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Zebrafish *sin3b* mutants are viable but have size, skeletal and locomotor defects

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Abstract

Background: The transcriptional co-repressor Sin3 is highly conserved from yeast to vertebrates and has multiple roles controlling cell fate, cell cycle progression, and senescence programming. Sin3 proteins recruit HDACs and other chromatin modifying factors to specific loci through interactions with transcription factors including Myc, Rest, p53 and E2F. Most vertebrates have two Sin3 family members (*sin3a* and *sin3b*), but zebrafish have a second sin3a paralogue. In mice, *sin3a* and *sin3b* are essential for embryonic development. Sin3b knockout mice show defects in growth as well as bone and blood differentiation.

Results: To study the requirement for Sin3b during development, we disrupted zebrafish *sin3b* using CRISPR-Cas9, and studied the effects on early development and locomotor behavior.

Conclusion: Surprisingly, Sin3b is not essential in zebrafish. *sin3b* mutants show a decrease in fitness, small size, changes to locomotor behavior, and delayed bone development. We did not detect a role for Sin3b in cell proliferation. Our analysis of the *sin3b* mutant revealed a more nuanced requirement for zebrafish Sin3b than would be predicted from analysis of mutants in other species.

Keywords

Sin3b; zebrafish; behavior; CRISPR; early development

Introduction:

Coordination of gene expression and cell cycle progression are essential for maintaining the balance between cell growth and differentiation during development. Sin3 is a transcriptional repressor that has key functions during development (David et al. 2008; Pennetta & Pauli 1998; Sharma et al. 2008; Shi & Garry 2012; van Oevelen et al. 2010; Yang et al. 2000), cell cycle progression (Balciunaite et al. 2005; David et al. 2008; Rayman

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et al. 2002; van Oevelen et al. 2008) and senescence programming (David et al. 2003; Grandinetti et al. 2009).

Sin3 was originally identified in yeast (Sternberg et al. 1987) and is conserved across the animal kingdom. The structure of Sin3 is comprised of three amphipathic alpha-helices (PAH1-PAH3)(Wang et al. 1990) and a histone deacetylase interaction domain following the third PAH3 domain (Kasten et al. 1997). Mammals have two homologs, Sin3a/b (Ayer et al. 1995; Halleck et al. 1995) that have both distinct and overlapping roles (David et al. 2008; Grandinetti & David 2008; van Oevelen et al. 2010). In zebrafish, the *sin3* family consists of three genes, *sin3aa*, *sin3ab* and sin3b. In many cases of zebrafish gene duplication events, subsequent subfunctionalization provides an opportunity to dissect functions for each homologue.

In general, Sin3b is not as broadly studied as Sin3a. Despite the absence of a DNA binding domain, Sin3b modulates transcription of a diverse set of genes by interactions with transcription factors including p53 (Bansal et al. 2011), E2F(Grandinetti & David 2008), Rest (Naruse et al. 1999), MNF-β (Yang et al. 2000), Fok1 (Shi & Garry 2012), Myc (Garcia-Sanz et al. 2014), and SMAR1 (Rampalli et al. 2005). Sin3b acts as a scaffold to recruit HDACs and other chromatin modifying enzymes that condense chromatin and repress transcription. Recruitment of HDACs is the most well characterized activity of Sin3, but additional proteins associated with Sin3 methylate DNA and histones and remodel nucleosomes (Silverstein & Ekwall 2004) . In addition to HDAC1/2 (Hassig et al. 1997; Laherty et al. 1997), the Sin3/HDAC co-repressor complex contains SAP18(Zhang et al. 1997), SAP30 (Zhang et al. 1998), MeCP2 (Bird et al. 1998), and RBP1 (Lai et al. 2001). While generally thought of as a powerful repressor, Sin3 activity is more nuanced and the protein is sometimes associated with active genes (Stadhouders et al. 2015). It has been suggested that recruitment of HDACs to actively transcribed genes may limit inappropriate transcription initiation or slow the rate of transcriptional elongation.

The influence of Sin3b extends beyond direct effects on chromatin landscapes, although these functions of Sin3b are poorly studied. In addition to counteracting Myc by promoting MxD mediated transcriptional repression of E-Box target genes (Hurlin & Huang 2006), Sin3b also mediates de-acetylation and destabilization of Myc (Garcia-Sanz et al. 2014). A second potentially important and novel non-nuclear role of Sin3b in modulation of neural activity has recently been identified (Vega et al. 2013). In this context, Sin3b regulates sodium currents by controlling trafficking of the sodium channel, Nav1.2. This finding reveals a largely unexplored function for Sin3b in the nervous system.

Sin3 is not essential for viability in *S. cerevisiae* (Sternberg et al. 1987) but *Drosophila sin3* and murine *Sin3a* and *Sin3b* mutants are lethal (Pennetta & Pauli 1998; Sharma et al. 2008; Cowley et al. 2005; David et al. 2008; Dannenberg et al. 2005). Mouse embryos lacking Sin3a die during the blastocyst stage (Cowley et al. 2005; Dannenberg et al. 2005), while *Sin3b* null mutants are late stage embryonic lethal (E 18.5). Mouse *Sin3b* mutants are smaller, present with diminished ossification, and poorly differentiated erythrocytes (David et al. 2008). Under serum starvation, MEFs derived from *Sin3b* knockout mice fail to arrest at G_0/G_1 and progress further into the cell cycle, but are unable to divide (David et al. 2008).

Disruption of *Sin3b* in hematopoietic stem cells impedes differentiation (Cantor & David 2017). Tissue specific ablation of Sin3b in muscle does not affect survival, but enhances phenotypes produced by disruption of *Sin3a* (van Oevelen et al. 2010).

To broaden the understanding of *sin3b* function during early development and assess its potential role as a Rest cofactor during neurogenesis, we employed the CRISPR–Cas9 system to disrupt zebrafish *sin3b*. The *sin3b* mutants are viable and fertile, which enables widespread analysis of the requirements for Sin3b at multiple developmental stages. We observed that *sin3b* mutants have defects in locomotor behavior, skeletal development and overall growth. However, we did not detect changes in cell proliferation in the mutants. These results reveal that Sin3b is required for aspects of normal development (growth, timing of bone development, normal locomotor kinetics) in zebrafish, but broader roles in cell cycle control and differentiation are likely supplied by other Sin3 family members.

