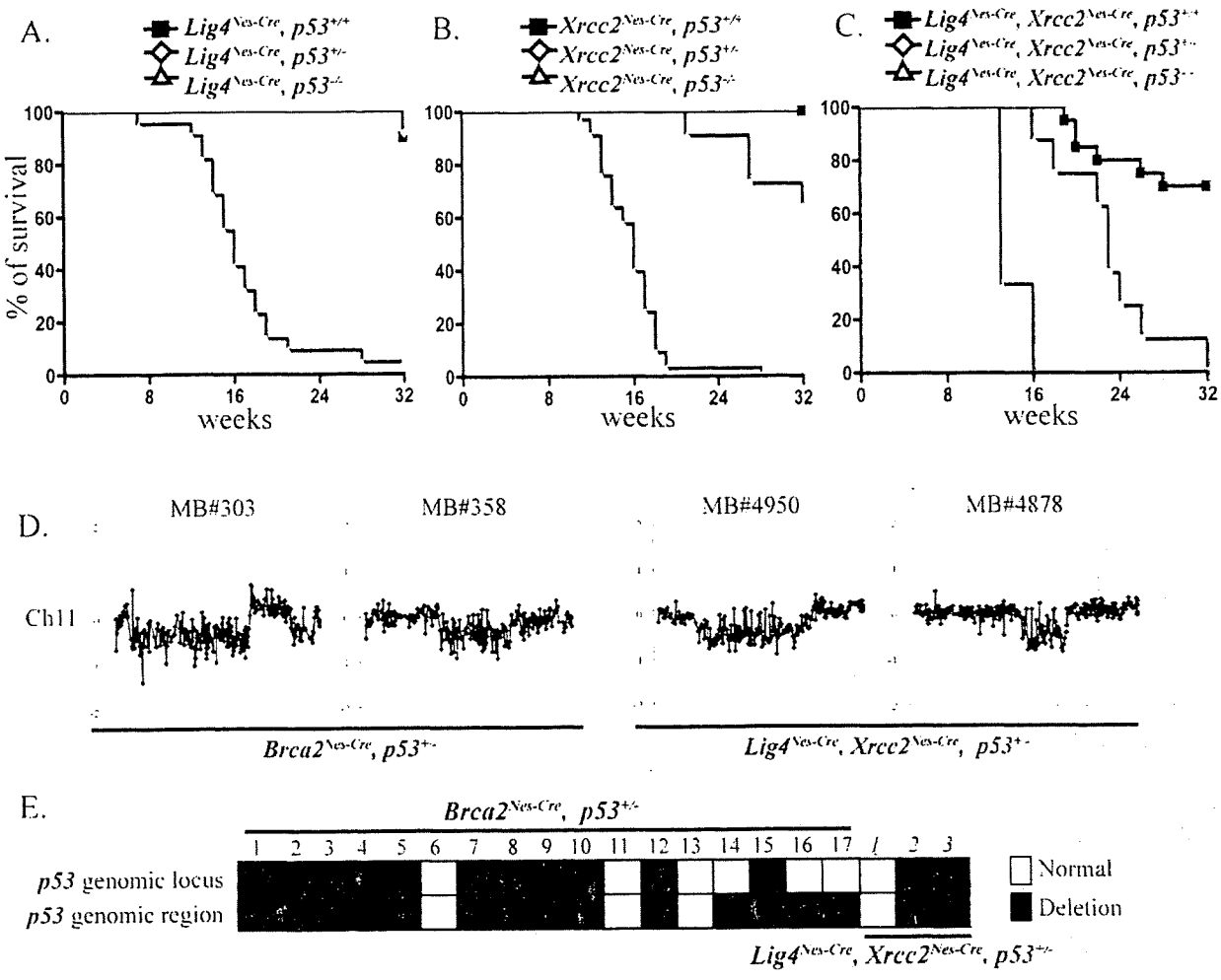

A.
Lig4Nes-Cre, p53+/+
Lig4Nes-Cre, p53+/-
Lig4Nes-Cre, p53-/-
% of survival
weeks
B.
Xrcc2Nes-Cre, p53+/+
Xrcc2Nes-Cre, p53+/-
Xrcc2Nes-Cre, p53-/-
weeks
C.
Lig4Nes-Cre, Xrcc2Nes-Cre, p53+/+
Lig4Nes-Cre, Xrcc2Nes-Cre, p53+/-
Lig4Nes-Cre, Xrcc2Nes-Cre, p53-/-
weeks
D.
MB#303
MB#358
MB#4950
MB#4878
Ch11
Brca2Nes-Cre, p53+/-
Lig4Nes-Cre, Xrcc2Nes-Cre, p53+/-
E.
Brca2Nes-Cre, p53+/-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 1 2 3
p53 genomic locus
p53 genomic region
Normal
Deletion
Lig4Nes-Cre, Xrcc2Nes-Cre, p53+/-

