"Actin-dependent re-localization of the *hb*, *Kr* and *eve* loci determines end of early competence in *Drosophila* neuroblasts"

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Abstract:

Neural stem cells produce specific cell types in a time-dependent manner passing through different competence windows. However, how these competence windows are regulated is largely unknown. Here we use embryonic Drosophila neuroblasts (NB) as a model system to investigate this question. These NBs produce specific cell lineages stereotypically and are characterized by sequential expression of transcription factor cascade Hb/Kr>Kr> Pdm>Pdm/Cas>Cas. It has been shown earlier that the competence to produce Hb-dependent early cell fates can be extended if Hb expression is prolonged. Similar results were observed in Cortex development of vertebrates where the Hb homolog Ikaros permits early cell fate competence to neural stem cells of this region. Here we show that in Drosophila continuous Hb-dependent activation of the Kr-locus which normally gets silenced after termination of its expression is necessary for the extension of NB competence window. Intriguingly this silencing of *Kr* gene locus is correlated with its unusual re-localization from nuclear periphery, a normally repressive subnuclear compartment, towards the interior and can be inhibited by continuous Hb activity. Strikingly, co-expression of Hb and Kr outside of the early competence window induces a reversion of *Kr*-locus position towards nuclear periphery correlating with ectopic production of early neuronal cell types at this late time of development. An opposite and Hb-independent re-localization pattern from the nuclear interior towards periphery has been described earlier for the *hb*-locus during early competence window. Here we observed similar re-localization of *eve* locus but in contrast to the *hb*-locus, this is also inhibited by continuous Hb expression. Interestingly, co-expression of Hb and Kr outside of the early competence window reversed *eve*-locus position towards nuclear interior, thus again correlating with NB competence. However, less is known about possible mechanisms required for such chromatin rearrangements and there are hints in a vertebrate system that nuclear ß-actin might play a role. Here I found evidence that oligomerization of nuclear ß-actin is involved in such events in *Drosophila*. This has been deduced from overexpression experiments using dominant negative non-oligomerizing NLS-GFP-actin which resulted in a deceleration of locus re-localizations whereas wild-type NLS-GFP-actin showed acceleration. Furthermore, over-expressing Exportin 6, a specific transporter of ß-actin out of the nucleus, leads to a block of *hb*, *Kr* and *eve* locus re-localization. Most importantly, late overexpression of Hb in this situation re-establishes the ability to induce early cell fates showing a causal relationship between gene locus re-localizations and NB competence. Furthermore, I could show the role of epigenetic factors Polycomb and HDAC1

in re-localization of these loci. Knockdown of these factors resulted in slow down or halt in the repositioning of loci. Together we propose that within the early competence window there are multiple nuclear actin dependent gene loci re-localizations in different directions suggesting ongoing epigenetic silencing which can be partly inhibited or even reverted by the combined activity of Hb and Kr.

Contents

List of Abbreviations:

°C	Degree celsius
ß-Gal	ß-Galactosidase
Bp	Basepair
BSA	Bovine Serum Albumin
Ca.	circa
Cas	Castor protein
cas	*castor*-gene
CDS	Coding sequence
CNS	Central nervous system
CO_2	Carbon dioxide
Chic	Chikadee Protein
DAPI	4′,6-Diamidin-2-phenylindol
DEPC	Diethylpyrocarbonate
DNA	desoxy ribonucleic acid
dNTP	Deoxy nucleosid triphosphate
Dpn	Deadpan Protein
Drosophila	*Drosophila melanogaster*
E.coli	Escherichia Coli
En	Engrailed protein
en	*engrailed*-gene
et al.	and others
Eve	Even-skipped protein
eve	*even-skipped*-gene
EtBr	Ethidiumbromide
FBS	Fetal Bovine Serum
FHB	FISH Hybridization Buffer
Fig	Figure
g	Gram
GMC	Ganglion mother cell
Hb	Hunchback protein
hb	*hunchback*-gene
kb	Kilobase
Kr	Krueppel protein
Kr	*Krueppel*-gene
l	Litre
LiCl	Lithiumchloride
LSM	Laser scanning microscope
M	Molar
mdp	Mean distance to the periphery
MeOH	Methanol
mg	Milligram
min	Minute
ml	Millilitre
mm	Milimetre
mM	Millimolar
mRNA	messenger-RNA
NaCL	Sodiumchloride
NB	Neuroblast
ng	Nanogram
NB	Neuroblast
NB7-1	Neuroblast 7-1

PBS	Phosphate buffered saline
PBT	PBS + Triton x 100
PcG	Polycomb group
Pc	Polycomb
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	Potential Hydrogenii
pHM	pre-Hybridization Buffer
PNS	Peripheral nervous system
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
ssDNA	Single stranded DNA
Svp	Seven-up-protein
svp	*seven-up*-gene
st.	Stage
Stg	String protein
tsr	*twinstar* gene
Tab.	Table
TAE	Tris-acetate-EDTA
UAS	upstream activating sequence
UTR	untranslated region
UV	ultraviolet
V	Volt
VNS	Ventral nervous system
Vol.	Volume
WT	Wildtype
w/v	Weight/Volume
µg	Microgram
µl	Microlitre

1. Introduction

The central nervous system (CNS) is a network of more than millions of individual nerve cells that control our actions, sense our surroundings, and define who we are. The functional units of the CNS are neurons which are unique in their ability to transmit and store information. Although these neurons are generated from a relatively small number of neural progenitors (stem cells) during development, it makes them one of the most important cell types in brain development from mammals to insects. These progenitors respond to different cues in order to produce different types of neural progenitors, and thereby different types of neurons and glia. Progress has been made recently in understanding the molecular mechanisms by which these individual progenitors can generate a sequence of different cell types and this phenomenon is termed as temporal patterning or specification (Jessel et al., 2000; Pearson and Doe, 2002). Studies of neurogenesis in multiple organisms show that the molecular players involved in such temporal specification are highly conserved. However, it is rather difficult to study such development in higher organisms due to longer life-cycle, the complexity of brain and availability of tools. On the other hand, *Drosophila melanogaster* (further termed as *Drosophila*) is serving as a powerful genetic model organism over a century to study and understand such molecular mechanism. This is due to the fact that, it has shorter life-cycle, the genome has been deeply sequenced and several molecular and genetic tools are well established. In this study, we have shed a light on the understanding regulation of progenitor competence and how these progenitors also undergo a change in chromatin architecture during embryonic neurogenesis in *Drosophila*.

1.1 Embryonic neurogenesis of *Drosophila melanogaster*

The CNS of *Drosophila* comprises of neural stem cells (neuroblasts, NBs) which divide in stem cell mode to form a chain of cells known as ganglion mother cells (GMCs). These GMCs further undergo another division to form two post-mitotic cells, which can be neuronal or glial in nature (Bossing et al., 1996; Schmid et al., 1999). The mechanisms regulating the spatiotemporal specification of neuroblasts have been well studied in the nerve cord that can be considered to be the *Drosophila* spinal cord. The nerve cord extends over three thoracic and eight abdominal segments. Each hemisegment initially contains the same invariant array of 30 neuroblasts that delaminate from the neuroectoderm during early embryogenesis (Doe,

1992; Technau et al., 2006). Each of the 30 neuroblasts inside an embryonic hemisegment can be identified both by its localization (Bate, 1976; Doe and Goodman, 1985) and the unique combination of spatial markers it expresses (Broadus et al., 1995; Doe, 1992). Based on this spatial information, NBs can be individually identified according to its row and column number (NB1-1, NB2-1, NB3-1, etc.). These NBs give rise to a set of progeny, which is specific and reproducible, and always generated in same temporal sequence. Such temporal identity of cells is autonomously regulated and linked to the sequential expression of certain transcription factors like Hunchback (Hb), Kruppel (Kr), Pdm1/Pdm2, Castor (Cas) and Grainyhead (Grh) further called temporal transcription factors (TTF, Fig 1). At the end of each time window, the expression of TTF is switched off in the NB but stays on in the GMC and its progeny. For two of these factors, *Hb* and *Kr*, it has been shown that they are indeed necessary and sufficient to specify the first two temporal fates in certain NB lineages (Isshiki et al., 2001; Novotny et al., 2002).

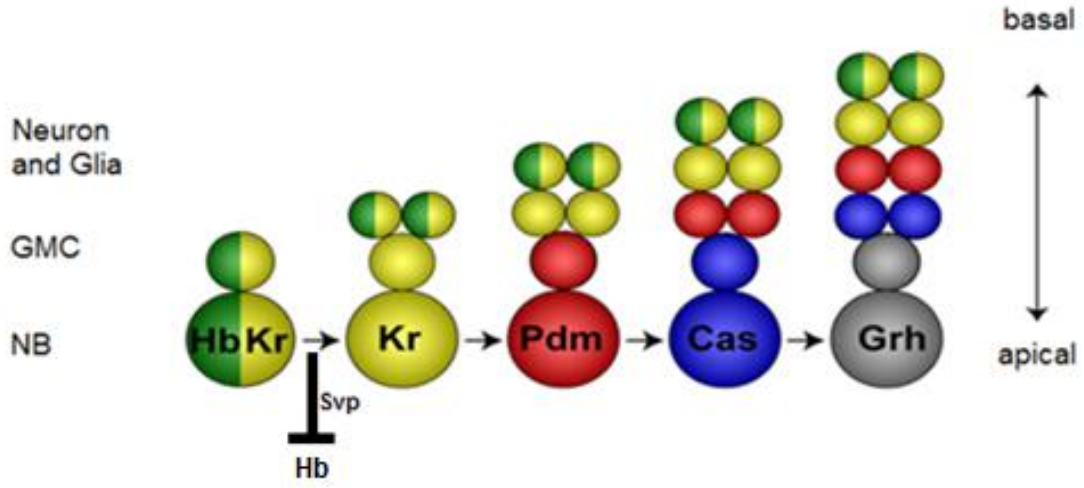


Fig.1 Principle sequence of expression of TTF in the *Drosophila* CNS. Svp represses Hb transcriptionally after second division.

The best characterized of these factors is Hb, which is a zinc finger transcription factor homolog of mammalian orphan receptor Ikaros (Elliott et al., 2008). The *hunchback* gene codes for the two transcripts *hb*-RA and *hb*-RB, which differ in their two UTRs. In addition to the characteristic for the protein zinc fingers, Hunchback has a number of other evolutionarily highly conserved sequences. While the exact function of most sections is still largely unknown, the D-Box offers an interaction domain for the ATPase dMi2. The binding

of dMi2 to Hunchback is required for the repression of target genes, such as the homeotic (HOX) genes in the development of Drosophila (Kehle et al., 1998). Hb is co-expressed along with Kr and is repressed one or two divisions later by Seven-up (Svp) a homolog of mammalian COUP-TF, so that Kr window is generated (Isshiki et al., 2001; Kanai et al., 2005; Kohwi et al.,2011; Mettler et al., 2006)(Fig 1). Intriguingly, recent work has shown that the COUP transcription factor 1 (COUP-TFI) and COUP-TFII nuclear receptors, which are orthologues of Svp, function as a 'timer' that switches progenitors from neurogenesis to gliogenesis. They are transiently expressed in neural progenitors near the end of the neurogenic phase, and their loss prolongs neurogenesis at the expense of gliogenesis. COUP-TF1 has also been implicated in the switch from early-born to late-born cortical neurons. Thus, Svp and the COUP-TF proteins seem to have conserved roles as switching factors in both *Drosophila* and mammalian neural progenitors (Naka et al., 2008; Faedo et al., 2008). Neurons born from the Hb-expressing NBs maintain *hb* transcription via a neuron-specific cis-regulatory element (Hirono et al., 2012). Similarly in vertebrates Ikaros, is expressed in young progenitors in which it specifies early-born retinal ganglion cell (RGC) identity (Elliott et al., 2008). In one of the next cell cycles, Kr is shut off and another factor, Pdm, is switched on, followed by Cas expression. The Krüppel transcription factor, in addition to the DNA-binding zinc fingers, has well-characterized activator and repressor domains (Licht et al., 1990, Licht et al., 1993, Sauer and Jackle, 1991), and a C-terminal dimerization domain (Sauer and Jackle, 1993) and a binding site for the co-repressor dCtBP (Nibu et al., 1998a). dCtBP is the *Drosophila* homolog to CtBP in humans with the same function as a repressor (Nibu et al., 1998b). Kr as a low-concentration monomer acts as a transcriptional activator, and at higher doses, heterodimers cause transcriptional repression (Sauer et al., 1995, Sauer and Jackle, 1993). In this context, the association of Krüppel DNA bound *hunchback* molecules can form a DNA-linked, transcriptional repressor complex (Sauer and Jackle, 1995) and Hunchback-dependent activation of gene expression can be inhibited in a tissue cell culture by co-expression of Krüppel (Zuo et al. , 1991).

The fifth and last transcription factor, the bHLH protein Grainyhead (Grh), which appears after Cas in neuroblasts. Grh is activated by Cas in most, if not all, neuroblasts, it can repress *cas*, and specifies different types of neural fates (Baumgardt et al., 2009). However, in contrast to the other TTFs, Grh is permanently expressed in neuroblasts once activated. It has been shown the existence of cross-regulatory interactions between these TTFs. Loss- and gain-of-function experiments have revealed the ability of a TTF to initiate activation of the next factor in the series. In addition, cross-repression motifs have been observed such that Hb

represses *pdm* and *cas* transcription, Kr represses *cas*, *Pdm* represses *Kr*, Cas represses *pdm*, and Grh represses *cas* (Baumgardt et al., 2009; Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kambadur et al., 1998; Tran and Doe, 2008). At the end of embryonic neurogenesis, NBs either undergo apoptosis, which is the case for the majority of NBs in the abdominal neuromeres, or they arrest in the G_1 phase of the cell cycle and become quiescent. The decision between apoptosis and quiescence is regulated intrinsically by the combined action of Hox proteins and temporal identity factors (Tsuji et al., 2008).

1.2 Regulation of NB competence

Several studies have demonstrated that in the course of development, mammalian NSCs transit through various competence windows during which they respond to given cues by generating specific types of progeny. For example, heterochronic transplantation experiments have shown that mouse NSCs will respond differently to the same extrinsic cue if applied at different developmental times (Desai and McConnell, 2000; Livesey and Cepko, 2001). Another example when Ikaros is ectopically expressed in older retinal progenitors *in vivo*, it can induce production of early-born neuronal identities, such as horizontal and amacrine cells, and suppress the production of the late-born Müller glia. However, Ikaros misexpression cannot generate early-born ganglion cells *in vivo*, suggesting that some but not all early progenitor competence can be restored (Elliott et al., 2008). Similarly, when *lin28* mRNA, a late retinal-progenitor microRNA target, is ectopically expressed in early progenitors, there is an increase in the $BRN3^+$ early-born ganglion cell type; however, there is no increase when *lin28* is expressed in late progenitors, which suggests that there is a restricted competence window in which early-born fate can be specified (La Torre et al., 2013). A similar mechanism exists in *Drosophila*, where recent work has shown that Hunchback, an Ikaros family zinc finger transcription factor, is sufficient and also necessary to decide the cell fate of early-born neurons in multiple neuroblast lineages and this has been well studied in the lineages NB7-1 and NB3-1(Fig 2). The NB7-1 delaminates from the neuroectoderm, together with nine other NBs, during the first segregation wave in late stage 8. The NB7-1 lineage consists of 16-22 neural cells, which arise from more than 20 GMCs (Bossing *et al*., 1996). NB7-1 produces the Even-skipped (Eve)-positive U1-U5 motoneurons during its first five cell divisions followed by a variable number of Eve-negative interneurons (Fig 2). Hb loss of function mutants lack early-born U1/U2 neurons, and prolonged Hb expression within this

NB will generate many additional Eve-positive U1-like neurons until end of embryogenesis, it has also been shown that along with continuous expression of Hb, Kr is also co-expressed (Isshiki et al, 2001; Novotny et al, 2002). Importantly, re-expression of Hb after the NBs fifth division has no effect (Pearson and Doe, 2003). This shows that NB7-1 loses its competence to generate Eve-positive motoneurons in response to Hb activity latest after five divisions and this is referred as 'neuroblast early competence window.' This is analogous to the activity of Ikaros in the vertebrate retina and cortex development (Elliott et al., 2008; Alsiö et al., 2013). Similarly, NB3-1 is known to generate the well-characterized RP1, RP4, RP3 and RP5 motoneurons (Bossing et al., 1996; Landgraf et al., 1997; Schmid et al., 1999). Hb and Kr also specify early temporal identity in NB 3-1 lineage, extending their role as multi-lineage temporal identity factors to a different spatial domain of the CNS (Tran and Doe, 2008). RP1/4 are Hb^{+} Kr^{+}, RP3 is Hb^{-} Kr^{+}, and RP5 is Hb^{-} Kr^{-} and these motoneurons additionally are also positive for $Hb9^{+}$ and $Islet^{+}$ markers (Tran and Doe, 2008). Further, it was shown that continuous *hb* expression in early time point of development led to the production of additional RP1/4 motoneurons (Tran and Doe, 2008). This proves that Hb is necessary and sufficient to specify early-born RP1/RP4 temporal identity within the NB3-1 lineage, resembling its role in specifying the first temporal identity in the NB7-1 lineage (Isshiki et al., 2001; Novotny et al., 2002).

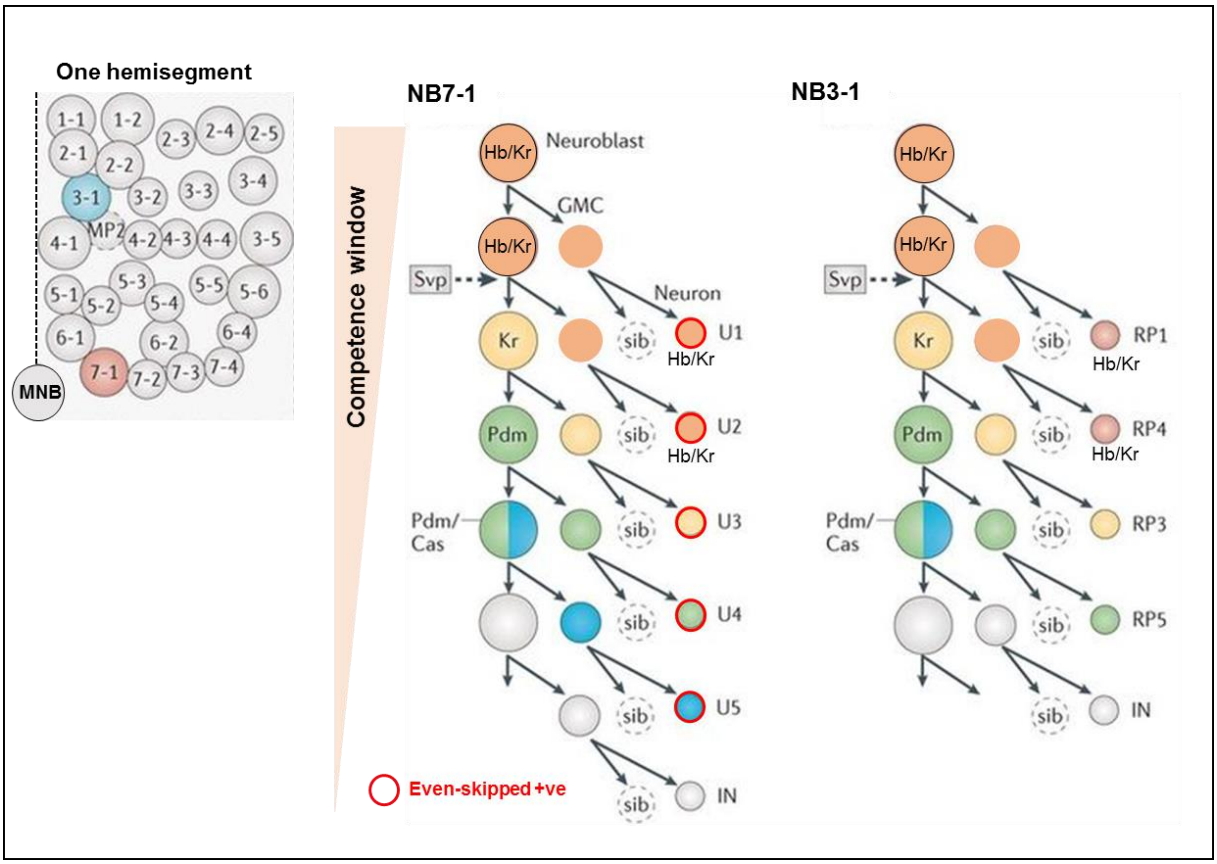


Fig.2 Schematic representation of NB7-1 and NB3-1 competence window in WT embryos. Cartoon to extreme left show one hemisegment of the embryo, where individual NB can be identified by its position where it is born. NB7-1 highlighted in pink and NB3-1 highlighted blue. The dotted line represents midline. (Image modified from Kohwi and Doe, 2013 review).

In case of both of these lineages, it was observed that they lose their ability to produce motoneurons after certain time point. In this study, we try to identify possible regulation that could result in gaining the competence back for NB7-1 to produce additional U motoneurons and NB3-1 to generate additional RP motoneurons.

1.3 Gene locus re-localization

Chromatin is mobile in the cell nucleus and undergoes movements that are best described as constrained diffusion. The cell nucleus is characterized by the existence of distinct structural and functional compartments (Fig 3). Mainly it consists of substructures that include the nuclear lamina, nucleoli, PML and Cajal bodies, and nuclear speckles (Fig 3). Genetic material within the genome is folded and packaged as higher ordered structures that are shown to play important role in gene regulation within the nucleus (Cremer T and Cremer C, 2001). Research from past decades has proved that, in the eukaryotic nucleus, the DNA double helix wraps around histone proteins in repeating units of nucleosomes to form chromatin, which in turn is folded into multi-level higher-order structures. Advances in microscopy techniques, in particular, those based on fluorescence in situ hybridization (FISH) and live-cell imaging of in vivo tagged genomic loci, have made it increasingly clear that eukaryotic genomes are non-randomly organized inside the nucleus. There is evidence showing non-randomness of the 3D organization of nucleus which consists of following aspects, 1) Different chromosomal territories (CT) are occupied by each chromosome. 2) The eukaryotic genome is divided into different euchromatin and heterochromatin regions (Fig 3). The heterochromatin is a region characterized by its transcriptionally repressed state and highly condensed structure, whereas euchromatin is transcriptionally active and less condensed. 3) Individual chromosomes, genes, and genomic elements undergo repositioning within the nucleus, which has been shown to correlate with genomic properties as well as with genomic functions such as transcriptional activity and replication timing (reviewed in Duan and Blau 2012). Interior of nucleus is considered euchromatic while lamina or periphery a compartment known to be involved in transcriptional repression, in order to experience a gene silencing is considered densely heterochromatic also there is an evidence in multiple organisms showing that repositioning of a gene to the nuclear lamina can cause transcriptional

repression (Fig 3) (Pickersgill et al., 2006). It has been suggested for a long time that this non-random distribution of heterochromatin and euchromatin has a function and that attachment of chromatin to the nuclear envelope is important to obtain the three-dimensional organization of the chromatin fibers (G Blobel, 1985). Furthermore, as mentioned currently repressed genes are often associated with heterochromatin and active genes are usually located around nuclear speckles (Lamond and Spector, 2003). However it is seen that the gene locus position can change in response to transcriptional activation (Parada and Misteli, 2002) and vice versa, the gene's transcriptional activity is affected by its position in the nucleus (Finlan et al., 2008). In 2006, Chuang et al. could visualize the dynamics of gene locus re-localization using constructed cell lines, which carry tandem gene arrays. These tandem gene arrays are under the control of an inducible promoter. The entire locus relocated from nucleus' periphery to the interior, immediately after the expression of the array was activated. Another example in vertebrate system, where it was shown that chromatin around key differentiation gene loci Pax6 and Irx3 undergoes both de-compaction and displacement towards the nuclear center coincident with transcriptional onset (Patel et al., 2013).

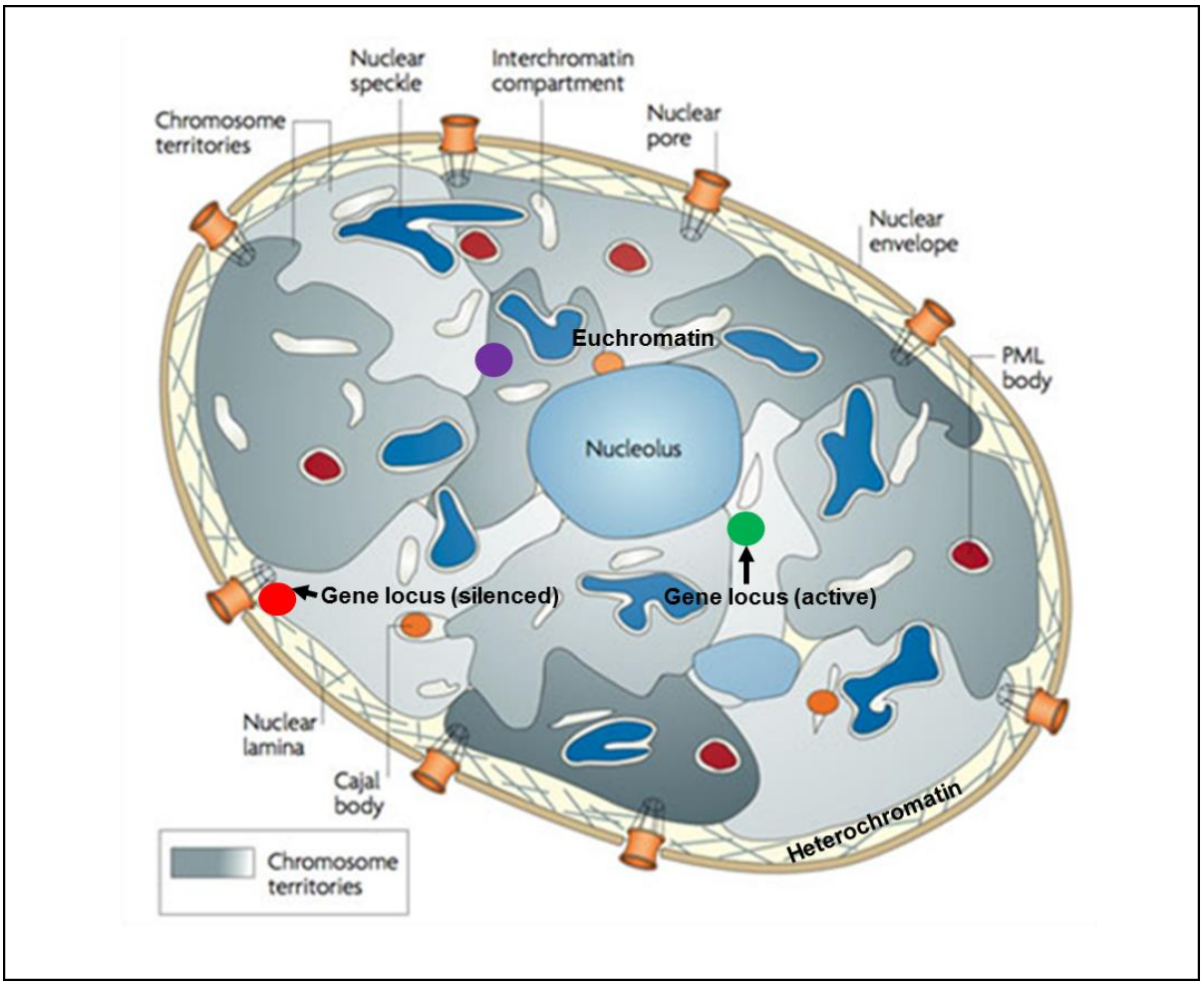


Fig.3 Cartoon representing structure and organization of nucleus (modified from Lanctot et al., 2007)

In 2013, Kohwi et al. found that the loss of competence after the fifth cell division correlates with the repositioning of the *hunchback* (*hb*) locus to the nuclear periphery. This repositioning takes place almost synchronously within the entire NB population at embryonic stage 9/10. the time when the *hb* gene locus moves strikingly correlates with the end of its early competence window in NB7-1 at late stage 12, three divisions after repressing the *hb* transcription. The transcription of the *hb* gene is consequently regulated on two distinct levels. First, it is terminated by Svp after the second cell division. Secondly, *hb* becomes permanently silenced by repositioning it to the nuclear periphery after the fifth division. In this study, we could observe a strong correlation between gene locus re-localization of *Kr* and *eve* with NB competence. *Kr* locus re-localizes in multiple NBs at the onset of competence starting in the periphery and is active transcriptionally with a final localization in the nuclear interior. In this study, we also observed that *eve* re-localizes from nuclear interior to periphery, similar to *hb* and potentially get silenced at end of competence window. Thus we could here observe a strong correlation between gene locus re-localization and NB competence.

1.4 Nuclear actin and its different roles in the nucleus

Actin is one of the most abundant proteins found in all eukaryotic cells. In the cytoplasm, actin has long been known to exist in a dynamic interplay between its monomeric form, G-actin, and its polymerized form F-Actin. F-actin is a complex cytoskeletal structure involved in a variety of conserved functions, including cell adhesion, cell shape, endocytosis, cytoplasmic transport and nuclear migration (Mishra et al., 2014 and Stricker et al., 2010). The existence of actin in the nucleus became evident only recently, but it plays a key role in this area as well, since it’s regulating both, chromosome organization and gene activity. Actin binds to all three polymerases and may be needed to form the active forms of RNA polymerase I and II (Grummt, 2006; Hu et al., 2004; Hofmann et al, 2004). There are many other functions of nuclear actin, e.g. editing and nuclear export of mRNAs and chromatin remodeling (Gieni and Hendzel, 2009; Skarp and Vartiainen, 2013).

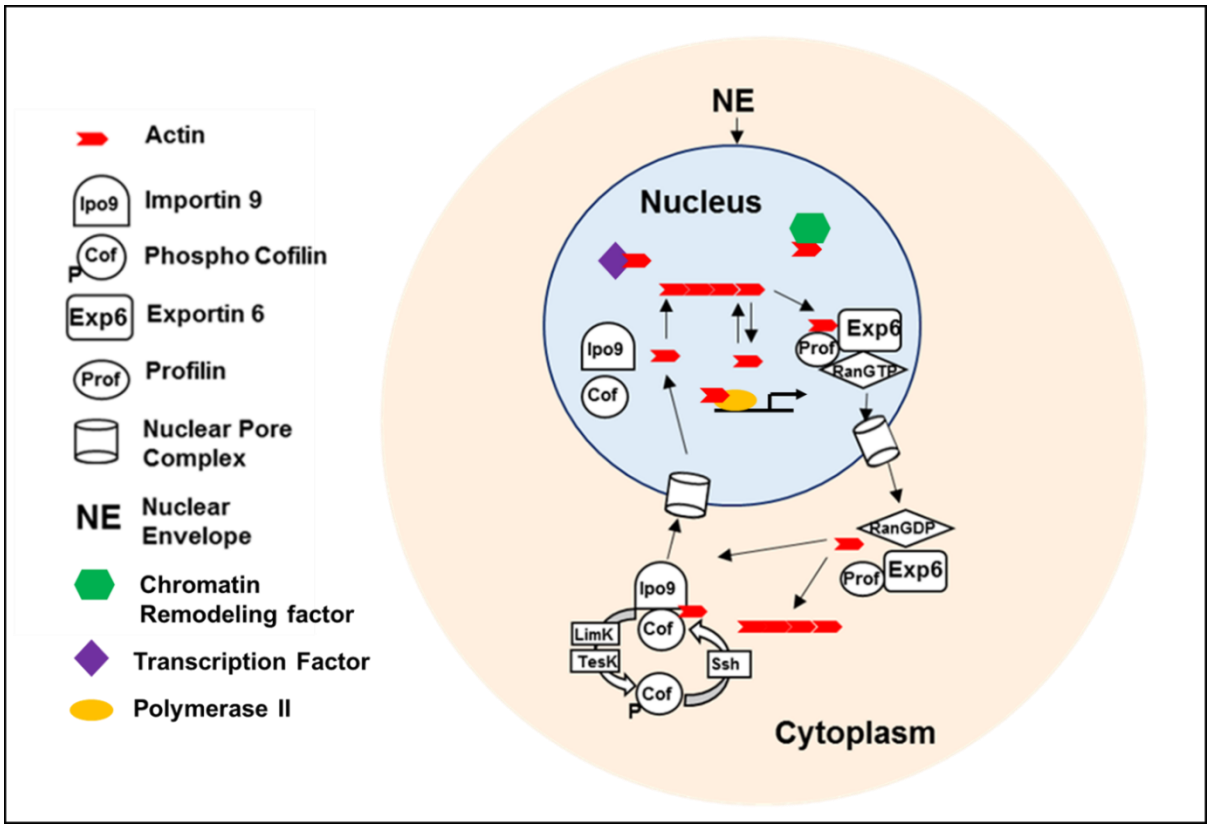


Fig.4 Multiple roles of actin within the nucleus and its nucleo-cytoplasmic shuttling model. Unphosphorylated cofilin and importin9 bind actin monomers in the cytosol and then translocate to the nucleus through nuclear pore complexes (NPC). In the nucleus, cofilin depolymerizes actin filaments to maintain the actin monomer pool. Actin monomers and filaments exist therefore in dynamic equilibrium. The export of actin through NPCs is mediated by Exportin6 together with profiling and RanGTP. The expression level of Importin is maintained via the mitochondrial assembly regulatory factor (MARF) and the copper transporter 1a (Ctrl1) (Dopie et al., 2015).

Additionally, it has been shown in *Drosophila* that actin does undergo constant nuclear and cytoplasmic shuttling with the help of actin-binding proteins. The function of Actin-binding-proteins, from which more than 30 have been identified in the nucleus (Castano et al., 2010, Weston et al., 2012) is most likely the regulation of actin dynamics by controlling the formation of monomeric and polymeric actin in the nucleus. This work is mostly based on the three ABPs Importin 9, Cofilin and Profilin. Fig. 4 shows a model for the nucleo-cytoplasmic transport of actin. Ranbp9 is the Drosophila homolog of Importin 9 which represents the relevant nuclear import factor for actin. The active maintenance of nuclear actin levels by this ABP is required for maximal transcriptional activity (Dopie et al., 2012). Ranbp9 acts together with Cofilin (twinstar (*tsr*) in *Drosophila*), enhances de-polymerization of actin by cutting F-actin (Paavilainen et al., 2004). It binds to G-actin and forms a heterodimer which is then imported into the nucleus (Dopie et al., 2015). The export of actin out of the nucleus and

into the cytoplasm is mediated by Profilin (Chickadee in *Drosophila*) together with Exportin 6 (Exp6) and Ellipsoid body (Ebo) in *Drosophila*. Exp6 forms a dimer with Profilin acting as cofactor of actin export and hence as a suppressor of actin polymerization in the nucleus (Stüven et al., 2003). The exchange between nuclear and cytoplasmic actin occurs at nuclear pore complexes (NPCs), macromolecular structures that form channels penetrating the double lipid bilayer of the nuclear envelope, connecting the nucleus and the cytoplasm. Nuclear actin filaments have been proposed to participate in many processes, including control of chromatin architecture, regulation of transcription, and intra-nuclear cargo transport. Specifically, such role of nuclear actin can be divided into following groups 1) It is involved in long-range intra-nuclear chromosomal movements (Chunag et al., 2006; Dundr et al., 2007; Khanna et al., 2014) 2) Increasing mechanical strength of nucleus (Boschak et al., 2006) 3) Assembly of nuclear regulatory components by creating small scaffolds (Huang et al., 2011; Belin et al., 2013). To further elaborate point (1) it has been shown in vertebrate's specialized role of nuclear actin in gene locus re-localization. For example, in inducible transgene Chinese Hamster ovary cells it was observed that tethering the transcription acidic activation domain (AAD) of the viral protein VP16 to a peripheral site led to the repositioning of the chromosome site from the nuclear periphery towards the nuclear interior and this repositioning was halted after expression of non-polymerizing mutant (G13R) and accelerated when nuclear actin was strongly polymerized (S14C) (Chuang et al., 2006). Also, Dundr et al (2007) revealed the occurrence of directed movement over a distance of 2-3μm by examining the interaction between the Cajal bodies (CBs) and U2 genes and they too could observe that expression of G13R actin mutant inhibited the repositioning of the locus. How nuclear actin does polymerize in S14C and how this polymerization is lost in G13R mutant? The answer lies in the process where nucleotide binding and the conformational changes occur upon ATP hydrolysis (Posern et al., 2002). For further examination of the role of actin/myosin involvement in the gene locus movement, Hu et al. (2008) chose ERα-mediated inter-chromosomal interactions. They treated E2-stimulated breast epithelial cells the drugs latrunculin, which blocks actin polymerization and jasplakinolide, which in turn inhibits actin de-polymerization. In both cases, the ERα-mediated inter-chromosomal interactions and even the activation of ERα target genes are prevented. These examples thus hint towards the direction that polymerization of nuclear actin is also important in this mechanism. In order to further support this phenomenon in our system we also generated flies, which express a dominant/negative (D/N) G14R (Glycine to Arginine) and a gain of function (GoF) mutated form of actin S15C (Serine to Cysteine), both lines tagged with GFP-NLS sequence, similar

to vertebrate system. Altogether it can be proposed that nuclear actin indeed has some role to play in a mechanistic way in re-localization of genes and in our case we also could see the similar function of nuclear actin which is discussed in details further in results and discussion section.

1.5 Role of epigenetic factors in gene silencing

Cell type expression programs are established within the genome in order to generate different cell identities from a single set of genomic sequences and this is achieved with help of epigenetic mechanism (Ptashne, M. 2007). Different modes of epigenetic regulation exist within the genome that controls transcriptional program in space and time, these include DNA methylation, post-translational modifications of histone tails etc. Changes in the nuclear organization are an important component among epigenetic mechanisms contributing to robust and stable gene silencing (Sexton and Cavalli, 2015). The evolutionarily conserved Polycomb Group (PcG) proteins are one of the most prominent epigenetic silencing systems, playing a central role in cell differentiation and the maintenance of cell identity. PcG proteins are chromatin-associated factors that locally modify chromatin through their histone-modifying activities to regulate their target genes. Furthermore, they participate in chromatin looping and long-range interactions between Topological Associated Domains (TADs) marked by trimethylation of histone H3 lysine 27 (H3K27me3), thereby regulating global genome architecture (Reviewed in Di Croce and Helin, 2013). The three main PcG complexes in vertebrates and *Drosophila* are Polycomb Repressive Complex 1 (PRC1) consisting of Polycomb (Pc), Polyhomeotic (Ph), dRING, and posterior sex combs (PSC) (Shao et al., 1999) Polycomb Repressive Complex 2 (PRC2) comprises of PRC2 contains three components: Enhancer of Zeste (EZ), Suppressor of zeste 12 (Suz12), Extra sex combs (Esc) and Pho-Repressive Complex (PhoRC). In simple words the function of these proteins is as follows: PRC2 first binds to chromatin and its catalytic subunit, EZH2, trimethylates H3K27. H3K27me3 is then recognized by the CBX component of PRC1, the E3 ligases RING1/2 then monoubiquitinates H2A on K119 which leads to chromatin compaction and pausing of RNAPII (Francis et al., 2004) thus leading to silencing of the gene. In a study related to change in competence in NB7-1 and NB3-1 it was shown that a loss-of-function mutation of the Polycomb repressor complex extends the competence of Kr to induce motoneurons, while the gain of function resulted in a loss, thus suggesting role of PcG proteins in restricting NB competence (Touma et al., 2012). Next, it has been seen that in Polyhomeotic (Ph) mutant

Even-skipped expression is significantly increased throughout the CNS, thus implying another hint towards epigenetic mediated silencing (Dura and Ingham, 1988). In this study we could observe loss of re-localization for all three gene loci i.e. *hb*, *Kr* and *eve* in RNAi knockdown of Polycomb (Pc), thus suggesting that it is indeed necessary for silencing of the gene locus.

Histone post-translational modifications play a central role in epigenetic gene regulation. There are various post-translational modifications studied like, acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, etc. Lysines at the N-terminal ends of the core histones are the predominant sites of acetylation and methylation. Histone protein acetylation refers to the post-translational addition of an acetyl moiety to the ε-amino group of a lysine residue. It has been shown that active genes are preferentially associated with highly acetylated histones whereas inactive genes are associated with hypoacetylated histones. There are two categories of enzymes Histone Acetyl Transferases (HATs) which promotes histone acetylation thereby maintaining the active state of a gene and next are Histone De-acetyl Transferases (HDACs) that remove acetyl groups from lysine residues resulting into a repressive state of a gene (Reviewed by Yang and Seto, 2007). In vertebrates, it has been shown that Class I HDACs, in particular (HDAC 1, 2, 3 and 8), are present in the nucleus and are responsible for maintaining a repressive state of chromatin. Five different HDACs such as Rpd3 (HDAC1), HDAC3, HDAC4, HDAC6, and Sir2 are known in *Drosophila* (Barlow et al., 2001). Recently, it was shown in vertebrates that nuclear actin and HDAC1 interacts with each other and nuclear actin is a critical mediator of class I HDAC function, monomeric form of nuclear actin was shown to decrease HDAC activity whereas polymerization of nuclear actin increased it. This observation thus pointed towards the notion that the local polymerization and de-polymerization of nuclear actin may be an elegant epigenetic regulator (Serebryannyy et al., 2016).

1.6 Research Aim

Stem cells or progenitor cells lose their ability to respond to internal or external cues in order to generate desired cell fates during aging termed as loss of competence. A similar mechanism exists in *Drosophila* where NB7-1 and NB3-1 lose their ability to produce motoneurons whilst aging. In this study, we try to identify factors responsible to regain such loss of competence. Sebastian Jansen showed that ectopic co-expression of Hb/Kr late in development could regain such loss for NB7-1 lineage; here I investigated if a similar mechanism exists in NB3-1 lineage. Further, I investigated the correlation between *Kr* and *eve* gene loci re-localization with NB competence, similar to *hb* locus (Kohwi et al., 2013). Next, the investigation was done to identify the possible mechanism behind gene locus re-localization. Next analysis was done if these loci are silenced by epigenetic regulators and if their re-localization is required for such phenomenon.

2 Material and Methods

2.1 Fly food media

Fly stocks were maintained on a standard food medium containing yeast flakes, soy flour, corn flour, malt extract and sugar beets syrup. Agar was added for solidifying the medium, Nipagin- and propionic acid served as preserving agent. Before use, the medium was sprinkled with dry yeast. Stocks were maintained at 25°C and transferred to fresh medium every two weeks. At this temperature, the generation time takes about ten days. Stocks currently not used for experiments were maintained at 18°C and transferred every four weeks. The generation time at this lower temperature is about 20 days.

For egg collections, 2,8% apple juice agar was used. Therefore 28g agar (Roth®) were dissolved in 1l apple juice and heated to boiling in a microwave. The medium was stirred until the agar was completely dissolved, and subsequently decanted into vials. The agar was allowed to cool down and solidify and the vials were then plugged with mite-proof foam plugs. Such as the vials with the standard medium, the apple juice agar was sprinkled with dry yeast, before used.

2.2 Fly stocks

Fly strains used during Ph.D. thesis are listed as follows in Table 1.

Tab. 1: Fly stocks

Denotation	Genotype	Reference
WT	Oregon-R-C	Bloomington (BL 5)
hb^{15}	hb^{15}, *hbp1*/TM6b-hb-lacZ	Institute of Genetics (Re-balanced with ftz-lacZ)
ebo^{678}	ebo^{678} (homozygous viable)	AG Prof. Staruß

chic^{221}	chic[221] cn[1]/CyO; ry[506]	Bloomington (BL 4892)
Gal4-Lines		
MTD-Gal4	P{w[+mC]=otu-GAL4::VP16.R}1, w[*]; P{w[+mC]=GAL4-nos.NGT}40; P{w[+mC]=GAL4::VP16-nos.UTR}CG6325[MVD1]	Bloomington (BL 31777)
insc-Gal4	w*; P{GawB}*insc*Mz1407	Bloomington (8751)
cas-Gal4	w;*cas*-Gal4	Institute of Genetics
en-Gal4	w;*en*-Gal4	Institute of Genetics
UAS-Lines		
UAS-*hb2,3*	w; UAS-hb; UAS-hb	Institute of Genetics
UAS-*hb;* UAS-*Kr*	w;UAS-Kr/Cyo;UAS-*hb*/TM6b	Institute of Genetics
UAS- *ebo*	n.a	AG Prof. Strauß
UAS-RNAi-HDAC1	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00607}attP2	Bloomington (BL 33725)
UAS-RNAi-Polycomb	y1 v1; P{TRiP.HMS00016}attP2/TM3, Sb1	Bloomington (BL33622)
UAS-NLS-GFP-G14R	w; UAS-NLS-GFP-G14R-*actin5C*	Institute of Genetics, self-generated
UAS-NLS-GFP-S15C	w; UAS-NLS-GFP-S15C-*actin5C*	Institute of Genetics, self-generated
UAS-RNAi-mCherry	y[1]sc[*]v[1];P{y[+t7.7] v[+t1.8]=VALIUM20- mCherry}attP2	Bloomington (BL 35785)
Balancer Stocks		
DTS4	w; TM3 ftz-lacZ/TM6b iab2 lacZ	O.Vef (Institute of Genetics)
Four Fold balancer	w;plum/cyo;cxd/Tm6b (Blue balancer)	O.Vef (Institute of Genetics)
DTS91^{1}	l(2)DTS91^{1} sna^{Sco}/CyO	O.Vef (Institute of Genetics)

2.3 Genetic crosses

For collecting F1 generation progeny from crosses, flies were transferred to fresh medium every three to four days. Vials with eggs were kept at 25°C for approximately 10 days, around which point the F1 progeny started hatching. Flies with the desired genotype were then collected for further crosses or experiments. To set up new crosses, freshly hatched female virgins of the Gal4 Fly strains were collected every 3 hours at 25°C. They were then crossed to males of the UAS fly stock. