Diploid S in both the SF4068-Vector and SF4068-HES1 cells was not altered, suggesting that HES1 did not alter the growth rate of SF4068 cells. Similar results were obtained when HES1 was expressed in SF4433 and SF3061 meningioma cell lines (Figure 1*D*). In all three cases, HES1 expression was associated with the appearance of tetraploid cells.

HES1-Induced Tetraploidy Is Associated with Features Indicative of Chromosomal Instability

Because tetraploidization is often an intermediate step in the process to aneuploidy and enhanced aneuploidy is indicative of chromosomal instability [21], we evaluated for features of chromosomal instability in HES1 stable cells. SF4068-HES1 cells showed nuclear atypia characterized by the presence of micronuclei, nuclear bridges, and elongated and lobulated nuclei (Figure 2, *A–E*), none of which were observed in SF4068-Vector cells (Figure 2*F*). Cells with nuclear atypia were typically positive for HES1 expression (Figure 2, *G–L*). We performed immunofluorescence using an antibody against α-tubulin to view mitotic spindle structures. SF4068-HES1 cells had an increased frequency of aberrant mitotic spindle structures, including multipolar spindles (Figure 2*M–O*), none of which were observed in control cells.

Both Activated Notch1 and Notch2 Induce Tetraploidy in Meningiomas

Whereas HES1 is a primary target of Notch signaling, recent studies have shown that other signaling pathways can regulate