Figure 2

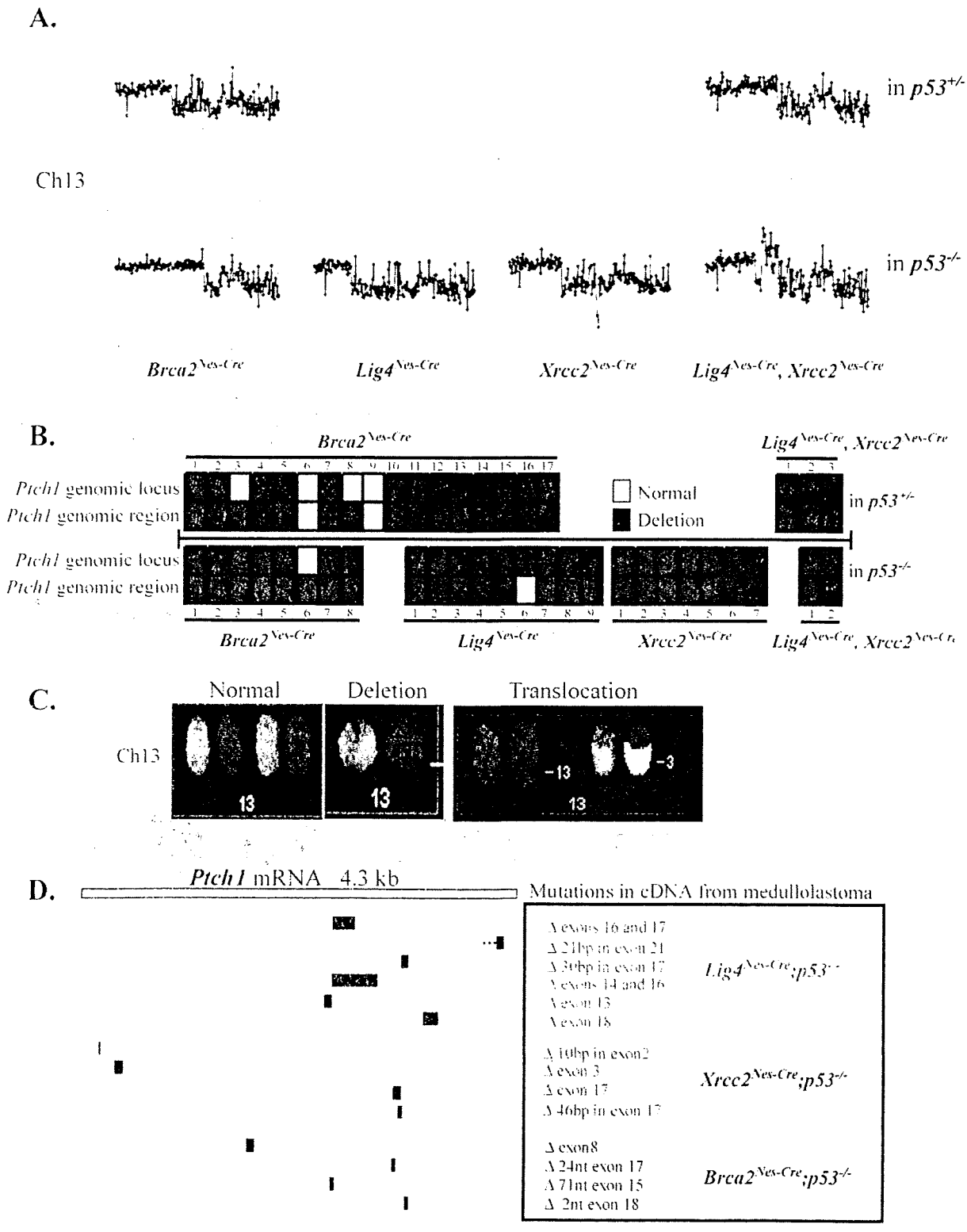

A.
in p53+/-
Ch13
in p53-/-
Brca2Nes-Cre
Lig4Nes-Cre
Xrcc2Nes-Cre
Lig4Nes-Cre, Xrcc2Nes-Cre
B.
Brca2Nes-Cre
Lig4Nes-Cre, Xrcc2Nes-Cre
Ptch1 genomic locus
Ptch1 genomic region
Normal
Deletion
in p53+/-
in p53-/-
C.
Normal
Deletion
Translocation
Ch13
13
-13
-3
D.
Ptch1 mRNA 4.3 kb
Mutations in cDNA from medullolastoma
Δ exons 16 and 17
Δ 21bp in exon 21
Δ 30bp in exon 17
Δ exons 14 and 16
Δ exon 13
Δ exon 18
Lig4Nes-Cre;p53-/-
Δ 10bp in exon2
Δ exon 3
Δ exon 17
Δ 46bp in exon 17
Xrcc2Nes-Cre;p53-/-
Δ exon8
Δ 24nt exon 17
Δ 71nt exon 15
Δ 2nt exon 18
Brca2Nes-Cre;p53-/-

Figure 3

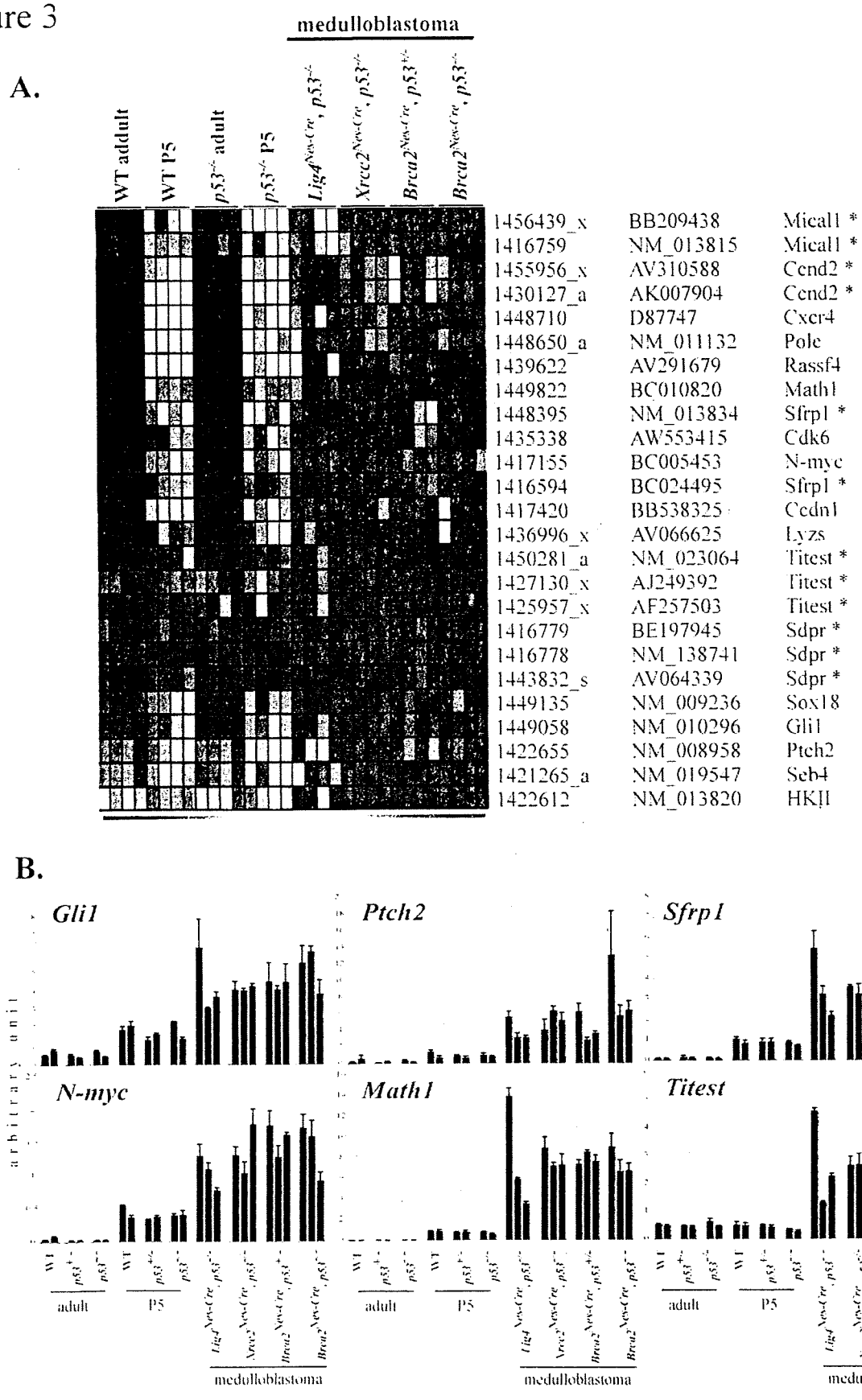

A.
medulloblastoma
WT addult
WT P5
p53-/- adult
p53-/- P5
Lig4Nes-Cre, p53-/-
Xrcc2Nes-Cre, p53-/-
Brca2Nes-Cre, p53+/-
Brca2Nes-Cre, p53-/-
1456439_x BB209438 Mical1 *
1416759 NM_013815 Mical1 *
1455956_x AV310588 Ccnd2 *
1430127_a AK007904 Ccnd2 *
1448710 D87747 Cxcr4
1448650_a NM_011132 Pole
1439622 AV291679 Rassf4
1449822 BC010820 Math1
1448395 NM_013834 Sfrp1 *
1435338 AW553415 Cdk6
1417155 BC005453 N-myc
1416594 BC024495 Sfrp1 *
1417420 BB538325 Ccdn1
1436996_x AV066625 Lyzs
1450281_a NM_023064 Titest *
1427130_x AJ249392 Titest *
1425957_x AF257503 Titest *
1416779 BE197945 Sdpr *
1416778 NM_138741 Sdpr *
1443832_s AV064339 Sdpr *
1449135 NM_009236 Sox18
1449058 NM_010296 Gli1
1422655 NM_008958 Ptch2
1421265_a NM_019547 Seh4
1422612 NM_013820 HKII
B.
Gli1
Ptch2
Sfrp1
N-myc
Math1
Titest
arbitrary unit
WT
p53+/-
p53-/-
adult
P5
Lig4Nes-Cre, p53-/-
Xrcc2Nes-Cre, p53-/-
Brca2Nes-Cre, p53+/-
Brca2Nes-Cre, p53-/-
medulloblastoma

Figure 4

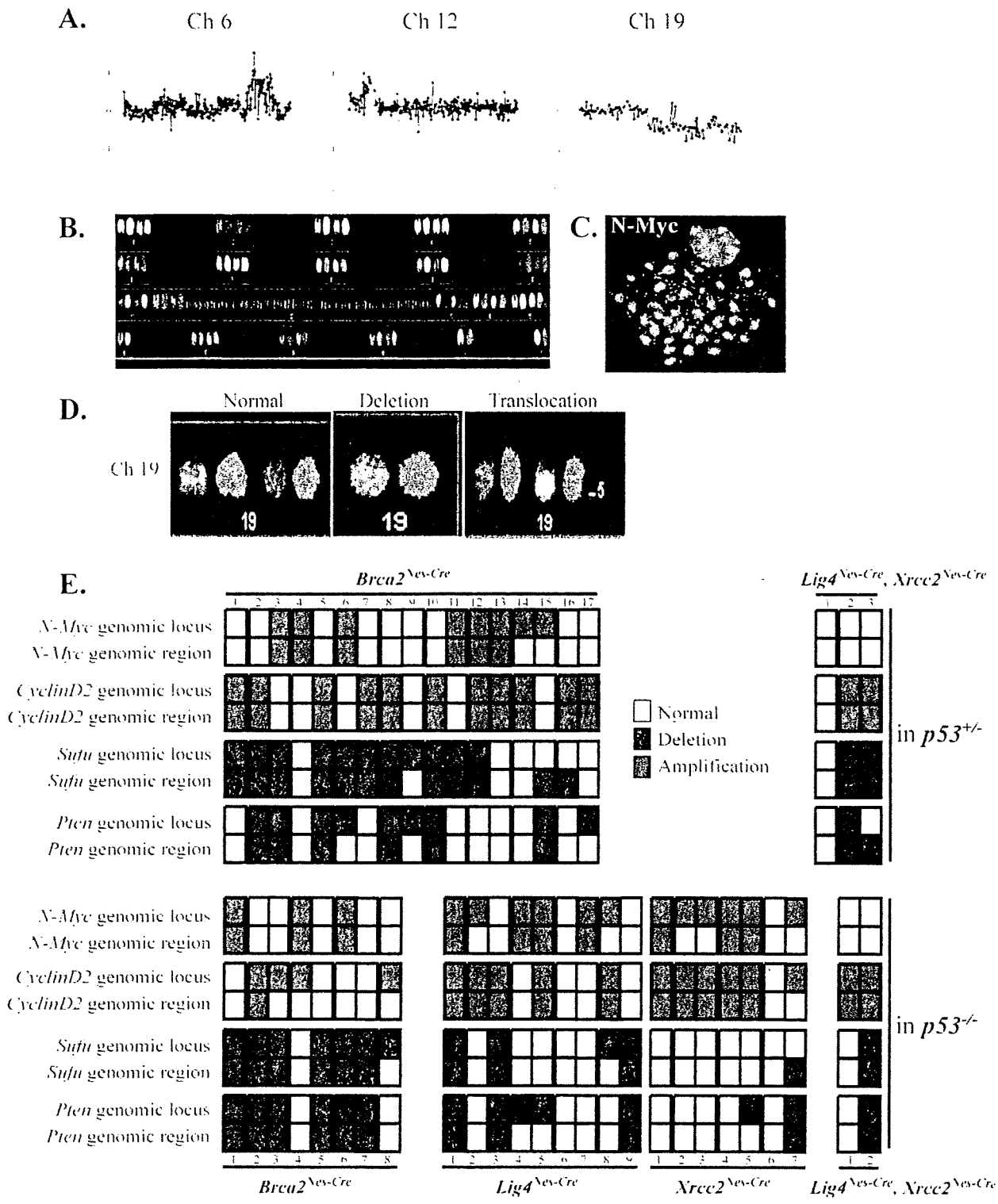
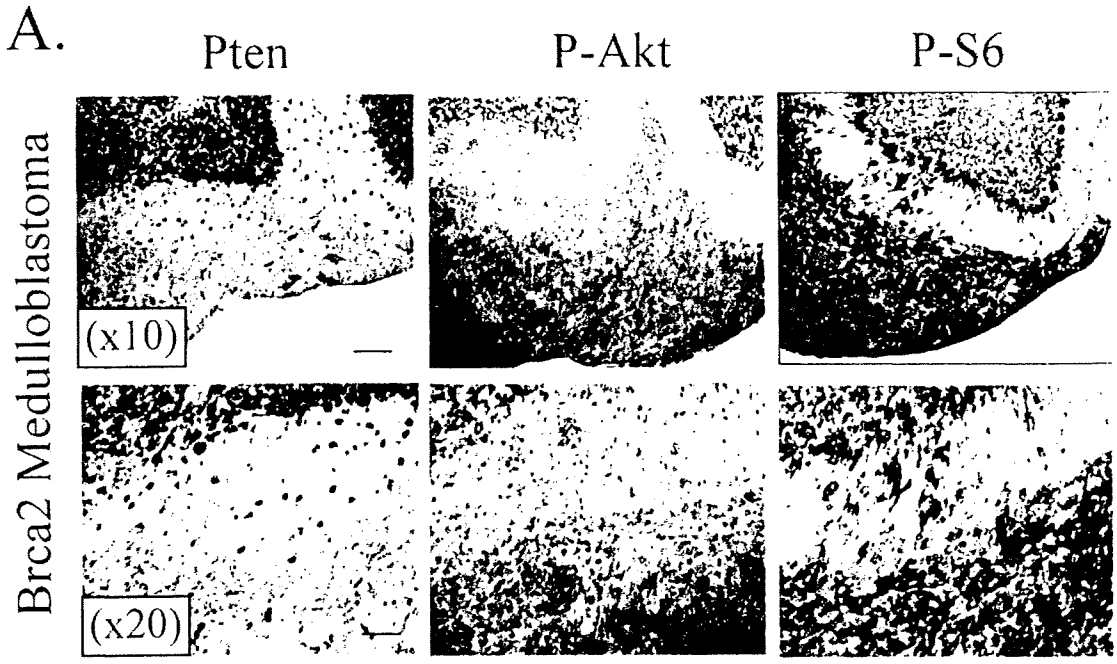
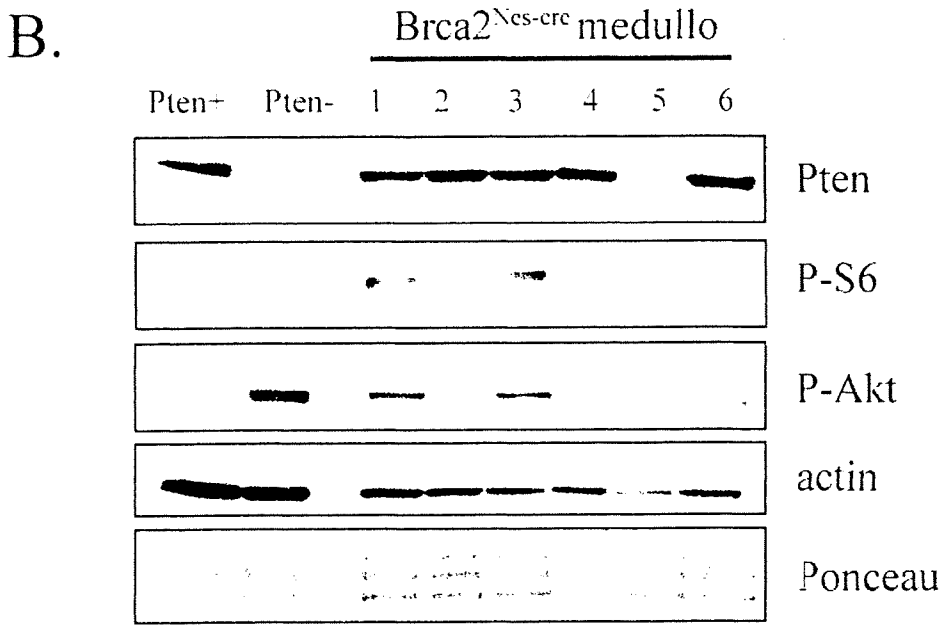

A.
Ch 6
Ch 12
Ch 19
B.
C.
N-Myc
D.
Normal
Deletion
Translocation
Ch 19
19
19
19
E.
Brca2 Nes-Cre
Lig4 Nes-Cre, Xrcc2 Nes-Cre
N-Myc genomic locus
N-Myc genomic region
CyclinD2 genomic locus
CyclinD2 genomic region
Sufu genomic locus
Sufu genomic region
Pten genomic locus
Pten genomic region
Normal
Deletion
Amplification
in p53+/-
in p53-/-
Brca2 Nes-Cre
Lig4 Nes-Cre
Xrcc2 Nes-Cre
Lig4 Nes-Cre, Xrcc2 Nes-Cre

Figure 5.





Supplementary Table 1. SKY analysis of medulloblastomas.