Results:

Domain structure of zebrafish *sin3b*

Yeast SIN3 has 3 PAH domains, a Sin3 C-terminal domain (originally described as PAH 4), and a histone deacetylase interacting domain (Interpro database). This domain structure is conserved in both Sin3a and Sin3b in mammals (Halleck et al. 1995). The zebrafish Sin3 family includes two *sin3a* paralogs, *sin3aa, sin3ab* and a single *sin3b* ortholog. These proteins are predicted to have identical domain structures consisting of 3 PAH domains, a histone deacetylase interacting domain and a Sin3 C-terminal domain (Figure 1A). Phylogenetic analysis of the zebrafish genes revealed two duplication events, an ancient one in the vertebrate lineage that generated *sin3a* and *sin3b* and one specific to teleost fish, which created the *sin3aa* and *sin3ab* paralogues (Figure 1E). On the amino acid level, zebrafish Sin3b is about 50% identical to Sin3aa and Sin3ab, while Sin3aa and Sin3ab are about 78% identical to each other. Overall zebrafish Sin3b is 31.31% identical to yeast SIN3 and 67.23% identical to human SIN3B. Similarity between the zebrafish and human Sin3b proteins is even greater within the functional domains. For example, the three PAH domain of zebrafish Sin3b are 86.5%, 74.6% and 88.9% identical to the corresponding human Sin3b PAH domains, while the zebrafish Sin3b histone deacetylase interacting domain is 80.0% identical to same domain of the human protein.

Targeting of zebrafish *sin3b*

To study the requirement for Sin3b during vertebrate development, we disrupted zebrafish *sin3b* using the CRSPR-Cas9 system (Chang et al. 2013; Hwang et al. 2013). Multiple lesions were recovered in the middle of the first PAH domain. We characterized three of these mutations; 2 that produce premature stop codons (*sin3b*^{Sbu65} and *sin3b*$^{Sbu66)}$ and one in frame deletion, *sin3b*^{Sbu67} (Figure 1C-D). Our studies primarily utilized the *sin3b*^{sbu65} allele (5bp deletion), which is likely a null allele because the frame shift in the first PAH domain eliminates key interaction domains.

Sin3b is required for normal growth and fitness.

Unlike the mouse *Sin3b* knockout, *sin3b*$^{sbu65/sbu65}$ embryos appear grossly normal throughout embryogenesis. However, adult *sin3b* mutants are not recovered at expected Mendelian ratios (Figure 1E) (P=0.00002) indicating reduced fitness of the mutants. Of adult offspring of *sin3b*$^{sbu65/+}$ intercrosses, only 27/167 (16.1%) were homozygous mutants and these were smaller in size then their siblings. To determine whether the growth deficits in the mutants stem from embryonic defects, we compared embryonic length at 6 dpf. No differences were detected between *sin3b* mutants and their siblings (Data Not Shown).

In the mouse, Sin3a is required for male germ cell development (Pelligrino 2012). Because both male and female adult zebrafish *sin3b*$^{sbu65/sbu65}$ mutants produce viable offspring, we conclude that zebrafish Sin3b is not essential for germ cell development or meiosis. The progeny of *sin3b*$^{sbu65/sbu65}$ mutants (maternal zygotic (MZ) *sin3b* mutants) lack maternal contribution of *sin3b* and do not show additional defects and present with a similar growth rate when compared to wild types (Data Not Shown). This indicates that maternally supplied *sin3b* does not have an overtly distinct function from zygotic *sin3b*.

Sin3b modulates larval locomotion in zebrafish

Sin3b has not been well studied in the developing nervous system but has potential key functions through its interactions with the Rest transcriptional repressor (Naruse et al. 1999; Nomura et al. 2005), sodium channels (Vega et al. 2013) and regulation of the circadian clock (Naruse et al. 2004). We therefore sought to determine whether Sin3b is also required to control spontaneous locomotion and evoked behaviors.

At six days, we compared spontaneous locomotion of MZ*sin3b* mutants (N=46) to related wild-type controls (N=46) (Figure 2). This analysis revealed that MZ*sin3b* mutants (an average of 1753 +/−120.3 (SEM) movements) initiated fewer movements than the related wild-type controls (an average of 2245 +/−113.61 movements) over 15 minutes (Figure 2A, P=0.0038) during the day. A repeated measure ANOVA evaluated movements over one-minute time intervals, identified a significant main effect of genotype and time, but no significant interactions between time X genotype (Table 1). On average, the related wild-type controls made 149 movements/min., while the MZ*sin3b* mutants made 116 movements/min (Figure 2B). A similar pattern of decreased activity in the MZ*sin3b* mutant larvae was observed with additional parameters of locomotion including distance traveled and duration of movements (Figure 2 C-F, Table 1). Locomotor behavior was also scored for zygotic *sin3b* mutants and a similar pattern of decreased locomotion was observed (Data not shown). Overall the loss of either zygotic or maternal and zygotic Sin3b during development results in hypoactivity.

Swim length and velocity were also analyzed in MZ*sin3b* mutants. Overall, the MZsin3b mutants show a significant decrease in both swim length (P=0.0073) and velocity (P=0.0066) when compared to wild-type controls (Figure 3 A-B). This indicates that the larvae tend to cover less distance and are slower when compared to wild-type controls. A similar trend was also observed for zygotic *sin3b* mutants (Data Not Shown).

We also investigated place preference of the larvae because larvae deficient for maternal *rest* mRNA present with atypical spatial preferences showing increased time spent near the wall at six days (Moravec et al. 2016). Wall preference of the larvae was analyzed at six days by calculating the percentage of time larvae spent in subdivisions of the circular wells. Unlike MZ*rest* mutants, both *sin3b* and *MZsin3b* mutants spent less time in the outer zone when compared to the controls. MZsin3b mutants spend an average of 91% of time in the outer well compared to the wild-type controls' 95% of the time (P=0.0003)(Figure 3C). These behaviors are similar to the phenotype of the Mecp2 mutant (Pietri et al. 2013), a protein that interacts with Sin3b (Bird et al. 1998).

Sin3b also interacts with CRY1, a core component of the circadian clock (Naruse et al. 2004). Therefor, we sought to determine whether the hypoactivity observed in *sin3b* mutants was a function of the time of day by assaying locomotor behavior at night. Overall larvae move less at night then during the day, but the locomotion parameters of number of movements, distance traveled and duration of movements were similar between the MZ*sin3b* mutants (n=24) and the related wild-type controls (n=24) (Figure 2C-D,G-H,K-L). The data indicates that MZ*sin3b* mutants have a normal behavior at night. These findings also suggest that the observed locomotor defects in *sin3b* mutants are only apparent at higher activity levels because similar phenotypes were not observed during the night when the animals move less.

Evoked behavior of *sin3b* mutants at six days

Because Sin3b interacts with Na_V channels, we sought to determine whether MZ*sin3b* mutants initiate normal responses to stimuli using a visual motor response (Emran et al. 2007). In this assay, a change in illumination from light to dark that causes a rapid transient spike in movements of wild-type larvae. Comparison of MZ*sin3b* mutants (n=46) to related wild-type controls (n=46) showed no significant difference in duration, distance traveled, swim length and velocity in response to the stimulus (Figure 3D-E). This suggests that evoked movements in *sin3b* mutants are comparable to wild-type controls.

rest target genes are not de-repressed in *sin3b* mutants

Because zygotic *rest* mutants also present with hypoactivity (Moravec et al. 2015) and Sin3b is a Rest corepressor, we sought to determine whether expression of Rest target genes (RE1 containing genes) is altered in MZ*sin3b* mutants using qPCR (Data Not Shown). None of the target genes were upregulated in MZ*sin3b* suggesting that either other Sin3 family members or other co-repressors supply the essential repressor activity during early development.

Growth of primary motor neurons in sin3b mutants

Zebrafish mutants with reduced larval locomotion sometimes present with changes in the growth of primary motor neurons (Granato et al. 1996). Furthermore, the density of primary motor neuron projections is influenced by Rest/Nrsf (Moravec et al. 2016). To investigate if the reduced larvae locomotion was caused by either changes to the growth of primary motor neurons or gross defects in trunk muscle, we preformed immunostaining of 30, 56 and 96 hpf MZ*sin3b* mutants and wild-type controls using Znp-1 (synaptotagmin-2) and at 56 hpf for Mf-20, (myosin) antibodies. Neither Znp-1 (Figure 4 A-C) or Mf-20 (Data Not Shown)

immunostaining revealed significant differences in the MZsin3b mutant compared to the WT control, which suggests that the reduced locomotor activity in the *sin3b* mutants has another origin.

Sin3b regulates bone development

We observed that adult *sin3b* mutants are often smaller in size then their siblings. Because embryonic bone differentiation defects were reported in *Sin3b* null mice (David et al. 2008), we examined bone maturation in *sin3b* mutant fish to determine if this correlated with the overall growth defect. Alcian/alizarin stain was performed on 15 dpf and one month old fish derived from a *sin3b*$^{sbu65/+}$ intercross to investigate the requirement for *sin3b* role in ossification in the zebrafish. At 15 dpf, wild-type larvae show variation in length and the rate of bone maturation (Parichy et al. 2009). Larvae from the intercross were staged according to a post embryonic staging series (Parichy et al. 2009). The larvae fell into 3 different categories; 4.3mm (minimal ossification of the spinal cord with no tail ray), 5.0 mm (over half the vertebral column is ossified and cartilage apparent in tail ray) and 6.2 mm (majority of the vertebral column shows ossification along with ossification starting in the tail ray) (Figure 5A-C). All nine of the *sin3b* mutants fell in to the 4.3 mm category while the *sin3b* heterozygotes and the related wild-type controls fell into all three of the categories (Figure 5A-D). The *sin3b* mutant juveniles were statistically smaller when compared to the related wild-type controls (P=0.0271)(Figure 5E).

At one month the *sin3b* mutants and controls showed comparable bone maturation (Figure 5G). However, at one month, *sin3b* mutants remain smaller compared to the related wild-type controls (P=0.0012)(Figure 5F). Based on these findings, we conclude that Sin3b controls the progression of bone maturation because *sin3b* mutants complete ossification later than their siblings. However, an overall size deficit persists in adult *sin3b* mutants.

The role of Sin3b in cell proliferation

In mice, Sin3b regulates cell cycle exit control under limiting conditions *in vitro* and contributes to the repression of E2F gene targets *in vivo* (David et al. 2008). In addition, Sin3b also interacts with key modulators of the cell cycle including p53 (Bansal et al. 2011) and E2F (Grandinetti & David 2008). To investigate the role of Sin3b in proliferation during zebrafish development we employed a single cell transplant assay and phospho-H3 immunostaining.

Transplants were performed by removing a single cell from a labeled sphere stage (4 hpf) donor embryo and placing it on the animal pole of a sphere stage wild type embryo. The number of progeny of transplanted cells, and fate of these cells were scored at 30 hpf. At 30 hpf, there was no significant difference between the number cells generated from donor cells originating from a *MZsin3b*$^{sbu65/sbu65}$ donors (n=18) or from related wild-type control donors (n=18), an average of 12.38 cells and 11.2 cells respectively (P=0.6295) (Figure 6B).

We also compared the fate of transplanted *sin3b* mutant cells to wild-type cells. Based on location and cell shape we sorted the cells into four categories, brain or spinal cord (blue arrow), eye (red arrow), epidermis (yellow arrow) or other (a group of cells located between the head and the yolk.)(Figure 6A). We observed that transplanted mutant and wild-type

cells gave rise to brain/spinal cord and eyes at the same rate, while we see a significant decrease in the amount of *MZsin3b*$^{sbu65/sbu65}$ donor cells entering the epidermis, 26% of wild type transplanted cells end up in the epidermis compared to 11% of *MZsin3b*$^{sbu65/sbu65}$ donor cells (p=0.0004)(Figure 6C). These transplants suggest that Sin3b does not have an effect on cell division but has a potential role in ectodermal cell fate.

We also performed phospho-histone H3 whole mount immunostaining to investigate proliferation of endogenous cells. Phosphorylation on H3 Ser10 occurs during chromosome condensation that is necessary for mitosis (Y. Wei et al. 1999). Whole mount immunostaining was performed at one, two, and four days post fertilization and the numbers of positive cells were counted (Figure 6D). Overall, no significant difference was noted between the *MZsin3b*$^{sbu65/sbu65}$ and the related wild-type controls at any of these time points (Figure 6E). This data is consistent with the single cell transplant data indicating that Sin3b does not have an effect on cell division during early development *in vivo*.

Discussion:

In the mouse, Sin3b has roles in the development of multiple lineages including blood, bone and muscle (David et al. 2008; Shi & Garry 2012; van Oevelen et al. 2010; Yang et al. 2000). The Sin3 family is essential for early development in *drosophila* (Pennetta & Pauli 1998) and mouse (Cowley et al. 2005; David et al. 2008). To examine the requirement for Sin3b during zebrafish development and assess its potential role as a Rest cofactor during neural development, we used the CRISPR-Cas9 system to disrupt *sin3b*. Despite the multiple roles for Sin3b during development, the zebrafish *sin3b* mutant is viable and the adults are fertile and produce viable offspring. However, adult *sin3b* mutant fish are smaller than their siblings and are recovered at sub-Mendelian rates indicating that the loss of Sin3b reduces fitness. In addition, we observe that mutant larvae display hypoactivity that appears distinct from possible roles of Sin3b as a mediator of the Rest transcriptional repressor. Lastly, we did not uncover any requirements for Sin3b in control of proliferation.

Does Sin3b function differ in the Zebrafish and Mouse?

On the surface the mouse and zebrafish *Sin3b* mutant phenotypes differ because zebrafish *sin3b* mutants are viable. However, we observe similar effects on bone development and growth. Zebrafish *sin3b* mutants present with delayed maturation of the skeleton (Figure 5), while the mouse mutants were interpreted as having a block in differentiation. The differing interpretations of the role of Sin3b in bone development could stem from minor differences in the cellular mechanisms of differentiation and activation of osteoclasts (Witten & Huysseune 2009) or more likely, because of our ability to more easily examine multiple developmental stages in the fish mutants. Alternatively, in zebrafish one of the *sin3a* paralogues may be sufficient for the final steps in bone differentiation. We cannot rule out the possibility that the delay in ossification is part of a broader maturation delay in the entire animal that also contributes to reduced overall size.

Sin3b tethers repressor complexes to transcription factors that play key roles in overall growth and cell cycle control including Myc/Mad, Rb/E2F and p53. Failure of any of these pathways could be central to the size and proliferation defects observed in *sin3b* mutants.

However, because p53 mutant zebrafish do not exhibit growth defects (Berghmans et al. 2005) and the retina is the primary tissue effected in zebrafish *rb* mutants (Gyda et al. 2012), we favor a failure of Sin3b repression of Myc or Myc targets as the most likely cause of the phenotypes.

In vitro experiments using *Sin3b* knockout cells indicated that Sin3b plays a role in cell cycle control under limiting conditions (David et al. 2008). We assayed proliferation by single cell transplants and by phospho-histone H3 assays. Both of these assays failed to reveal significant functions for Sin3b *in vivo* proliferation during early development of zebrafish. However, we cannot rule out the presence of subtler defects and careful analysis of the hematopoietic lineage in conditional *sin3b* mutant mice revealed that Sin3b is required for the maintenance of stem cell quiescence (Cantor & David 2017). Furthermore, Sin3b dependent control of senescence has also been shown in an oncogene driven pancreatic cancer model (Rielland et al. 2014). The single cell transplant assay also allowed us to investigate cell fate decisions of the *sin3b* mutant cells. *Sin3b* mutant cells are less likely to acquire an epidermal fate (Figure 6). Previous work has shown functional link between Sin3a and c-Myc for epidermis development (Nascimento et al. 2011). Sin3b has also been shown to regulate the levels of Myc in cell culture (Garcia-Sanz et al. 2014). The single cell transplants data suggest that Sin3b might play a role in regulating epidermal development.

Even though similar phenotypes are observed in the zebrafish and mouse Sin3b mutants, the disparity between the role of *sin3b* during early development might stem from a compensatory mechanisms involving either *sin3aa*, *sin3ab*. In the mouse, Sin3a and Sin3b can not compensate for each other (David et al. 2008; Grandinetti & David 2008; van Oevelen et al. 2010), but in zebrafish, additional subfunctionalization may have occurred as a result of the gene duplication that generated *sin3aa* and *sin3ab*.

Sin3b modulates locomotion

Sin3b interacts with multiple neuronal proteins, including Rest (Naruse et al. 1999; Nomura et al. 2005). In zebrafish, Rest fine tunes neural gene expression (Kok et al. 2012) and regulates locomotor behavior (Moravec et al. 2016; Moravec et al. 2015). While MZ*sin3b* mutants and the zygotic *rest* mutants (Moravec et al. 2015) both present with hypolocomotion, disruption of *sin3b* also alters swim length and swimming velocity. Furthermore, the observations that Rest target genes are not derepressed (Data Not Shown) and that primary motor neurons architecture is unaltered (Figure 4) in the absence of Sin3b leads us to conclude that Sin3b is dispensable for Rest mediated transcriptional repression during early development. These findings are consistent with recent results that suggest that Sin3b has a minimal role in Rest repression in P19 cells (Halder et al. 2017)

Sin3b regulates Na_v-channel activity by directly binding to Na_V channels and reduce the gating charge associated with sodium channel activation (Vega et al. 2013). Zebrafish $Na_v1.6a$ mutants show diminished spontaneous and touch-evoked escape behaviors during development (Low et al. 2010). MZ*sin3b* mutants phenocopy the reduced locomotion phenotype, but show normal evoked movements suggesting that sodium channel function in this context is not compromised when Sin3b is absent.

In conclusion, the zebrafish *sin3b* mutant provides the ability to assess the role of *sin3b* in many tissues and life stages. Our analysis revealed that even though *sin3b* plays a role in bone maturation rate, complete ossification is ultimately achieved. While Sin3b is required for normal growth and mutants show reduced fitness, we found no evidence for a role in cell cycle control *in vivo*. Furthermore, modulation of locomotor behavior by Sin3b appears to be independent of its interaction with Rest. A fuller analysis of the Sin3b function will require assessment of *sin3b* phenotypes in contexts where the activities of the *sin3a* paralogues are compromised to identify redundant activities.

Experimental Procedure:

Sin3b gene targeting

Sin3b was disrupted using the CRISPR–Cas9 system, Guide RNA target site: GGGCACCCGGACCTTGTCCT. gRNAs were generated using Ambion *MegaScript T7* kit. Guide RNA (100pg) was co-injected with 130pg of Cas9 mRNA into the cell of one-cell embryos. Fish were genotyped using primers: F:TGACACACCAGGAGTTATCAACA, R:GGAATTTCAATCCGATACCC

Alcian and Alizarin staining

Embryos or larvae were fixed with 4% PFA for 2 hours (lying flat) at room temperature and then placed in 100% methanol for storage until needed. Fish were immersed in trypsin solution (0.1% trypsin in 30% saturated sodium borate) for 12 hours at 4 degrees for larvae and room temperature for older fish, washed for 10 minutes with 100mM Tris PH 7.5/10mM $MgCl_2$, Alcian stain (0.02%Alcian/10mM $MgCl_2$) was applied for 30 minutes with rocking. After Alcian staining, fish were washed with 80%, 50% and 25 % EtOH/100mM Tris PH7.5/ 10mM $MgCl_2$ for 10 minutes each while rocking,. Following the washes, fish were bleached (3%H_20_2/.1%KOH) until melanophores were no longer visible (45 minutes to 2 hours). After bleaching two 10 minutes washes of 25% glycerol/75% 0.1% KOH were preformed followed by staining with .01% Alizarin Stain/10% glycerol/0.5% KOH for 30 minutes while rocking. Destain was performed in 50% glycerol/0.1% KOH for 10 minutes with rocking. Fish were then transfered to 75%glycerol/0.1%KOH until equilibrated and photographed in 100% glycerol.

Single-cell transplants

Single-cell transplants were performed as previously described with minor modifications (Martin & Kimelman 2012). $Sin3b^{sbu65/sbu65}$ and WT donor embryos were injected with 2% fluorescein dextran at the 1-cell stage. At sphere stage, a single cell was removed and deposited in the animal pole of an uninjected wild-type sphere stage host. The embryos were visualized under a fluorescent dissecting microscope after transplantation to confirm single cell transplantation. At 30 hpf, host embryos were imaged on a DMI6000B inverted fluorescent microscope equipped with a DFC360 FX camera (Leica). Green transplanted cells were counted and assigned tissue types based on morphology and position. Fluorescent and DIC images were overlaid using ImageJ.

Immunostaining

ZPN1 and MF20 immunostaining procedures were preformed as previously described (C. Wei et al. 2013). For the Phospho-H3 staining, embryos were fixed in 4% PFA overnight at 4°C, permeabilized in acetone at −20°C for 1hr and Proteinase K (10 μg/mL) for 45 minutes. Blocking was performed in 3% goat serum in PBS with 0.1% Triton X-100 and 1% DMSO for 2 hours at RT. Embryos were incubated in p-Histone 3 (Ser 10) rabbit polyclonal IgG primary antibody (Santa Cruz Biotechnology, #sc-8656-R) (1:200) at 4°C overnight. Embryos were rinsed in PBT with 1% Triton for 3 hours at room temperature, then placed in Alexa Fluor© 594 Donkey Anti-Rabbit IgG (H+L) secondary antibody (1:500) diluted overnight at 4°C. Embryos were either cleared in glycerol overnight or imaged immediately in PBT using a Zeiss stereo fluorescence microscope.

Behavioral Testing Apparatus and Paradigms:

The Visual-Motor-Behavioral Assay and the testing apparatus were previously described (Moravec et al. 2015). All behavioral assays were performed between 1 to 5 PM and approved by the Stony Brook University IACUC. Statistical analyses were conducted as previously described (Moravec et al. 2015) using SPSS, version 21 and Graphpad software. Outliers were detected using the Grubs test and removed from analysis. Significance was defined as less than 0.05 and trending was defined as 0.099 to 0.05. All error bars represent standard error.

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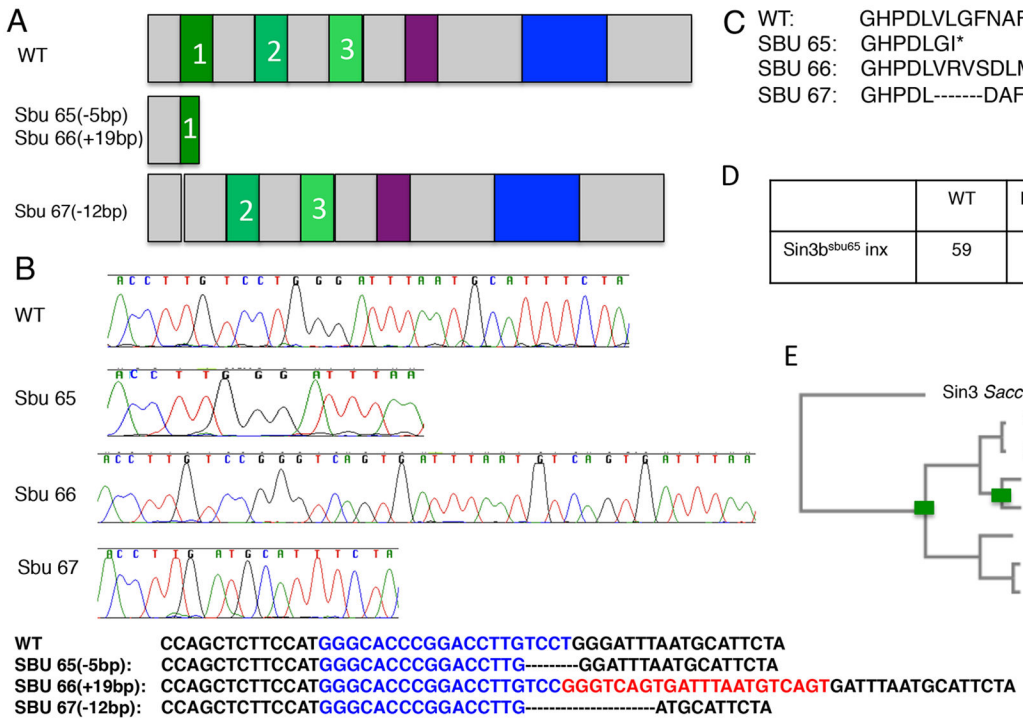
- Disruption of zebrafish *sin3b* reduces fitness but many mutants survive to adulthood and are fertile.
- Zebrafish sin3b mutants show delayed bone maturation, small size and locomotor defects.
- Overall cell proliferation rates are not altered by the loss of Sin3b.



	WT	Heterozygote	Mutant
Sin3b^{sbu65} inx	59	81	27

Figure 1: CRISPR/Cas9 Targeting of zebrafish *sin3b*

A) Domain structure of wild-type Sin3b and truncated proteins produced by Sbu 65, Sbu 66, and Sbu 67 are illustrated beneath. Green = PAH domains Purple = histone deacetylation interacting domain Blue= Sin3, C-terminal B) Sequence chomatograms and alignments from wild-type, Sbu 65, Sbu 66, and Sbu 67 C) Predicted protein sequence of the SBU alleles aligned with a wild-type D) Survival table for a sin3b^{sbu65}inx. E) Phylogenetic tree for the Sin3 family, the green square represent duplication events.

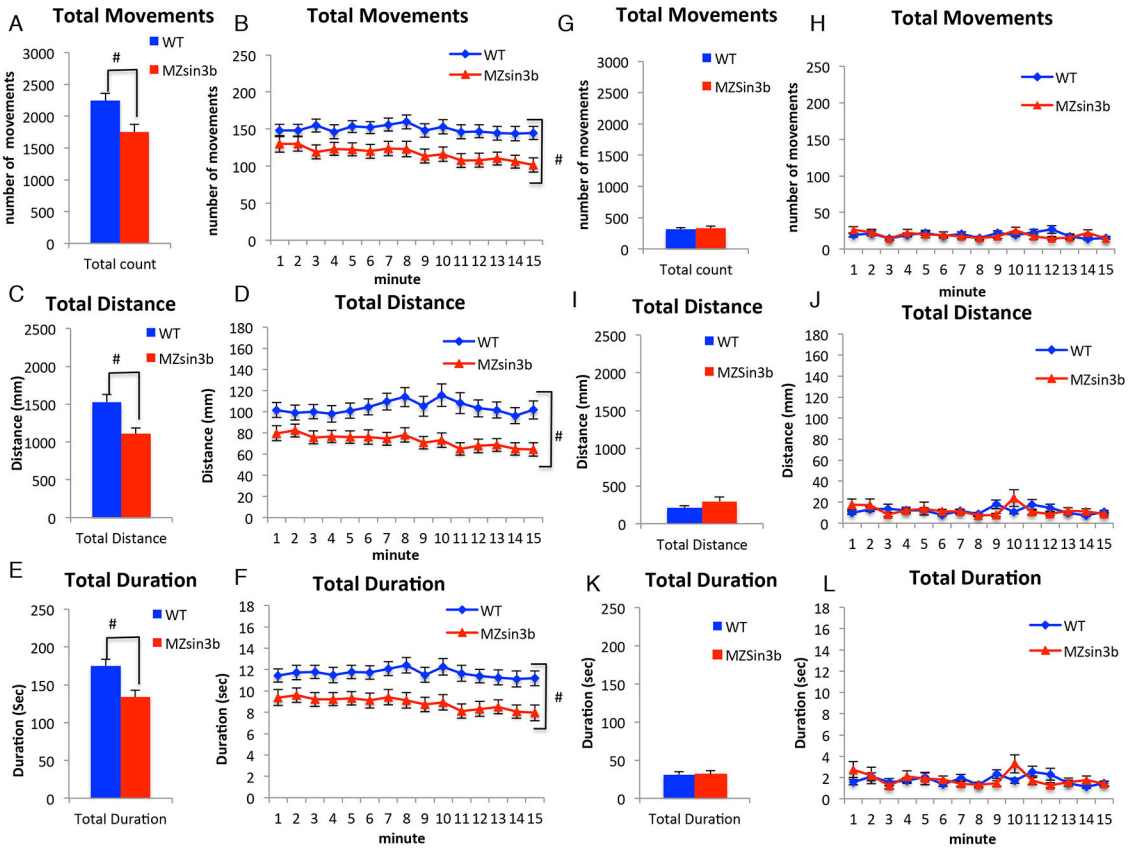


Figure 2: Larvae lacking *sin3b* are hypoactive during the day at 6dpf

A-F) Wild type controls (N=46) out perform MZ*sin3b* mutants (N=46) during the day in total movements (A-B), distance traveled (C-D), and duration of movements (E-F) at 6dpf.G-H) Wild type controls (N= 24) have similar locomotion at night when compared to MZsin3b mutants (N=24) in total movements (G-H), distance traveled (I-J), and duration of movements (K-L) at six dpf.

Genotype P<.05

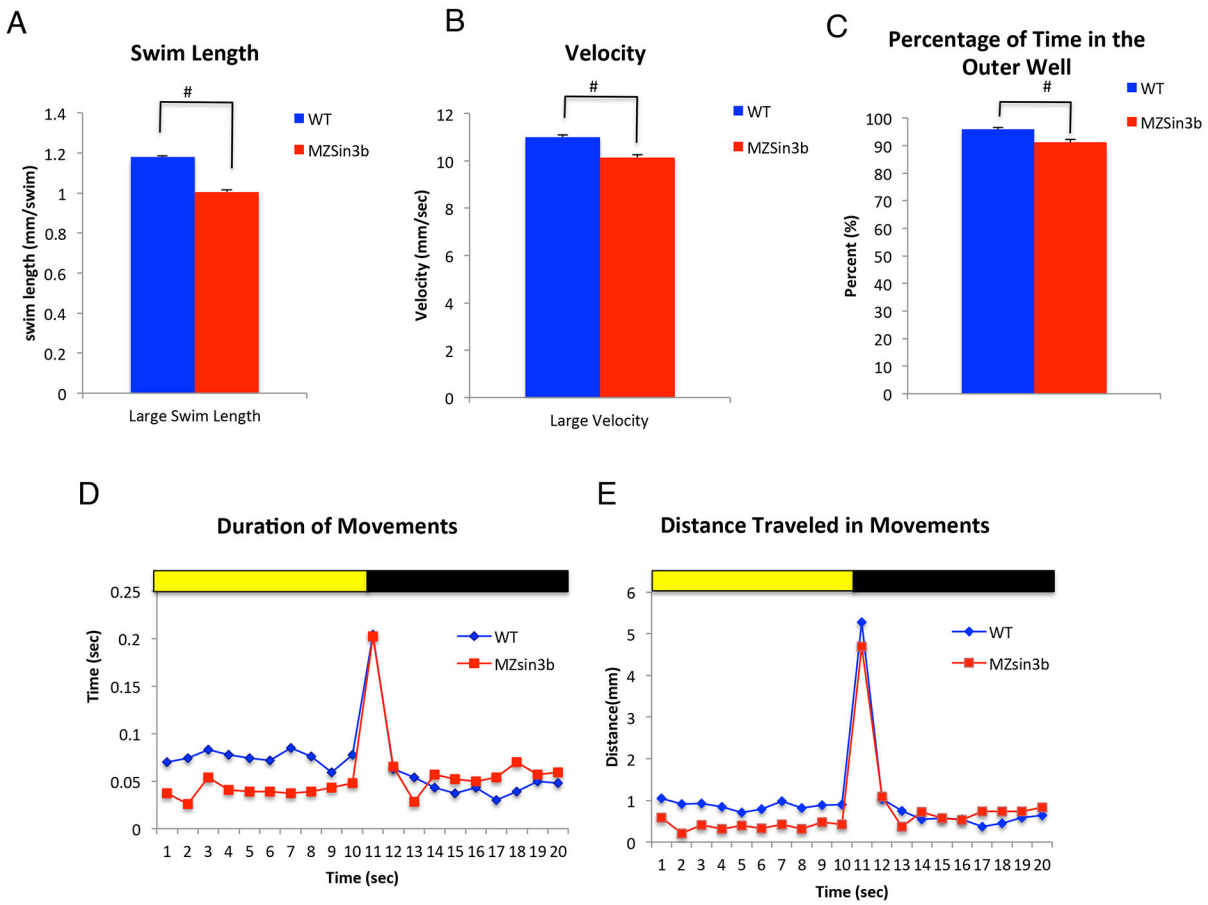


Figure 3: Decrease in swimming kinetics and a spatial preference were observed in MZ*sin3b* mutant

A-B) MZ*sin3b* mutants(N=46) have a smaller large swim length (A) and a slower velocity (B) then related wild-type controls(N=46). C) Quantification of percentage of time spent in the outer well over 15 minutes MZ*sin3b*(N=46) spend significantly less time in the outer well when compared to controls(N=46). D-E) Comparison of duration (D) and distance (E) of movements between MZ*sin3b*(N=46)_ and related wild-type controls (N=46) showed no difference in visual motor assay. Yellow and black rectangles represent the lighting state (on/off).

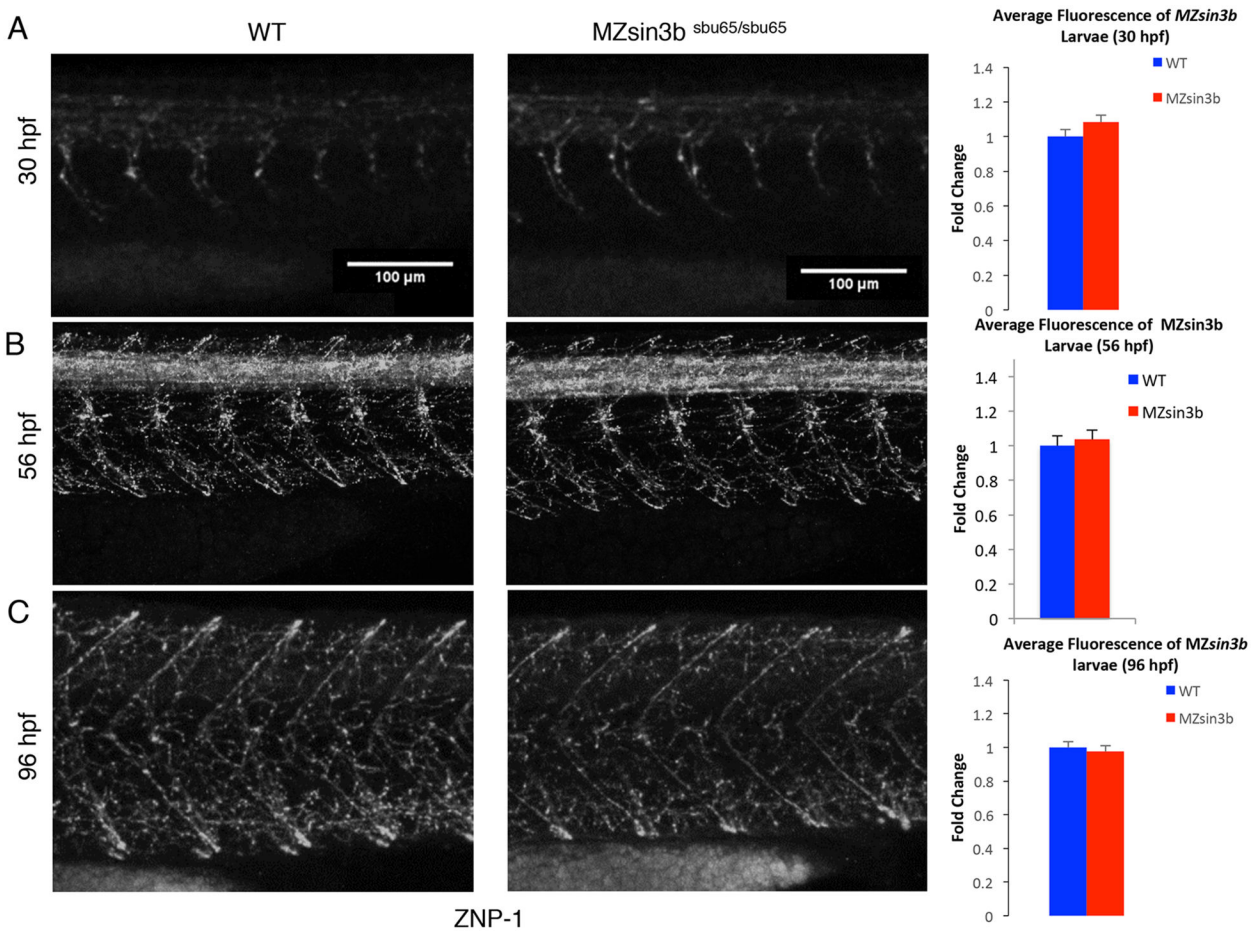


Figure 4: Sin3b does not regulate primary motor neuron development

znp-1immunohistochemistry on whole mount zebrafish embryos at 30 hpf,56 hpf and 96 hpf to label primary motor neurons. Confocal images were acquired from the truck using the yolk extension as a landmark (10um stacks). A-C) No changes were apparent in primary motor neuron architecture or fluorescence was observed MZsin3b $^{sbu65/sbu65}$ mutant(N=3-4) embryos when compared to wild-type controls(N=3-4) per a time point.

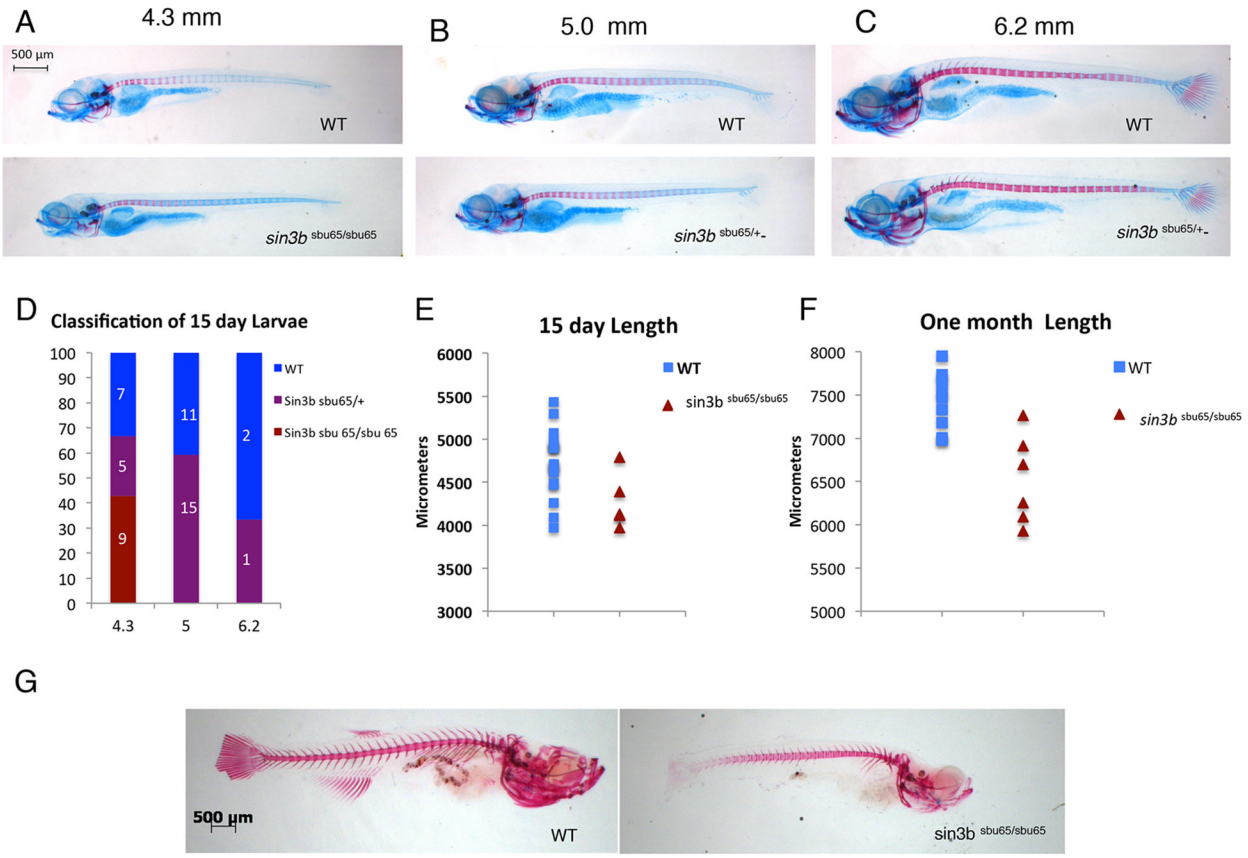


Figure 5: *sin3b* mutants showed delayed ossification when compared to sibling wild-type controls

A) At 15 days *sin3b* mutants show less mature bone when compared to the sibling wild-types and heterozygotes based on juvenile staging classification. B) Number of fish of each genotype that fall in each classification. All the *sin3b* mutants are classified as 4.3 mm. C-D) The *sin3b* mutants are smaller then sibling wild-type controls at 15 days (C) and one month (D). E) Both the *sin3b* mutant and the sibling wild-type controls show complete ossification at 30 days.

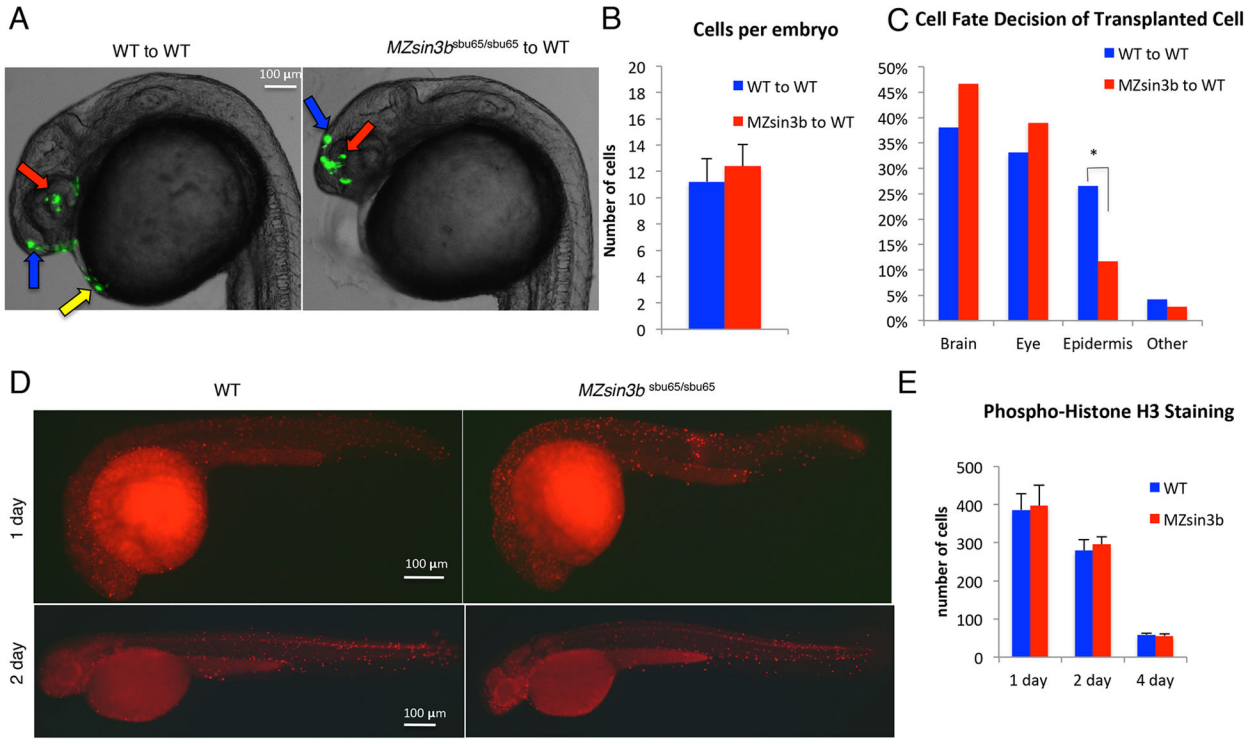


Figure 6: Sin3b mutants show no changes in cellular proliferation

A-C) Transplantation of single MZ*sin3b* (N=18) cells show no difference in cellular proliferation in and these cells are less likely to adopt an epidermis cell fate then controls(N=18). A) Embryos at 30hpf with the transplanted cells expressing GFP. Blue = neural cells Red= eye Yellow=epidermis. B) Graph showing the average number of cells counted at 30 hpf. C) Graph showing the cell fate choices of the transplanted cells. D-E) No difference in phosph-histone H3 staining is observed between mutants and controls at 1 day, 2 day or 4 day.

Table 1:

Two-way ANOVA with repeated-measure design to compare genotypes in 1 min intervals during spontaneous movement

Variable	Genotype		Time		Time X Genotype	
Day	**F value**	**P value**	**F value**	**P value**	**F value**	**P value**
Total Movements	8.387883	0.004741	2.695970	0.008358	1.109130	0.355267
Total Distance	11.321811	0.001132	2.558165	0.009858	0.762709	0.633606
Total Duration	12.651823	0.000604	2.127956	0.034201	1.106171	0.357021
Night						
Total Movements	0.507489	0.507489	1.605384	0.073767	1.231582	0.248165
Total Distance	0.132895	0.717924	1.528661	0.176661	1.827624	0.102166
Total Duration	0.028852	0.866190	1.797273	0.088514	1.993562	0.056730