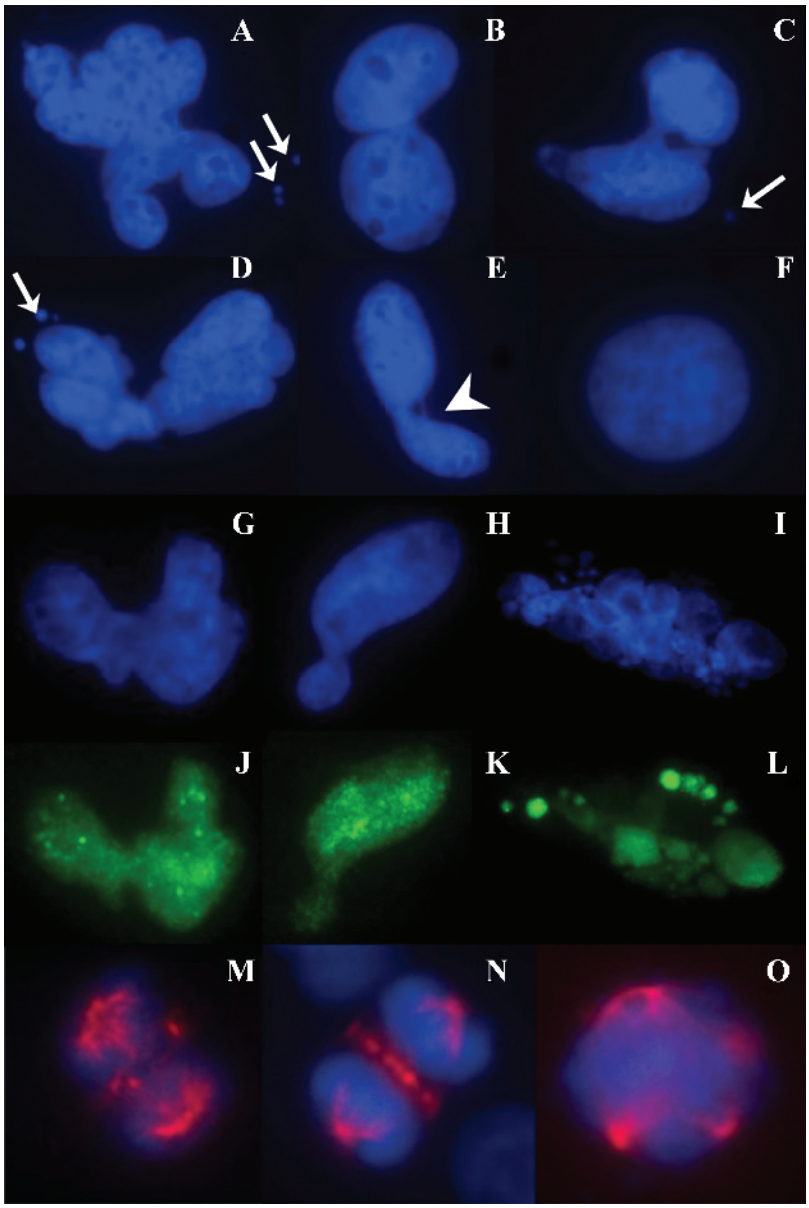

Figure 2. Tetraploidy in meningioma is associated with features of chromosomal instability and mitotic spindle anomalies. DAPI staining (blue stain) was used to show that SF4068-HES1 cells were associated with an increased frequency of nuclear atypia (A–E) characterized by the presence of micronuclei (arrows), elongated and lobulated nuclei, and nuclear bridges (arrowhead), not found in SF4068-Vector cells (F). Individual nuclei exhibiting atypia were HES1-positive (green stain; compare G–I with J–L). Staining with α-tubulin (red stain, M–O) revealed aberrant mitotic spindles, including multipolar spindles (O) in SF4068-HES1 cells.

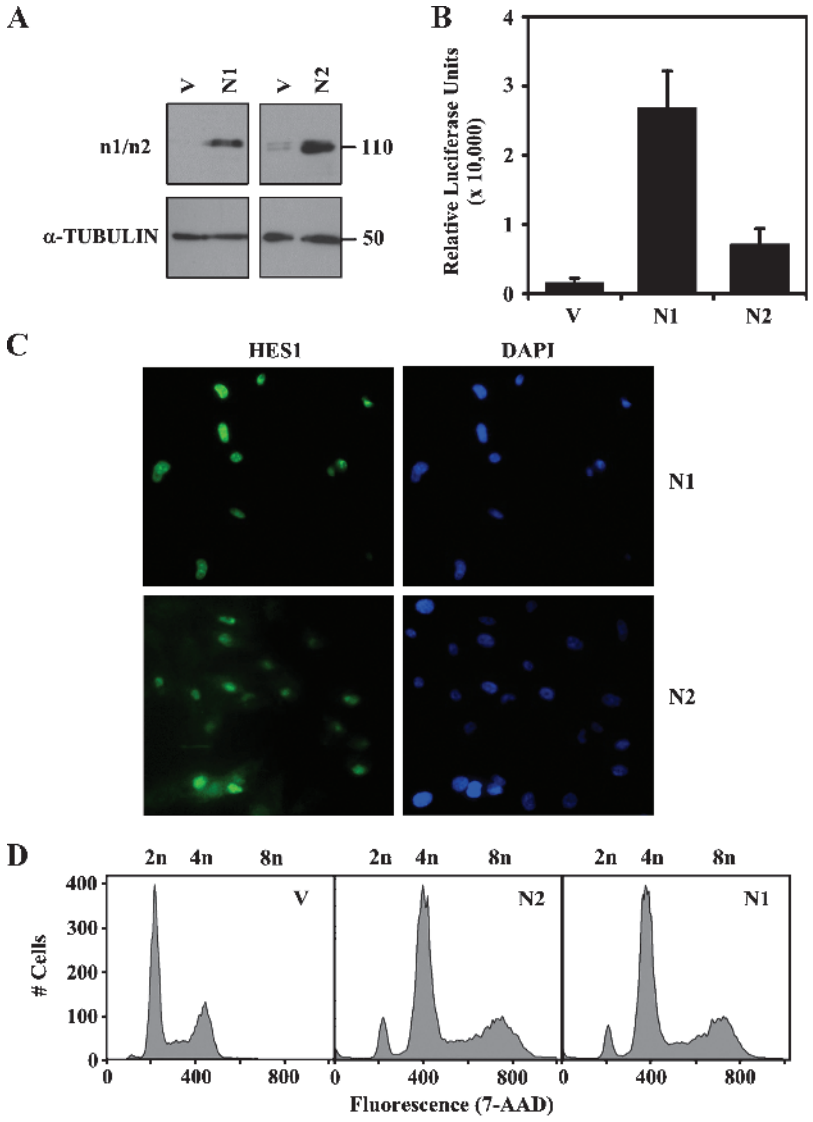

Figure 3. Activated Notch1 and Notch2 induce endogenous HES1 expression and tetraploidy in meningioma cell lines. (A) Western blot analysis of SF4068-Vector (V), SF4068-N1ICD (N1), and SF4068-N2ICD (N2) cells using monoclonal antibodies specific for Notch1 (n1) or Notch2 (n2). α-Tubulin was included as a loading control. (B) Activity of the HES1 promoter was determined using the luciferase reporter assay. Relative luciferase units (*y*-axis) in SF4068-Vector (V), SF4068-N1ICD (N1), and SF4068-N2ICD (N2) cells are plotted. (C) Immunofluorescence using the HES1 polyclonal antibody (left panels) showed induction and nuclear localization of endogenous HES1 in SF4068-N1ICD (N1) and SF4068-N2ICD (N2) stable cell populations. Nuclei were counterstained with DAPI (right panels). (D) Flow cytometric analysis for total DNA content by 7-AAD was performed in SF4068-Vector (V), SF4068-N1ICD (N1), and SF4068-N2ICD (N2) cells. The number of individual cells (*y*-axis) is plotted against total DNA content (*x*-axis). The 2n, 4n, and 8n peaks are indicated above the plots.

HES1 expression in a Notch-independent fashion [22]. In addition, different Notch receptor and ligand homologs can have different, nonredundant and sometimes opposing functions [23,24]. We had previously shown that meningiomas express both the Notch1 and Notch2 proteins [11]. We therefore evaluated whether Notch1 and/or Notch2 could induce tetraploidy in SF4068 cells by exogenously expressing the activated intracellular domains of Notch1 (N1ICD) and Notch2 (N2ICD; Figure 3*A*). Both SF4068-N1ICD and SF4068-N2ICD stable cell populations induced HES1 promoter activity (Figure 3*B*), and endogenous HES1 protein expression (Figure 3*C*) compared with SF4068-Vector cells. Both N1ICD and N2ICD expression was associated with the appearance of tetraploid cells in SF4068 (Figure 3*D*). Although the amplitude of induction of HES1 promoter activity by N1ICD and N2ICD was different, most cells in these stable cell populations were positive for nuclear HES1, and there was no difference in the incidence of tetraploidy. The N1ICD- and N2ICD-associated tetraploid cells exhibited features of chromosomal instability (data not shown). Similar results were obtained when N1ICD and N2ICD were exogenously expressed in SF4433 meningioma cells (data not shown). Thus, both Notch1 and Notch2 receptor homologs have similar functions in altering the ploidy of meningioma cells.

Tetraploid Cells Are Viable and Have Slightly Higher Rates of Spontaneous Apoptosis

Tetraploid cells have different fates depending on the cell type and the genetic context. In cell lines with an intact tetraploidy checkpoint, they are cell cycle–arrested and eliminated, whereas in others,

stable propagation of tetraploid cells is observed. In addition, tetraploid cells have altered apoptotic rates and response to apoptotic agents when compared with their diploid counterparts [25]. To evaluate whether tetraploid meningioma cells generated by expression of HES1 were viable and not undergoing massive cell death, we sorted the diploid G_1 (2n) and tetraploid G_2 (8n) populations and passaged them separately in culture (Figure 4*A*). The ploidy of the tetraploid cells was evaluated after 10 passages in culture. Greater than 90% of the cells were still tetraploid (Figure 4*B*). Thus, the tetraploid cells were viable and propagated stably. Next, we compared the apoptotic rates of tetraploid cells to diploid cells. Whereas only 0.5% of the SF4068-Vector diploid cells were spontaneously apoptotic, 4% of the SF4068-HES1 tetraploid cells were spontaneously apoptotic (Figure 4*C*). Thus, tetraploid meningioma cells had slightly elevated rates of spontaneous apoptosis.

Tetraploid Cells Acquire More Chromosomal Abnormalities Compared to Diploid Cells

We used SKY to assess the frequency of generation of numerical and structural chromosomal abnormalities in isolated SF3061-HES1 tetraploid cells compared with diploid cells. We chose SF3061 for these experiments because SF4068 and SF4433 cells have been immortalized with the *E6/E7* oncogenes, which are known to enhance genomic instability [26,27]. SKY analysis confirmed that the tetraploid G_2 (8n) population had double the DNA content of SF3061-Vector cells (Figure 5*A*). The translocations present in SF3061-Vector cells at P8 were considered baseline (Table 1 and Figure 5). These metaphases contained 9.2 translocations per metaphase and an average of 42.8 chromosomes per metaphase. In contrast, tetraploid cells at the same passage had 19.3 translocations per metaphase and an average of 89.3 chromosomes per metaphase (Table 1). Thus, both the translocations per metaphase and number of chromosomes per metaphase were slightly more than double in the tetraploid cells when compared with the corresponding diploid cells. In addition, tetraploid cells had acquired 23 translocations that were not found in the corresponding diploid metaphases. After 11 further passages in culture, tetraploid cells had acquired an average of 28.3 additional chromosomes per metaphase, whereas this number was unchanged for the diploid cells (Table 1). Moreover, tetraploid cells had acquired 137 total translocations, whereas diploid cells had only acquired 3. As an additional control, we analyzed the translocations in isolated SF3061-HES1 diploid cells at P19. Similar to the SF3061-Vector metaphases, these diploid metaphases contained 8.9 translocations per metaphase and an average of 42.3 chromosomes per metaphase. Representative SKY karyograms of diploid and tetraploid metaphase at P19 are shown in Figure 5. The tetraploid metaphase contained several new translocations that were not found in diploid metaphases such as t(2;10), t(6;4), t(10;3). In addition, this metaphase had many numerical abnormalities such as six copies of chromosome 4, 14, 16, 19, 21, and 22, six copies of the t(3;8) translocation, and three copies of the t(17;20) and the t(11;13) translocation. Thus, tetraploid cells generated by HES1 expression had a higher frequency of both numerical and structural chromosomal abnormalities when compared with diploid cells, suggesting that Notch signaling enhanced chromosomal instability in meningioma cells.

Discussion

The contribution of aberrant Notch signaling to meningioma tumorigenesis is unknown. We show that expression of activated Notch1, Notch2, and HES1 induce tetraploidy associated with features of chromosomal instability in meningioma cell lines. In addition, we show that tetraploid cells exhibit chromosomal instability developing a higher number of numerical and structural chromosomal aberrations.

Tetraploidy occurs commonly in many cancers and is often an intermediate step in the process to aneuploidy, a hallmark of most cancers and an end feature of chromosomal instability [28]. Usually, cells with tetraploid DNA content arise as an early step in tumorigenesis and precede the formation of aneuploid cells. This has been

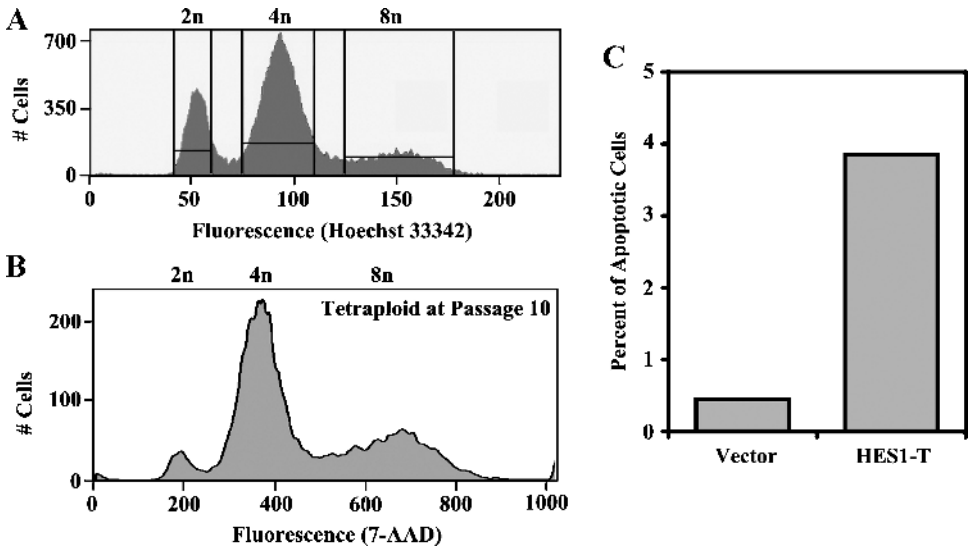

Figure 4. Isolated tetraploid cells are viable but have higher spontaneous apoptotic rates compared with diploid cells. (A) Viable diploid G_1 (2n) cells were separated from tetraploid G_2 (8n) cells using Hoechst Staining and flow cytometry and propagated in culture. (B) After 10 passages in culture, the ploidy of the tetraploid cells was measured by 7-AAD staining for total DNA content and flow cytometry. (C) The percentage of apoptotic cells (*y*-axis) in SF4068-Vector (Vector) and isolated tetraploid cells from SF4068-HES1 (HES1-T) are shown.