Mi-2/NuRD complex protects stem cell progeny from mitogenic Notch signalling

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Abstract

To progress towards differentiation, progeny of stem cells need to extinguish expression of stem cell maintenance genes. Failures in such mechanisms can drive tumorigenesis. In Drosophila neural stem cell (NSC) lineages, excessive Notch signalling results in supernumerary NSCs causing hyperplasia. However, onset of hyperplasia is considerably delayed implying there are mechanisms that resist the mitogenic signal. Monitoring the live expression of a Notch target gene, $E(spl)mγ$, revealed that normal attenuation is still initiated in the presence of excess Notch activity so that re-emergence of NSC properties occurs only in older progeny. Screening for factors responsible, we found that depletion of Mi-2/NuRD ATP remodeling complex dramatically enhanced Notch-induced hyperplasia. Under these conditions, $E(spl)mγ$ was no longer extinguished in NSC progeny. We propose that Mi-2 is required for decommissioning stem cell enhancers in their progeny, enabling the switch towards more differentiated fates and rendering them insensitive to mitogenic factors such as Notch.

Introduction

An important property of many stem cells is their ability to undergo asymmetrical divisions, generating one progeny cell that retains stem cell identity while the other is routed towards differentiation. Critically, the stem cell properties must be rapidly shut off in the latter to ensure it transitions to a different state. Failure to do so effectively can result in reactivation of stem cell programmes in the progeny, leading to hyperplasia and tumorigenesis. For example, a subset of the cells in many solid tumours (e.g glioblastomas, neuroblastomas) have characteristics of stem cells, deploying programmes that normal stem cells would use for tissue development and repair to support the development and progressive growth of the tumour (Azzarelli et al., 2018; Lathia et al., 2015; Reya et al., 2001; Singh et al., 2004). How some cells reacquire stem cell properties and what events are required to ensure that daughter cells are normally programmed towards differentiation are currently unclear.

Neural Stem Cells, known as neuroblasts (NBs) in Drosophila, have served as a powerful model to identify mechanisms that regulate asymmetrical stem cell divisions. Similar to mammalian neural stem cells, NBs divide asymmetrically to generate a larger daughter cell that
retains stem cell identity and a smaller daughter cell that will follow a more differentiated fate (Homem et al., 2015; Knoblich, 2008; Sousa-Nunes et al., 2010). After entering quiescence at the end of embryogenesis, NBs resume divisions in larval stages, upon receiving proper nutrition signals from their adjacent glial cells (Chell and Brand, 2010; Sousa-Nunes et al., 2011). In each division, key cell fate determinants such as Prospero, Numb and Brat are segregated into the smaller progeny cell. Together these factors help switch off the stem cell programme and their loss disturbs the balance between self-renewal and differentiation (Bello et al., 2006; Betschinger et al., 2006; Choksi et al., 2006; Hirata et al., 1995; Ikeshima-Kataoka et al., 1997; Knoblich et al., 1995; Lee et al., 2006b; Rhyu et al., 1994). For example, Numb inhibits Notch activity, and the loss of Numb or other mechanisms giving excess Notch signalling can lead to hyperplasia (Bowman et al., 2008; Lee et al., 2006a; Wang et al., 2006; Weng et al., 2010).

However, certain lineages, so-called Type I lineages, appear resistant to the ectopic Notch activity as the majority of their daughter cells continue to be routed towards differentiation even in these conditions.

Type I and Type II NBs can be distinguished based on their mode of division as well as the differential expression of specific molecular markers. In Type I lineages, the smaller daughter, so called Ganglion mother cell (GMC), divides only once to generate two post-mitotic neurons or glia cells. In Type II lineages the smaller progeny of the NBs become intermediate neural progenitors (INP), which then divide 3-6 times asymmetrically to self-renew and to generate GMCs that, like the GMCs derived from Type I NBs, then divide into two post-mitotic neurons or glia cells (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009; Kang and Reichert, 2014; Knoblich, 2008). Both Type I and Type II neuroblasts exhibit high levels of Notch activity and express key targets deadpan (dpn), Enhancer of split-HLHmγ (E(spl)mγ) and klumpfuss (klu), that are shut off in the progeny (Almeida and Bray, 2005; Berger et al., 2012; San-Juán and Baonza, 2011; Xiao et al., 2012; Zacharioudaki et al., 2016, 2012). Type II lineages are highly sensitive to excessive Notch activity, which brings about ectopic expression of stem cell markers in INPs to drive NB-like behaviour and tumour formation (Bowman et al., 2008; Wang et al., 2006; Weng et al., 2010; Zacharioudaki et al., 2016, 2012). In contrast, Type I lineages only become hyperplastic under extreme conditions. Many factors have been found that enhance the sensitivity of Type II lineages to hyperplasia (Bayraktar and Doe, 2013; Berger et al., 2012; Eroglu et al., 2014; Koe et al., 2014; Liu et al.,...
2017; Weng et al., 2010; Xiao et al., 2012; Xie et al., 2016) however few of these moderate the onset of hyperplasia in Type I lineages and it remains unclear how ultimately Type I progeny cells become engaged to re-initiate a stem cell program.

Monitoring the emergence of hyperplasia in NB lineages with constitutive Notch activity we show that prolonged exposure is required before NB-like tumour cells are formed in Type I lineages. Furthermore, these NB-like cells arise from a reversion of the post-mitotic progeny, rather than from newly born GMCs. Reasoning that there are intrinsic mechanisms that attenuate stem cell programmes and Notch activity in NB progeny, we screened for genes whose depletion accelerated tumourigenesis of Type I NB lineages. To do this we used RNAi knock-down and focused on genes that have been associated with tumour formation in human neuroblastomas. Our results reveal that Mi-2, and other members of the NuRD ATP-remodelling complex, suppress the emergence of stem-cell characteristics in Notch induced conditions. We propose Mi-2/NuRD complex normally acts to decommission enhancers that are active in the stem cells, so that they become refractory to Notch and other signals in the stem cell progeny.
Results

NB lineages are initially resistant to excessive Notch signalling

Prolonged expression of constitutively active Notch (N\textit{Δecd}) in NB lineages results in their overproliferation and the formation of many NB-like cells expressing key targets such as \textit{dpn}, \textit{E(spl)γ} and \textit{klu} (Bowman et al., 2008; Wang et al., 2006; Weng et al., 2010; Xiao et al., 2012; Zacharioudaki et al., 2016, 2012). To better characterise the onset and progression of Notch driven hyperplasia, constitutive active Notch (\textit{N\textit{Δecd}, here referred to as Nact}) was expressed for a short (8 hours), medium (24 hours) or long (48 hours) period of time in larval Type I NBs (via grhNB-Gal4 Gal80ts) to produce nerve cords bearing supernumerary Dpn-expressing cells (Figure 1A,B). In the early stages of Notch driven hyperplasia (8 hours), only a small proportion of Type I lineages turned hyperplastic (22%, Figure 1C) and these contained only a few extra cells expressing stem cell markers. Testing a panel of such stem cell markers revealed that \textit{E(spl)γ} and \textit{dpn} were the first to be expressed and, of the two, \textit{E(spl)γ} appeared to be the earliest as there was a subset of cells in hyperplastic lineages that express \textit{E(spl)γ-GFP} only (2±0.2; Figure 1A,D; Figure 1-figure Supplement 1). Slightly more cells per lineage expressed both \textit{E(spl)γ-GFP} and Dpn (6.6±0.5; Figure 1A,D). Nevertheless, it is striking that relatively few Type I lineages exhibit ectopic expression of these direct Notch targets even after 8 hours of exposure to active Notch.

Continued expression of Nact for 24 hours led to an increase in the number of Type I lineages with ectopic NB markers (36.3%; Figure 1A,C), as well as to an expanded number of cells per lineage with ectopic \textit{E(spl)γ-GFP} only (4.6±0.6; Figure 1A,D) and with both \textit{E(spl)γ-GFP} and \textit{dpn} expression (18.8±1.1; Figure 1A,D). However, it was only with more prolonged Notch activity (48 hours) that the majority of lineages became hyperplastic (89.3%; Figure 1A,C) with a large fraction of the cells in each lineage expressing stem-cell like markers so that large regions were occupied by NB-like cells (Figure 1A). Notably, the cells that acquired stem cell characteristics were intermediate in size between a GMC and a NB, suggesting that they do not arise from a symmetrical division of a pre-existing NB. Furthermore, the NBs themselves continued to divide asymmetrically even in the presence of excessive Notch signaling (Figure 1-figure Supplement 2). To rule out the possibility that the change in tumourigenic potential was due to the age of the NBs rather than the time of exposure to Notch activity, we
also performed experiments where we varied the time of onset of exposure. This yielded identical results, i.e. the extent of hyperplasia correlated with the duration of exposure not the developmental stage at which the NBs were exposed (Figure 1-figure Supplements 3).

In summary, even after 24 hours of exposure to Notch activity, only a few Type I stem cell lineages become hyperplastic and these contain only a small number of cells expressing the “early” stem cell identity markers, $E(spl)\text{m}_\gamma$ and $dpn$. This differs from the situation in Type II lineages, which undergo much more severe overproliferation and suggests that there is a mechanism conferring resistance to Notch in the Type I NB progeny. With more prolonged Notch activity, this resistance is overcome and a large majority of cells acquire stem-cell like markers (Figure 1B).

**Hyperplasia involves NSC progeny re-acquiring stem cell properties.**

Notch activity drives additional cells to acquire NB-like characteristics. However, it is unclear whether this arises at the time of NB division, so that the GMC retains a stem cell fate, or whether the neuronal progeny re-acquire stemness. To distinguish between these possibilities, we analysed the expression of $E(spl)\text{m}_\gamma$-GFP in NB lineages in real time, by culturing NBs from normal brains and Notch-driven hyperplastic brains (after 24 hours with Nact). NBs were imaged continuously for 10 to 14 hours and their progeny tracked following each division to determine whether or not they maintained or reacquired $E(spl)\text{m}_\gamma$-GFP expression. In these conditions, normal NBs underwent asymmetric cell divisions and $E(spl)\text{m}_\gamma$-GFP expression was rapidly extinguished in the GMC progeny (Figure 2A,C,E and Figure 2-video 1). In the NB itself the levels fluctuated, with a defined temporal pattern through the cell cycle where the highest expression occurred as the NBs entered and exited mitosis (Figure 2A,C,E and Figure 2-video 1).

In the presence of high levels of Notch activity, the NBs continued to undergo asymmetric cell divisions and the expression of $E(spl)\text{m}_\gamma$-GFP was extinguished in the GMC, albeit with slightly slower kinetics than in normal conditions (Figure 2B,D,F and Figure 2-video 2). After a few hours however, some of the NB progeny reacquired $E(spl)\text{m}_\gamma$-GFP expression. The reappearance of $E(spl)\text{m}_\gamma$-GFP only occurred in progeny born before the onset of imaging (Figure 2B,D,F and Figure 2-video 2), indicating that it must occur in cells that had been born
from the NSC >8 hours earlier. As the GMC divides after ~4 hours, it is likely that the cells regaining \( E(spl)\gamma-GFP \) are progeny of a GMC division. Furthermore, in most cases they were intermediate in size between NBs and GMCs, suggesting that these changes precede the expression of \( E(spl)\gamma-GFP \). Subsequent to expressing \( E(spl)\gamma-GFP \), the cells adopted an asymmetric mode of division and only the larger daughter cell retained expression (see P2 in Figure 2B and D). These profiles indicate that NB-like cells arise from a reactivation of the stem cell programme in the progeny, rather than an expansion of the stem cells themselves, and that the normal process of stem cell fate attenuation is initiated but subsequently overcome.

**Mi-2 attenuates the response to Notch in NSC lineages.**

To identify the mechanisms that attenuate the response to Notch, delaying the transformation towards tumourigenesis, we screened for genes whose depletion by RNAi was sufficient to accelerate Notch induced tumourigenesis. The candidates were primarily drawn from the pool of genes that are frequently mutated in human solid brain tumours (e.g. neuroblastomas and glioblastomas) based on genome-wide sequencing studies (Bosse and Maris, 2016; Chmielecki et al., 2017; Huether et al., 2014; Lin et al., 2016; Parsons et al., 2011; Pugh et al., 2013; Wu et al., 2014). To probe function in the context of Notch induced tumours we used GrhNB-Gal4 to drive Nact in combination with RNAi lines targeting candidate genes and monitored the consequences on Dpn. Expression was restricted to a 24-hour window, using Gal80ts, a condition where constitutively Nact produces only mild hyperplasia, as described above (Figure 1A).

From a total of 124 genes screened in this way, only a small proportion (13.7%) modified the Notch induced hyperplasia. Of those, the most striking effect was seen with the knock down of Mi-2, a member of the NuRD ATP-remodelling complex. This significantly enhanced the number of Dpn-positive (\( \text{Dpn}^{\text{+ve}} \)) cells elicited by Nact: the total number of Dpn expressing cells was enhanced 2.7-fold when Mi-2 was depleted compared to Nact alone (1322±71 vs 485±27; Figure 3A and D). In contrast, Mi-2 depletion alone was not sufficient to bring about any increase in Dpn+ve cells (161±2 vs 158±2), despite that this treatment robustly extinguished Mi-2 protein levels in the NB lineages (Figure 3- figure supplement 1A). Similar effects, where Mi-2
depletion enhanced the hyperplasia from Nact, were obtained using a range of different Gal4 lines that drive expression in the NB and its early born progeny (Figure 3- figure supplement 1B,C,E,F). No such effects were seen when active Notch and Mi-2 RNAi, were co-expressed in more mature neuronal progeny (Figure 3- figure supplement 1D,G), indicating there is a limited window during which they can revert these cells to NBs.

As Mi-2 is a core member of the NuRD chromatin remodelling complex, we next asked whether loss of other members of this complex would exhibit similar effects in the Notch driven hyperplasia. Knock down of MTA-like (Figure 3B) or Caf1-p55 (Figure 3C) both produced a 1.4-fold enhancement of Notch induced hyperplasia (Figure 3E,F). The fact that these phenotypes are relatively weak, compared to that from depleting Mi-2 may be technical, due to differences in knock-down or protein stability, or it may indicate that the other subunits, which are involved in protein-protein interactions, are less critical than the catalytic Mi-2. Nevertheless the results suggest that Mi-2 functions in attenuating Notch activity via its participation in the NuRD complex.

To investigate whether the increase in Dpn^+ve cells occurs because Mi-2/NuRD complex is necessary to switch off the response to Notch in GMCs, we performed live imaging of E(spl)mγ-GFP in NSC lineages with reduced Mi-2 with and without Nact. Depletion of Mi-2 alone had no discernible effect on E(spl)mγ-GFP in GMCs: it was extinguished as in normal conditions (Figure 4-figure supplement 1 and Figure 4-video 1). However, in the context of Nact there was a striking persistence of E(spl)mγ-GFP in the GMCs in a substantial proportion of lineages (27.8%; Figure 4A-E and Figure 4-videos 2,3). Thus, the newly born GMCs retained high levels of E(spl)mγ-GFP for the full duration of the movie, while the NB underwent several rounds of division (Figure 4A-D and Figure 4-videos 2, 3). Furthermore, when E(spl)mγ-GFP expressing progeny then divided, both daughter cells retained E(spl)mγ-GFP unlike the situation in the presence of Nact alone, where the acquisition of E(spl)mγ-GFP in the progeny was linked to them adopting asymmetrical divisions. These data demonstrate therefore that Mi-2/NuRD complex has an important role in suppressing the ability of the stem cell progeny to respond to Notch and suggests that it contributes to the shut-down of stem cell promoting genes.

Mi-2 loss leads to de-repression of E(spl)-C genes
To investigate further whether Mi-2 is important for attenuating the response of Notch-regulated enhancers, we analysed its effects at the E(spl)-Complex in Kc cells, where we have previously analysed the changes in expression and in the recruitment of the Notch pathway transcription factor, Suppressor of Hairless [Su(H)], in the presence and absence of Notch activity (Skalska et al., 2015). Down-regulating Mi-2 by treating cells with RNAi resulted in a robust increase in the expression of E(spl)mβ, E(spl)m3 (Figure 5A-C). These effects were similar to those seen when the co-repressor Hairless was depleted (Figure 5C,D and Figure 5-figure Supplement 1A). Furthermore, loss of Mi-2 in the context of conditions where Notch signalling was activated, led to an enhanced induction of the E(spl) genes that are normally up-regulated in these conditions (Figure 5C). Similar effects were observed for 3 other Notch regulated genes in Kc cells, which were up-regulated following depletion of Mi-2 (Figure 5-figure Supplement 1B). Thus, loss of Mi-2 leads to de-repression of E(spl) genes and of other Kc cell Notch targets in Notch off conditions and enhances their activation in Notch on conditions.

The effects of Mi-2 depletion are similar to those when Hairless is removed. Thus, one model is that Mi-2 is normally recruited by the Su(H)/Hairless corepressor complex to shut down target enhancers. If Hairless is required to recruit Mi-2, the combined knock down should produce similar effects to knockdown of Hairless alone. In contrast, concomitant knock down of both Mi-2 and Hairless led to a much greater de-repression of E(spl)mβ and E(spl)m3 than from either knock down alone, suggesting the two factors work independently to repress these genes (Figure 5E and Figure 5-figure Supplement 1C). In agreement, no interaction between Su(H) and Mi-2 was detected (Figure 5F) arguing that Mi-2 acts independently of the SuH-Hairless co-repressor complex to attenuate the expression of E(spl) genes. A second hypothesis was that Mi-2 reorganises the chromatin at target enhancers, preventing enhanced recruitment of Su(H) in the presence of Nact (Kreher et al., 2017). However, loss of Mi-2 had no effect on Su(H) recruitment in either Notch off or Notch on conditions (Figure 5G) neither did it affect the levels of H3K27 trimethylation, the repressive histone modification deposited by Polycomb complexes, or of H3K27ac (Figure 5-figure Supplement 1D,E).

These data raise the question whether the Mi-2 complex acts directly at the target enhancers or exerts its effects indirectly. We therefore examined the overlap between Notch regulated enhancers and Mi-2 bound regions based on previous genome-wide profiling of Su(H).
(Skalska et al., 2015) and Mi-2 (Ho et al., 2014) by chromatin immunoprecipitation in Kc cells. Strikingly, 72% of Su(H) bound regions overlapped with Mi-2 occupied sites. These included significant enrichments for Mi-2 at the E(spl)mβ and E(spl)m3 regulatory regions in the E(spl)-C (Figure 5A) which we verified by ChIP qPCR (Figure 5H). Thus Mi-2 is present at Notch regulated enhancers suggesting it has a direct effect on their activity.

In summary, Mi-2 binds directly to Notch target genes and exerts a repressive effect on them, even in Kc cells, but it appears to do so independently of Su(H) and Hairless. This implies that other factors must be involved in its recruitment.

**Zfh1 cooperates with Mi-2 in stem cell lineages.**

If Mi-2 recruitment is not dependent on Su(H), this implies that other DNA-binding transcription factors are required. Likely candidates in the NB lineages include transcription factors that are required to promote differentiation or that have been linked to Mi-2. The former include Prospero, which is asymmetrically segregated and accumulates in the nuclei of the GMCs, and Lola, which is highly expressed in stem cell progeny and is necessary to prevent their de-differentiation (Southall et al., 2014). The latter include Tramtrack, a BTB zinc-finger protein shown to bind directly to Mi-2 (Murawsky et al., 2001; Reddy et al., 2010), and Zfh1, a zinc-finger-homeodomain repressor whose homologue ZEB2 cooperates with NuRD complex to antagonise Notch in Schwann cells (Wu et al., 2016). We therefore tested whether depletion of any of the candidate factors was sufficient to enhance Nact induced hyperplasia in a similar way to Mi-2. Of those tested, only two factors significantly enhanced the hyperplasia, namely Prospero and Zfh1, indicating that they were plausible candidates to recruit Mi-2 (Figure 6A,B and Figure 6-figure Supplement 1A, B).

If Prospero or Zfh1 are involved in attenuating the activity of stem cell genes via their recruitment of Mi-2, their ectopic expression in NBs might promote their differentiation in a Mi-2 dependent manner. Indeed, expression of either Prospero or Zfh1 was sufficient to significantly reduce the number of Dpn+ve cells, suggesting they have suppressed NB identity (Figure 6C,D and Figure 6-figure Supplement 1C,D). Furthermore, depletion of Mi-2 partially overcame the effects of Zfh1 overexpression, restoring the number of NBs to close to normal levels (Figure 6B...
and D). In contrast, Mi-2 depletion failed to rescue the effects from Prospero over-expression (Figure 6-figure Supplement 1C,D). Finally, if Zfh1 antagonizes the ability of Notch to promote expression of stem cell genes, its ectopic expression in NBs with excessive Notch should prevent the formation of hyperplasia. In agreement, Zfh1 overexpression attenuated the Notch induced hyperplasia, an effect that was partially abolished by loss of Mi-2 (Figure 6-figure Supplement 2A-B). Together these data argue that Zfh1 cooperates with Mi-2 to suppress stem cell identity.

If Zfh1 is involved in shutting down the stem cell programme with Mi-2, it should be present in the NSC progeny during the period where they are resistant to ectopic Notch activity. Indeed, using a fly line in which the endogenous Zfh1 is fused to GFP (Albert et al., 2018), Zfh1 was detected in the progeny that are located in proximity to the NB and in the Asense labelled GMCs, albeit at lower levels (Figure 6E) in Type I NB lineages. In contrast, Zfh1 was absent from the NB and INPs in Type II lineages albeit these cells exhibited strong or moderate Mi-2 levels respectively (Figure 6-figure Supplement 2C). Together the results point to Zfh1 as an important intermediary in recruiting Mi-2/NuRD complex to shut off stem cell enhancers in the NB progeny of Type I lineages. However it is likely that different factors will recruit Mi2 in other contexts where it is required to tune Notch responsive enhancers.
Discussion

In order to progress towards differentiation, stem cell progeny need to shut off the transcription of genes involved in maintaining the self-renewing stem cell programme. Our results demonstrate the importance of Mi-2/NuRD chromatin complex in making this switch in *Drosophila* NB lineages, specifically in Type I lineages. Under normal conditions, these stem cell progeny are remarkably resistant to ectopic Notch activity, only reverting to a more-stem cell like fate after prolonged exposure to a constitutively active Notch. However, when Mi-2 or other members of the NuRD complex were depleted, progeny cells reverted with much higher frequency when exposed to high levels of Notch activity, leading to rapid onset of hyperplasia. Thus we propose that Mi-2/NuRD complex functions to decommission the stem cell enhancers in the differentiating progeny rendering them resistant to stem cell promoting factors like Notch (Whyte et al., 2012). As the iterative use of signaling pathways, including Notch, is important throughout development to generate different tissue and cell types, it is likely that NuRD will be widely deployed in such developmental transitions to decommission enhancers in readiness for subsequent lineage decisions.

The fact that expression of Nact fails to sustain expression of even its direct target genes, *E(spl)mγ-GFP* and *dpn*, in the progeny GMCs argues that their enhancers have become refractory. As the multi-protein NuRD complex contains both the Mi-2/CHD4 ATP-dependent chromatin remodelling and histone deacetylase subunits it could curtail enhancer activity by promoting an increase in local nucleosome density at these enhancers or by depleting H3K27 acetylation, as occurs in mouse embryonic stem cells where Mi-2 enables recruitment of Polycomb repression complexes (Reynolds et al., 2012b). Based on the results from analysing chromatin changes following Mi-2 knock-down in cultured cells, we favour the former model. No change in H3K27ac or H3K27me3 was observed at Notch target enhancers despite the fact that the genes were de-repressed. Furthermore, there was no evidence for eviction of the key transcription factor Su(H) from these enhancers. This is more compatible with a model where NuRD remodelling perturbs the recruitment of Mediator and PolII to restrict initiation from these genes (Bornelöv et al., 2018).

Although originally considered a repressive complex, NuRD is found at most sites of active transcription in embryonic stem cells, where it appears to fine-tune gene expression as well as
having a major role in allowing cells to exit self-renewal (Bornelöv et al., 2018; de Dieuleveult et al., 2016; Miller et al., 2016; Reynolds et al., 2012a, 2012b). It remains unclear what triggers the changes that enable this transition towards differentiation. One possibility is that the first step towards decommissioning or repurposing these enhancers involves removal of positive factors that keep a “check” on NuRD activity with the consequence that NuRD predominates to increase local nucleosome density (Whyte et al., 2012). At the same time, the up-regulation of differentiation factors that favour NuRD complex recruitment can enhance such a switch. This is the mechanism we propose for Zfh1, which is up-regulated in the NB progeny and whose ability to suppress the stem cell programme when ectopically expressed appears to require Mi-2. Depletion of Zfh1 in the context of ectopic Nact also enhanced hyperplasia, similarly to depletion of Mi-2. Although there are caveats to using RNAi for establishing epistatic relationships, the data argue that Zfh1 and NuRD act together to render enhancers of genes such as E(spl)γ-GFP and dpn refractory to Notch activity. This is similar to the role proposed for the mammalian homologue ZEB2 during Schwann Cell differentiation, where it engages the NuRD complex to antagonise Notch and Sox2 (Wu et al., 2016). Indeed, a missense mutation in the Zeb2 that abolished its association with the NuRD complex rendered it incapable of promoting Schwann Cell differentiation (Wu et al., 2016).

Other factors have also been shown to block responsiveness to Notch. For example eyeless/Pax6 is switched on in the progeny of old Type II NBs and makes them refractory to Notch (Farnsworth et al., 2015) and Ikaros expression during T-cell differentiation shapes the timing and repertoire of Notch target genes (Zhang et al., 2012). Whether these factors rely on Mi-2 remains to be explored but as Ikaros was found to associate with the Mi-2/NuRD complex, it could rely on similar mechanism to Zfh1/Zeb2 (Sridharan and Smale, 2007). Likewise, PROX 1, the mammalian homologue of Prospero, interacted with NuRD complex to suppress Notch pathway in colorectal cancer cells. We propose therefore that different factors will be involved in recruiting Mi-2 depending on the cell-type. For example, in Kc cells the Su(H) bound regions are enriched for the binding motif for Tramtrack, which has been shown to physically interact with Mi-2 (Murawsky et al., 2001) and is thus a good candidate. Conversely, the fact that Type II neuroblast lineages are exquisitely sensitive to ectopic Notch activity argues that these cells lack factor(s) that recruit Mi-2 to the target enhancers. Indeed Zfh1 is not expressed in the intermediate progeny of these lineages and it has been shown that an alternate mechanism,
involving the repressor earmuff/dFezf is deployed to repress the competence of INPs to respond
to Notch targets and thus their dedifferentiation into NBs (Janssens et al., 2014; Koe et al.,
2014). The fact that prolonged Notch activity can ultimately reprogramme progeny towards the stem
cell fates, driving hyperplasia even in the presence of Mi2, argues that NuRD is not fully
silencing target genes. This fits with the model that NuRD is involved in fine-tuning
transcriptional responses (Bornelöv et al., 2018). Thus, at enhancers where NuRD predominates
it would increase nucleosome density rendering the enhancer less accessible so that higher
amounts of signal are required to overcome it. If active Notch accumulates in the older progeny,
it could reach a critical threshold to win-out over NuRD. Alternatively, Mi2/NuRD may only be
required during the acute phase of transitions and be replaced at later times with other modes of
repression that can be more easily overcome by Notch activity. The fact that Zfh1 expression is
not sustained in older progeny fits with this model. However, distinguishing these possibilities
will require an understanding of the real time dynamics of the chromatin changes at target loci in
individual progeny.
## Materials and Methods

### Key Resources Table

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<td>Alexander Brehm, Marburg, Germany</td>
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Drosophila genetics

Drosophila stocks are described in FlyBase and were obtained from the Bloomington, Vienna or Kyoto Stocks Centres unless otherwise indicated. Over-proliferating third instar larval CNSs were generated by crossing UAS-N\textDeltaecd; UAS-wRNAi flies with tub-Gal80ts; grh-Gal4 flies to drive expression in most NSCs (Prokop et al., 1998). Crosses were kept at 18°C for 7 days, then shifted to 30°C for 8h, 24 h or 48h prior to dissection.

Flies for the live imaging experiments were of the following genotypes:

Control: UAS-LacZ, E(spl)m\gamma-GFP; H2Av-RFP,

Overexpressing active Notch: UAS-N\Deltaecd, E(spl)m\gamma-GFP; H2Av-RFP

Depletion of Mi-2 in presence of Nact: UAS-N\Deltaecd, E(spl)m\gamma-GFP; UAS-Mi-2RNAi

All above flies were crossed to tub-Gal80ts, H2Av-RFP; grhNB-Gal4 flies. These crosses were kept at 18°C for 7 days, then shifted to 30°C for 24 h prior to dissection and brain dissociation.

For knock down of members of the NuRD complex or differentiation TFs in NSCs, the following RNAi lines were initially combined with UAS-N\Deltaecd or UAS-LacZ and then crossed to tub-Gal80ts; grhNB-Gal4 flies: UAS-w RNAi (BL35573), UAS-Mi-2 RNAi (BL33419), UAS-MTA1-like RNAi (BL33745), UAS-Caf1p-55 RNAi(BL34069), UAS-zfh1 RNAi (BL29347), UAS-Pros RNAi (BL26745). Crosses were kept at 18°C for 7 days, then shifted to 30°C for 24 hours prior to dissection. Transgenic Flies carrying the putative Notch-regulated enhancers (NREs) in Cyclin E and pathetic (Djiane et al., 2013) were also crossed with the above genotypes to assess the effects of Mi-2 knockdown in other Notch target genes in Nact NB lineages.

For overexpression of TFs in NBs, the following lines: UAS-lacZ (control), or UAS-zfh-RB (made by Antonio Postigo (Siles et al., 2013); BL6879) were combined with UAS-Mi-2 RNAi or UAS-w RNAi (control) and crossed to insc-Gal4; tub-Gal80ts. UAS-ProsYFP (Choksi et al., 2006) or UAS-LacZ were combined with UAS-Mi-2 RNAi or UAS-w RNAi (control) and crossed to tub-Gal80ts; grhNBGal4. The progeny were kept at 18°C for 7 days and transferred to 30°C for 24 (, Pros) or 48 (Zfh1) hours prior to dissection.

Flies for assessing changes in NB asymmetric mode of division were of the following genotypes:

Control : UAS-LacZ; Ase-mcherryPonLD, (Derivery et al., 2015)

Overexpressing active Notch : UAS-N\Deltaecd; Ase-mcherryPonLD
These flies were crossed to *tub-Gal80ts; grh-Gal4* flies. Crosses were kept at 18°C for 7 days, then shifted to 30°C for 24 h prior to dissection.

Flies with a CRISPR engineered insertion of GFP into the *zfh1* locus, (*Zfh1-GFP* (Albert et al., 2018)) were used to visualise the expression of *Zfh1*.

**Immunofluorescence**

Fixation and immunohistochemistry of larval tissues were performed according to standard protocols. Primary antibodies were guinea pig anti-Dpn (1:2000) courtesy of Christos Delidakis; mouse anti-Mira [1:100, (Ohshiro et al., 2000)]; mouse anti-Pros MR1A (1:50, DHSB); rabbit anti-Ase (1:5000, courtesy of Y.N.Jan); rabbit anti-Grh (1:2000, gift of Christos Samakovlis); rabbit anti-Mi-2 (1:10,000, courtesy of Alexander Brehm (Kreher et al., 2017)). Mouse, rabbit or guinea pig secondary antibodies were conjugated to Alexa 488, 555, 568, 633 or 647 (Molecular Probes) or to FiTC, Cy3 or Cy5 (Jackson ImmunoResearch). Samples were imaged on a Leica TCS SP8 confocal microscope (Cambridge Advanced Imaging Center (CAIC), University of Cambridge).

**Live imaging of NBs**

Larval brains were dissected in dissection media (1.4M NaCl, 26mM KCl, 4mM NaH₂PO₄, 120mM NAHCO₃ and 50mM Glucose). Brains were dissociated in collagenase solution [2mg/ml (Sigma, C0130)] for 15 min, rinsed with culture media [Schneider medium (GIBCO 21720-024), 1mg/ml Glucose (D-L- glucose monohydrate) with 10% FBS (F4135), 2.5% fly extract, 1mg/ml human insulin (Sigma, I9278) and 1x Antibiotic Antimycotic (GIBCO, 15240-062)] and transferred into microcentrifuge tubes with 40μl/brain culture media. Brains were subsequently sheared mechanically and the emerging dissociated cells were placed into Poly-D-Lysine coated plates. Cells were left to rest for 30 min prior to imaging for 10-14 hours (Pampalona et al., 2015) on a Leica TCS SP8 confocal microscope (Cambridge Advanced Imaging Center (CAIC), University of Cambridge).

**Live imaging of whole brain explants**
Whole brains were dissected from 3rd instar larvae in Schneider’s medium and cultured according to an improved protocol for long term culturing and imaging of larval brains (Cabernard and Doe, 2013; Hailstone et al., 2017). Live imaging was performed for 26 hours in an inverted Olympus FV1200 confocal microscope (Ilan Davis Group, Department of Biochemistry, University of Oxford).

**Cell tracking and time-lapse movie image analysis**

Cells were segmented in 3D from the his2Av-RFP signal using a combination of median filtering, thresholding, active contour segmentation and 3D watershed to separate nuclei in contact. They were then tracked over time by finding the closest neighbour in a 12px radius and allowing to search in the 5 previous frames. The mean intensity in the green channel (from E(spl)γ-GFP) was obtained from the overlap with the tracked nuclei. Cells that were not correctly tracked were manually corrected afterwards and mother-daughter cells assigned. To account for possible differences in fluorescence levels between movies, mean intensity values were normalised to an average of the whole movie. The tracking and posterior analysis codes were implemented in Matlab (2018a, Mathworks) and R respectively and the scripts are available at http://github.com/juliafs93/CellTracker.

**Cell culture**

Drosophila Kc 167 cell line was used. They were obtained from the Drosophila Genomics Resource Centre (https://dgrc.bio.indiana.edu/Home), the community repository for Drosophila cell lines. These cells are not susceptible to Mycoplasma. Kc cells were cultured at 25°C in Schneider’s media (GIBCO S0146), supplemented with 5% FBS (Sigma, F9665) and 1x Antibiotic Antimycotic (GIBCO, 15240-062). For Notch activation, Kc cells were treated with 4mM EGTA in PBS for 30 min. EGTA destabilises the Notch negative regulatory region, exposing the site for ADAM proteases which renders the reminaig transmembrane fragment a substrate for γ-secretase cleavage and release of NICD (Gupta-Rossi et al., 2001; Ilagan et al., 2011; Krejčí and Bray, 2007).

**Mi-2 RNAi in Kc cells**
dsRNA for Mi-2, H or GFP (control) was prepared as following: dsRNA was transcribed with MEGAscript T7 Transcription Kit (AMB 13345) using 500bp PCR fragments specific for each gene flanked by T7 promoter sequences. The newly synthesised dsRNA was treated with DNase, purified with phenol chloroform extraction, precipitated with isopropanol, re-suspended in DEPC water and annealed at 45°C for 1h and 15min. dsRNA was subsequently stored at -20°C.

For single Mi-2 RNAi, H RNAi or GFP RNAi treatment: In a 6-well plate, the medium from Kc cells (2 x 10^6 cells per well) was replaced with 20µg dsRNA diluted in 600 µl OptiMEM media [Gibco/Life technologies (now part of Thermo-Fisher), 51985-026] for 30 min and 1.5ml of tissue culture media were subsequently added according to standard protocols. Changes in RNA levels were assessed 72 hours later. For double knockdown, Kc cell medium was replaced with 10µg dsRNA of each gene diluted in 600µl OptiMEM media for 30min. 1.5ml of tissue culture media were subsequently added according to standard protocols. mRNA levels were measured again after 72 hours.

**RNA isolation and qPCR**

Kc cells were harvested in Tri reagent and incubated for 10min. RNA was extracted using phenol chloroform and precipitated in isopropanol overnight at -20°C. RNA was resuspended in DEPC treated water and treated with DNase (Ambion,DNA-free kit, AM1906) to remove genomic DNA. The equivalent of 2µg of RNA was reverse transcribed with random primers [Oligo(dT)15 Primers (Promega C1101)] using M-MLV reverse transcriptase (Promega M531A). The cDNA products were subsequently diluted 1:5 and 1µl was used as a template in each quantitative PCR reaction. Quantitative PCR was performed using LightCycler® 480 SYBR Green I Master PCR Kit (Roche 04707516001) with a Roche Light Cycler. Samples were normalised to Rpl32. All primers are listed in Table 1.

**Table 1: PCR primers for RNA analysis:**

<table>
<thead>
<tr>
<th>Primer Name (for RNA)</th>
<th>Primer Sequence</th>
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<tr>
<td>mβ coding sequence for</td>
<td>AGAAGTGAGCAGCAGCCATC</td>
</tr>
<tr>
<td>mβ coding sequence rev</td>
<td>GCTGGACTTGAAACCGCACC</td>
</tr>
<tr>
<td>m3 coding sequence for</td>
<td>CGTCTGCAGCTCAATTAGTC</td>
</tr>
<tr>
<td>m3 coding sequence rev</td>
<td>AGCCCACCCACCTCAACCAG</td>
</tr>
<tr>
<td>mδ coding sequence for</td>
<td>AGGATCTCATCGTGACACC</td>
</tr>
<tr>
<td>mδ coding sequence rev</td>
<td>CAGACTTCTTGCCCATGATG</td>
</tr>
<tr>
<td>mα coding sequence for</td>
<td>TCCCAAATGCTCGCCTTTAGA</td>
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Chromatin immunoprecipitation

ChIP experiments were performed as previously described (Krejcí and Bray, 2007; Skalska et al., 2015) except for Mi-2 ChIP where a combination of 1% formaldehyde with 1mM EGS (ethylene glycol bis(succinimidyl succinate)) was used for cross-linking, (15 min at room temperature). A plate with 15×10⁶ cells was used as a starting material for each ChIP and the following antibodies were used: goat α-Su(H) (Santa Cruz Biotechnology sc15813; 12μl), rabbit α-H3K56ac (Active Motif 39281; 3μl), rabbit α-H3K27ac (Abcam ab4729; 10μl from 0.1μg/μl), rabbit α-H3K27me3 (Millipore, 07-449; 1μl), rabbit α-H3 (Abcam ab1791; 1μl), rabbit-a-Mi-2, (courtesy of Alexander Brehm; 2μl). Regions of enrichment were analyzed by qPCR using the primers in Table 2.

### Table 2: Primers for ChIP – qPCR:

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<td>mβ rev</td>
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<tr>
<td>m3 for</td>
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<tr>
<td>m3 rev</td>
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Co-Immunoprecipitation and Western blot

For detecting protein levels, control, Mi-2RNAi and H-RNAi treated Kc cells (~8×10⁶ cells) were harvested and incubated in Lysis Buffer (50mM Tris pH7.5, 150mM NaCl, 10% glycerol, 0.5% triton X-100, Complete protease inhibitors (Roche)) on ice for 30min. Upon centrifugation, cell debris was pelleted and the supernatant with the nuclear protein extracts was collected and

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<tr>
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denatured in SDS-sample buffer (100mM Tris-Cl, pH6.8, 20% Glycerol, 4% SDS, 0.025%
Bromophenol blue, 2% β-mercaptoethanol).

For co-immunoprecipitations, 15x10⁶ cells from Hairless-GFP or a control GFP-expressing
stable Kc cell line were incubated in IP Lysis buffer (50mM Tris-HCL pH8, 150mM NaCl, 10%
Glycerol, 0.5% Triton X-100, and proteinase inhibitor cocktail) on ice for 40min. Cell debris was
pelleted via centrifugation and the supernatants were incubated with 100μls of Protein G agarose
beads (Roche) for 1 hour at 4°C for preclearing. Rabbit anti-GFP (1:1.000, Invitrogen A11122)
was then added to the protein extracts and incubated overnight at 4°C. 60μl of protein G-Agarose
beads were added and incubated with the protein extracts for 2 hours at 4°C before washing five
times in IP buffer. The pelleted beads were then resuspended in SDS-sample buffer.

Total denatured proteins or immunoprecipitated proteins were resolved by 7% SDS—
polyacrylamide gel electrophoresis (BioRad) and transferred to nitrocellulose membrane.
Membranes were blocked in TBTM (TBS, 0.05% tween, 3% milk) for 1 hour prior to an
overnight incubation at 4°C with primary antibodies (Rabbit anti-GFP 1:2000, Invitrogen
A11122; Rabbit anti-Su(H) 1:400 Santa Cruz sc-28713; Rabbit anti-Mi-2 1:10000 courtesy of A.
Brehm; rat anti-tubulin 1:2000). Following 3 washes of 15 min at RT in TBT (TBS + 0.05
Tween), membranes were incubated with horseradish peroxidase-conjugated secondary
antibodies (1:2000, HRP Goat anti-rabbit or 1:2000, HRP Goat anti-rat) for 1 hour at RT then
washed 3 times for 15 min in TBT. Bound antibodies were detected by the Amersham ECL
detection system (GE Healthcare Life Sciences) and documented on X-Ray film.
Acknowledgements

We are grateful to Judith Pampalona for introducing us to the methods for NB cultures, to Martin Hailstone, Lu Yang and Ilan Davis for help with whole brain long term cultures and to Zoe Pillidge for advice on the molecular experiments in Kc cells. We acknowledge the Bloomington Stock Center, the VDRC Stock Center and the Developmental Studies Hybridoma Bank for Drosophila strains and antibodies and we thank Christos Delidakis and Alexander Brehm for antibodies. We appreciate the many valuable discussions with other members of the Bray lab during this project and we thank Torcato Martins, Jonty Townson and Kat Millen for critical reading of the manuscript. This work was funded by a program grant from the MRC to SJB and by studentship from the Wellcome Trust for JF-S.

Competing interest

The authors declare that they have no competing interests.


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Figure Legends and Figure Supplement Legends

Figure 1: Delayed onset of hyperplasia in NB lineages expressing constitutively active Notch. (A) Expression of stem cell markers in wild type (grhNBGal4 \textgreater\textgreater\textless LacZ, E(spl)m\textgamma\textgreater\textless GFP) ventral nerve cords (VNC) and in VNCs where NSC lineages were exposed to constitutive Notch activity (Nact; (grhNBGal4\textgreater\textgreater\textless N\Delta\textless ecd; E(spl)m\textgamma\textless GFP). Gal80ts was used to control the onset of expression to provide 8, 24 or 48 hours of Gal4. E(spl)m\textgamma\textless GFP (green or white) and Dpn (blue or white) two Notch responsive genes expressed in NSCs become upregulated in longer exposure times, High levels of Nicd (red) are present at even the earliest time-point. Red arrowheads indicate normal lineages, yellow arrowheads indicate hyperplastic lineages, yellow arrows indicate E(spl)m\textgamma\textless GFP\textsuperscript{\textgreater\textless}, Dpn\textsuperscript{\textless\textless} progeny. Scale bars: 25 \textmu m. (B) Schematic representation of NB lineages at different times of Nact exposure; NBs, large green cells with grey nucleus, GMCs yellow and neurons grey. Ectopic NB-like cells are depicted as intermediate sized green cells. (C) Percent of lineages that were hyperplastic following 8h, 24h and 48h of Nact expression. Box represents IQR, black line indicates median and whiskers indicate \pm 1.5\times IQR. N=15, 3 experiments (D) Number of cells per hyperplastic lineage that are E(spl)m\textgamma\textless GFP\textsuperscript{\textgreater\textless} Dpn\textsuperscript{\textless\textless} (green) or E(spl)m\textgamma\textless GFP\textsuperscript{\textgreater\textless} Dpn\textsuperscript{\textless\textless} (blue) in 8 or 24h Nact expression. Box represents IQR, black line indicates median and whiskers indicate \pm 1.5\times IQR. N=120, 3 experiments.

Figure 1- figure supplement 1: E(spl)m\textgamma and Dpn are among the earliest NB markers expressed by progeny of NB lineages exposed to constitutively active Notch. (A) Control NB lineages (grhNBGal4 Gal80ts\textgreater\textless LacZ; E(spl)m\textgamma-GFP) or NBs exposed to constitutively active Notch (grhNBGal4 Gal80ts\textgreater\textless Nact; E(spl)m\textgamma-GFP) for 8 h or 24 h in relatively young animals (72ALH). Note that E(spl)m\textgamma-GFP (green) and Dpn (red) are among the earliest markers expressed by NB-like cells, Grh (blue) appears shortly after (B) Control NB lineages or NB lineages expressing Nact. Note that m\textgammaGFP (green) and soon afterwards Dpn (red) are among the earliest markers expressed by NB-like cells whereas Mira (blue) is expressed many hours later in NB-like cells. See lineages in yellow squares or progeny pointed by yellow arrows or red arrowheads.

Figure 1_figure supplement 2: Stem cells with constitutively active Notch divide asymmetrically. (A) Expression of Dpn, Mira (blue) and Pon (red) in Control NB lineages (grhNBGal4 Gal80ts\textgreater\textless LacZ; Ase-mcherryPonLD) or NB lineages exposed to Nact (grhNBGal4
Gal80ts>Nact; Ase-mcherryPonLD) for 24 h. Note that Mira (blue) becomes localized asymmetrically into the progeny of mitotic NBs (see NBs with magenta asterisks) in lineages with excessive Notch signaling as in control NBs. (B) Live imaging of NB lineages in larval brain expressing>NΔecd (grhNBGal4 Gal80ts at the permissive temperature for 24 hours), with an example of dividing NB. After mitosis, re-emerging NB is larger and maintains E(spl)mγ-GFP expression whereas progeny GMC is smaller and rapidly loses E(spl)mγ-GFP (green). Histone-RFP (white) is used to monitor nuclei. Purple circles indicate dividing NB and its emerging progeny. Time is depicted below each panel, scale bar 15 μm. (C) Graph summarizing nuclear volume of tracked NB before and after division and of newly born GMCs. Note that the large size of the NB is maintained whereas newly born GMC is smaller.

**Figure 1-figure supplement 3:** The onset of hyperplasia in NB lineages expressing constitutively active Notch is delayed irrespectively of the age of the animal. (A) Expression of Dpn (white) in NB lineages exposed to Nact (grhNBGal4 Gal80ts > NΔecd; E(spl)mγ-GFP) in two distinct time windows of larval life (72ALH vs 96ALH). Gal80ts was used to control the onset of expression to provide Gal4 driven Nact expression for either 24 or 48 hours. Scale bars: 50 μm. Note that the extent of hyperplasia was very similar between younger and older stages, for a given period of exposure. (B) Box and whiskers diagram, percent of hyperplastic lineages following 24h and 48h of Nact expression in young (72ALH) vs older (96ALH) stages. Box represents IQR, black line indicates median and whiskers indicate ±1.5×IQR. N=15, 3 experiments (D) Number of cells per hyperplastic lineage that are Dpn+ve following 24 or 48h Nact expression in young vs older stages. Box represents IQR, black line indicates median and whiskers indicate ±1.5×IQR. N=80, 2 experiments

**Figure 2:** Live-imaging of E(spl)mγ-GFP expression in cultured NB lineages.

(A, B) Time points from time lapse movie of (A) wild type (>LacZ) and (B) Nact expressing NB lineage, Histone-RFP (red, H2Av-RFP) marks all nuclei, corresponding E(spl)mγ-GFP levels in grey scale below. E(spl)mγ-GFP is rapidly extinguished in NB progeny in wild-type and in Nact expressing lineages but appears in some progeny after a delay. Time is indicated below each panels and cartoons summarize the cell populations in each lineage. NBs are show in darker shades of green, GMCs in yellow, newly born GMCs in pale green and neurons in grey. NB-like
cells are shown in green. Coloured asterisks indicate cells coming from the same ancestor. White arrows indicate cells arising from a mitotic division. Numbers correspond to different cells indexed in the following diagrams. Scale bar 25 μm. (C, D) Bar chart diagram showing the progression over time of each cell in a wild type NB lineage (C) or of selected NB progeny over time from one Nact expressing lineage. The diameter of the bar represents cell size and the colour represents expression levels of E(spl)mγ-GFP according to the scale (Blue, low to yellow, high). Dashed lines indicate mitotic events and the emerging daughter cells. (E, F) Diagrams depicting changes in the intensity of E(spl)mγ-GFP in NBs and their progeny over time from control (E) and Nact-expressing (F) NB lineages. Note the decay in E(spl)mγ-GFP levels in the newly born GMCs in both control and Nact lineages and the re-expression of E(spl)mγ-GFP in an older Nact progeny P2.

Figure 3: Mi-2 depletion exacerbates Notch induced tumorigenesis.

(A) Depletion of Mi-2 increases the number of hyperplastic Dpn+ve cells (white) caused by Nact expression in type I lineages. Expression of Dpn in wild type (>LacZ;>w-Ri, first column), Mi-2 depleted only (>LacZ;>Mi-2-Ri; BL33415, 2nd column), Nact expressing (>NΔecd;>w-Ri; 3rd column) and Nact with Mi-2 depleted (>NΔecd;>Mi-2-Ri, 4th column). (B-C) Loss of MTA-like (B) or Caf1p55 (C) increases the number of hyperplastic Dpn+ve cells (white) caused by Nact expression. Expression of Dpn (white) in Nact expressing lineages (1st column; >NΔecd;>w-Ri) and in Nact with MTA-like or Caf1p55 depleted (2nd column; >NΔecd;>MTA-like-Ri; or >NΔecd;>Caf1p55-Ri). (D) Number of Dpn+ve cells per VNC induced by expression of Nact was significantly increased by depletion of Mi-2; whereas depletion of Mi-2 alone had little effect. (E-F) Number of Dpn+ve cells per VNC induced by Nact was significantly increased upon knock down of MTA-like (E) or Caf1p55 (F). Scatter dot plots where narrow black lines represent IQR, wider black line indicates median and whiskers indicate ±1.5×IQR. (* P<0.05, **0.001<P<0.05, ***P<0.001, ****P<0.0001, t-test). N=25-28 for each genotype tested; light and darker shades indicate data points from the three independent experiments. (G) Schematic depiction of NuRD Complex, with subunits tested here in dark orange.
Figure 3- figure supplement 1: Mi-2 depletion in NBs, GMCs or newly born progeny exacerbates Notch induced tumorigenesis but Mi-2 depletion in older neurons does not. (A) Mi-2 knockdown leads to severe depletion of Mi-2 protein levels. Mi-2 protein (red) levels in VNCs of the indicated genotypes: Nact alone (grhNBGal4Gal80ts >NAecd; >w-Ri), Mi-2 knockdown in Nact (>NAecd; >Mi-2-Ri) or Mi-2 knockdown alone (>LacZ; >Mi-2-Ri). Dpn (green) and Mira (blue) mark NBs. (B) Depletion of Mi-2 in NBs using insc-Gal4 leads to an increase of the number of hyperplastic Dpn$^\text{+ve}$ cells in Nact conditions. Expression of Dpn (green or white) is shown in VNCs with Nact alone (>NAecd; >w-Ri) and with knock down of Mi-2 in combination with Nact (>NAecd; >Mi-2-Ri). Ase (red) marks NBs and GMCs whereas Mira (blue) marks NBs. (C) Depletion of Mi-2 in GMCs and newly born NSC progeny using cas$^{GMR71C09}$-Gal4 leads to an increase in the number of hyperplastic Dpn$^\text{+ve}$ cells in Nact conditions. Dpn (white) in wild type (>LacZ; >w-Ri), Nact expressing (>NAecd; >w-Ri) knock down of Mi-2 only (>LacZ; >Mi-2-Ri Bloomington line #33415), and knock down of Mi-2 in Nact expressing (>NAecd; >Mi-2-Ri) lineages. Ase (red) marks NBs and GMCs whereas Mira (blue) marks NBs. (D) Neither Nact nor Mi-2 depletion nor combination of Nact with Mi-2 depletion in mature neurons via nsyb-Gal4 lead to increase in Dpn$^\text{+ve}$ cells. Dpn (white) in knock-down of Mi-2 only (>LacZ; >Mi-2-Ri; Bloomington line #33415), Nact expressing (>NAecd; >w-Ri) and knock down of Mi-2 in Nact expressing (>NAecd; >Mi-2-Ri) lineages. Ase (red) marks NBs and GMCs whereas Mira (blue) marks NBs. (E) Quantification of the number of Dpn$^\text{+ve}$ cells per VNC under the conditions indicated. When Mi-2 was depleted in GMCs using cas$^{GMR71C09}$-Gal4 in Nact conditions compared to Nact alone the number of Dpn$^\text{+ve}$ cells per VNC was significantly increased. VNCs from WT or Mi-2 knockdown backgrounds exhibit normal number of Dpn$^\text{+ve}$ cells. Box represents IQR, black line indicates median and whiskers indicate ±1.5×IQR. (*P<0.05, ****P<0.001, t-test) (F) Quantification of the number of Dpn$^\text{+ve}$ cells per VNC under the conditions indicated, where Mi-2 was depleted in NBs using insc-Gal4 in Nact conditions compared to Nact alone. Box represents IQR, black line indicates median and whiskers indicate ±1.5×IQR. (*P<0.05, ****P<0.001, t-test). (G) The number of Dpn$^\text{+ve}$ cells per VNC did not change where Mi-2 was depleted in neurons using nsyb-Gal4 in Nact conditions compared to Nact alone or Mi-2-depletion alone and was indistinguishable from wild-type (circa 150-160 Dpn$^\text{+ve}$ cells). Box plots, where Box represents IQR, black line
indicates median and whiskers indicate ±1.5×IQR. (* P<0.05, **P<0.001, ***P<0.001, ****P<0.0001, t-test).

**Figure 3**_figure supplement 2: Mi-2 depletion enhances the activation of Notch regulated genes (A-B) Depletion of Mi-2 in NB lineages that receive excessive Notch signalling results in an increase in the expression of the Notch regulated enhancers from pathetic (A; path[NRE]-GFP, green) (A) or CycE (B; CycE[NRE]-GFP, green) as indicated. Expression of Dpn (red) and Notch reporters (green) in wild type (control), Nact expressing (>Nact; >w-Ri) and Nact with Mi-2 depleted (>Nact; >Mi-2-Ri). Hyperplastic lineages with low/no (arrows) or high (arrowheads) levels of Notch reporter expression are indicated. (C) Grany head (Grh) and Miranda (Mira) are upregulated when Mi-2 is depleted in NB lineages expressing Nact; Dpn\(^{\text{\text{\textsuperscript{\text{\text{\text{\text{ve}}}}}}}}\) (green), Grh\(^{\text{\text{\text{\text{\text{ve}}}}}}\) (red) and Mira\(^{\text{\text{\text{\text{\text{ve}}}}}}\) (blue) in the indicated genotypes.

**Figure 4:** Expression of E(spl)\(\gamma\)-GFP in GMCs following Mi-2 depletion in Nact expressing lineages.

(A, B) Time points from two different time lapse movies of Nact Mi-2-RNAi NB lineages (dissociated from larval brains with grhNBgal4 Gal80ts at the permissive temperature for 24 hours). E(spl)\(\gamma\)-GFP remains at high levels in emerging GMCs. Upper panels, bright field images of NB and its progeny combined with Histone-RFP (red, H2Av-RFP) to monitor cell cycle stages; lower panels, expression of E(spl)\(\gamma\)-GFP (greyscale). Time is depicted below each panel along with cartoons of the lineages where NBs are in darker shades of green, GMCs with low expression of E(spl)\(\gamma\)-GFP in light green. Numbers correspond to different cells indexed in C, D. Scale bar 25 \(\mu\)m. (C, D) Bar chart depicting progression of each cell in a Nact Mi-2 RNAi NB lineage, bar thickness indicates cell-size and the colour represents E(spl)\(\gamma\)-GFP levels according to the scale (blue low levels, yellow high levels). Dashed lines mark mitotic events linking to the emerging daughter cells. (E) Bar diagram depicted the percent of lineages with newly born GMCs that retain expression of E(spl)\(\gamma\)-GFP in WT, Nact expressing and Nact expressing with compromised Mi-2. (F) Levels of E(spl)\(\gamma\)-GFP during one division cycle in NBs from WT, >Nact and >Nact, Mi-2 RNAi. E(spl)\(\gamma\)-GFP levels are high in interphase, immediately after mitosis in early G1 and before mitosis in late G2. However in >Nact, Mi-2 knockdown, the second phase of high E(spl)\(\gamma\)-GFP is lost. (G) Plot of E(spl)\(\gamma\)-GFP levels in
newly born GMCS in WT, >Nact and >Nact; Mi-2 RNAi. Note that E(spl)mγ-GFP levels decay in newly born GMCS in WT and >Nact but are maintained in >Nact; Mi-2 RNAi lineages.

**Figure 4** figure supplement 1: Expression of E(spl)mγ-GFP in GMCS upon Mi-2 depletion in NB lineages. (A) Time points from a time lapse movie of LacZ; Mi-2-Ri NB lineages (dissociated from larval brains with grhNBGal4 Gal80ts at the permissive temperature for 24 hours). E(spl)mγ-GFP is switched off in emerging GMCS as in WT lineages. Upper panels, bright field images of NB and its progeny combined with Histone-RFP (red, H2Av-RFP) to monitor cell cycle stages; lower panels, expression of E(spl)mγ-GFP (black). Time is depicted below each panel along with cartoons of the lineages where NBs are in darker shades of green, emerging GMCS with low expression of E(spl)mγ-GFP in light green and GMCS with no expression of E(spl)mγ-GFP in yellow. Asterisks indicate progeny from same ancestor cell. Numbers correspond to different cells indexed in B. Scale bar 25 μm. (B) Bar chart depicting progression of each cell in a Mi-2-Ri NB lineage, bar thickness indicates cell-size and the colour represents E(spl)mγ-GFP levels according to the scale (blue low levels, yellow high levels). Dashed lines mark mitotic events linking to the emerging daughter cells. (C) Plot of E(spl)mγ-GFP levels in NBs, newly born GMCS and progeny in >LacZ; Mi-2-Ri. Note that E(spl)mγ-GFP levels decay in newly born GMCS in >LacZ; Mi-2-Ri lineages.

**Figure 5**: Loss of Mi-2 leads to de-repression of E(spl)-C genes. (A) Genomic region spanning E(spl)-C locus with graphs depicting Mi-2 bound regions in S2 cells (purple) (Kreher et al., 2017) and Kc cells (magenta) (modEncode; Ho et al., 2014), Su(H)-bound regions in Kc cells (cyan) (Skalska et al., 2015) and chromatin signatures from Kc cells (Skalska et al., 2015). Gene models are depicted in black. (B) Levels of Mi-2 protein are reduced upon knockdown of Mi-2 via RNAi in Kc cells for 3 days compared to knockdown of GFP or Hairless via RNAi. Anti-tubulin is a control for loading. (C, D) Fold change in RNA levels in Kc cells upon knockdown of Mi-2 (C) or Hairless (D) compared to control conditions (con: GFP knockdown) and to cells with Notch activation (Nact). Note that E(spl)mβ and E(spl)m3 are both significantly de-repressed in Notch-off state and show even higher increase in expression in Notch-on state. (E) Fold change in RNA levels in Kc cells upon combined knockdown of Mi-2 and Hairless compared to control conditions and single knockdown of Mi-2 or Hairless. Note
additive effects on $E(spl)m\beta$ and $E(spl)m^3$ de-repression in combined knock-down conditions. (F) Mi-2 does not directly interact with Hairless. Immunoprecipitations with anti-GFP from Kc cells expressing Hairless-GFP or MCP-GFP as control, Su(H) is co-purified with Hairless but not Mi-2. (G) Enrichment of Su(H) is indicated at $E(spl)$-C or regions in Kc cells as revealed by ChIP in control (grey) or Mi-2 knockdown (for 3 days, green) conditions in Notch off (light shading) and Notch active (EGTA treatment 30 min; dark shading). Su(H) recruitment in Notch active and in control conditions is not altered by knockdown of Mi-2. (H) Enrichment of Mi-2 at indicated positions in $E(spl)$-C or other regions in Kc cells as revealed by ChIP. (P values: *0.01 < P < 0.05, ** 0.001 < P < 0.01, ***P < 0.001, Multiple t-tests)

**Figure 5-figure supplement 1: Effects of Mi-2 depletion on histone modifications at the $E(spl)$-C locus** (A) Fold change in RNA levels in Kc cells upon knockdown of Mi-2 or Hairless compared to control conditions (con: GFP knockdown). Mi-2 or Hairless knock down was performed for 3 days prior to the experiment. Note that $Mi$-2 and $H$ are both downregulated indicating that the knockdown strategy was successful and $E(spl)m\delta$, $E(spl)m\gamma$, $E(spl)m\alpha$ and $E(spl)m7$ are all significantly de-repressed in Notch-off state. (B) Fold change in RNA levels in Kc cells upon knockdown of $Mi$-2 compared to control conditions (con: GFP knockdown) and to cells with Notch activation (Nact). Note that other Notch regulated genes such as CG12290, CG17119, $Notch$ and regular (rgl) are de-repressed in Notch-off state and have enhanced expression in Notch-on state (C) Fold change in RNA levels in Kc cells upon combined knockdown of $Mi$-2 and $Hairless$ compared to control conditions and single knockdown of $Mi$-2 or $Hairless$. Note that $Mi$-2 and $H$ are downregulated in both single and double conditions indicating that the knockdown strategy was successful. (D) Enrichment of Histone3 is indicated at $E(spl)$-C or regions in Kc cells as revealed by ChIP in control (grey) or $Mi$-2 knockdown (for 3 days, green) conditions in Notch-off (light shading) and Notch-on (EGTA treatment 30 min; dark shading). H3 distribution in Notch active and in control conditions is not altered by knockdown of $Mi$-2. (E) Enrichment for various histone modifications such as H3K27acetylation, H3K27trimethylation and H3K56acetylation is indicated at $E(spl)$-C locus in Kc cells as revealed by ChIP in control (grey) or $Mi$-2 knockdown (for 3 days, green) conditions in Notch-off (light shading) and Notch-on (EGTA treatment 30 min; dark shading). No change in the levels of these modifications was detected following knockdown of $Mi$-2. (P values: *0.01 < P < 0.05, ** 0.001 < P < 0.01, ***P < 0.001, Multiple T tests).
Figure 6: Cooperation between Zfh1 and Mi-2 in NB lineages.

(A) Zfh1 depletion enhances the number of Dpn$^{+ve}$ cells (green or white channels) caused by Nact over-expression. Ase marks NBs and GMCs, Pros marks progeny. (B) Scatter dot plot, the number of Dpn$^{+ve}$ cells per VNC in >Nact; >zfhlRi (N=24, 3 experiments) was significantly increased compared to >Nact; >wRi larvae (N=34, 3 experiments). (C) Overexpression of Zfh (>zfhl-PB; w-Ri) results in decreased number of Dpn$^{+ve}$ cells per VNC (N=31, 3 experiments) compared to wild type (>LacZ; w-Ri; N=17, 3 experiments) or Mi-2 knockdown alone (>LacZ; >Mi-2-Ri; N=25, 3 experiments). Concomitant loss of Mi-2 (>zfhl-PB; >Mi-2Ri; N=33, 3 experiments) rescues Zfh1-induced loss of NBs. Ase and Pros as in (A). (D) Scatter dot plot with number of Dpn$^{+ve}$ cells in each condition quantified. (E) zfhl-GFP expression in type I NB lineages. Zfh1 is expressed in newly born neurons and at low levels in GMCs (yellow arrowheads). Ase marks neuroblasts and GMCs whereas Pros marks older neuronal progeny in NB lineages.

Figure 6-figure supplement 1: Prospero does not cooperate with Mi-2 in NB lineages. (A) Depletion of Pros enhances the number of Dpn$^{+ve}$ cells (green or white channels) caused by Nact over-expression, Ase marks NBs and GMCs, Pros marks progeny. (B) Scatter dot plot, the number of Dpn$^{+ve}$ cells per VNC in >Nact; >Pros-Ri (N=24, 3 experiments) was significantly increased compared to >Nact; >w-Ri larvae (N=27, 3 experiments). (C) Overexpression of Pros (>YFP-Pros; w-Ri) results in decreased number of Dpn$^{+ve}$ cells per VNC (N=15, 4 experiments) compared to wild type (>LacZ; >w-Ri; N=17, 4 experiments) or Mi-2 knockdown alone (>LacZ; >Mi-2-Ri; N=16, 4 experiments). Concomitant loss of Mi-2 (>YFP-Pros; >Mi-2-Ri; N=14, 4 experiments) does not rescue Pros-induced NB loss, instead it further decreases the number of Dpn$^{+ve}$ cells. Ase marks NBs and GMCs, Mira marks NBs. (D) Quantification of Dpn$^{+ve}$ cells in each condition, where shading of symbols indicates different experimental replicates for each genotype, with mean and SEM indicated by black lines.

Figure 6- figure supplement 2: Zfh1 resists Notch induced tumorigenesis. (A) Ectopic expression of Zfh1 reduces the number of Dpn$^{+ve}$ cells (green or white channels) caused by Nact overexpression. This effect is partially rescued by the loss of Mi-2. Ase marks NBs and GMCs, Pros marks progeny. (B) Quantification of Dpn$^{+ve}$ cells per VNC in each condition, where shading of symbols indicates different experimental replicates for each genotype, mean and SEM
indicated by black lines. >Nact; >zfh1-PB, >w-Ri (N=26, 3 experiments) was significantly
decreased compared to >Nact; >LacZ, >w-Ri larvae (N=11, 3 experiments) whereas it remained
unaltered in >Nact; >zfh1-PB, >Mi-2-Ri (N=20, 3 experiments). (C) Mi-2-YFP and zfh1-GFP
expression in type II NB lineages. Mi-2 is expressed in high levels in the Type II NB and lower
levels in INPs whereas Zfh1 is expressed only in some neurons. Yellow outlines of Type II
lineages. Dpn (red) and Mira (blue) mark neuroblasts and INPs.

Figure 7: Model summarizing the role of Mi-2 in decommissioning Notch responsive
enhancers in NB progeny.

The presence of Zfh1 and Mi-2 favours the decommissioning of enhancers from E(spl) and other
Notch target genes in GMCs (yellow) to ensure their expression is switched off. Notch is on in
NBs (green) and off in GMCs (yellow cells) due to asymmetrical segregation of Numb (ref).
GMCs divide to produce two post-mitotic neuronal cells (grey). In NB lineages with constitutive
Notch activity (Nact), the presence of Mi-2 at enhancers, recruited by Zfh1 (and potentially by
other factors too), is sufficient to attenuate Nact, so that E(spl) and other target genes are
switched off in GMCs. In a few NB progeny the effects of Mi-2 are overcome with time, and
E(spl) genes are up-regulated as they revert to NB-like cells. Depletion of Mi-2 in NB lineages
expressing Nact severely compromises enhancer decommissioning so that E(spl) and other
Notch target genes are upregulated in many of the GMCs. The majority of the NB progeny
acquire an NB-like fate.
SUPPLEMENTARY MATERIAL: Legends for videos

**Figure 2-video 1:** Time lapse movie of control (>LacZ) NB lineage with a duration of 10 hours. Histone-RFP (red, H2Av-RFP) on the left marks all nuclei. E(spl)mγ-GFP levels are depicted in greyscale on the right. Time (in min) is indicated on the top left. Scale bar 25μm. E(spl)mγ-GFP is extinguished in NB progeny.

**Figure 2-video 2:** A 14-hour time-lapse movie of Nact expressing NB lineage. Histone-RFP (red, H2Av-RFP) on the left marks all nuclei. E(spl)mγ-GFP levels are depicted in greyscale on the right. Time (in min) is indicated on the top left. Scale bar 25μm. Note that although E(spl)mγ-GFP is extinguished from all NB newly born progeny, it re-appears later in some progeny.

**Figure 4-video 1:** Time-lapse movie of Mi-2-RNAi NB lineage with a duration of 10 hours. Bright field series of images of NB and its progeny with histone-RFP (red, H2Av-RFP) to mark all nuclei are shown on the left. E(spl)mγ-GFP levels are depicted in greyscale on the right. Time is indicated in min on the top left corner. Scale bar 25μm. Note that E(spl)mγ-GFP is rapidly extinguished from emerging NB progeny similar to wild type lineages.

**Figure 4-video 2:** Time lapse movie of Nact Mi-2-RNAi NB lineage with a duration of 10 hours. On the left, bright field images of NB and its progeny with histone-RFP (red, H2Av-RFP) marks all nuclei. On the right, E(spl)mγ-GFP levels are depicted in greyscale. Top left, time is indicated in min. Scale bar 25μm. Note that E(spl)mγ-GFP is not extinguished from emerging NB progeny.

**Figure 4-video 3:** Time lapse movie of a larger Nact Mi-2-RNAi NB lineage with a duration of 10 hours. Bright field images of NB and its progeny combined with histone-RFP (red, H2A-RFP) to mark all nuclei are depicted on the left. E(spl)mγ-GFP levels are depicted in greyscale on the right. Time is indicated in min on the top left. Scale bar 25μm. Note again that E(spl)mγ-GFP remains high both in emerging and older NB progeny.
A | >LacZ; myGFP | >Nact myGFP

72ALH, 8h @30C  |  72ALH, 24h @30C |  72ALH, 48h @30C

myGFP Nicd Dpn | myGFP Nicd Dpn | myGFP Dpn

B

0h  |  72 h ALH

8h  |  24h |  48h

>C

% hyperplastic lineages

0  |  50  |  100  |  150

0  |  50  |  100  |  150

D

# cells / hyperplastic lineage

0  |  10  |  20  |  30

0  |  10  |  20  |  30

my^+veDpn^-ve  |  my^-veDpn^-ve
Figure 1 Supplementary Figure 1
Figure 1 Supplementary Figure 2

A

> LacZ; Ase-mcherryPonLD for 24h

> Nact; Ase-mcherryPonLD for 24h

B

Interphase Mitosis End of Cytokinesis Interphase

C

Nuclear volume (um^3)

NB before division NB after division GMC

Figure 1 Supplementary Figure 2
Figure 3
Figure 3 Supplementary Figure 1
Figure 3 Supplementary Figure 2
Supplementary Figure 1

Figure 4 Supplementary Figure 1
Figure 5 Supplementary Figure 1
Figure 6
Figure 6 Supplementary Figure 1
**WT**

- Notch ON
- E(spl)
- **NSC**
- **GMC**

- Mi-2 decommissions enhancers:
  - resets targets in Notch off progeny

**>Nact**

- Nact
- E(spl)
- **E(spl)**
- **NSC**
- **GMC**

- Mi-2 counteracts Notch: few progeny switch on stem cell programme

**>Nact; >Mi-2-Ri**

- Nact
- E(spl)
- **E(spl)**
- **NSC-like**

- Absence of Mi-2: Notch drives many cells into stem cell programme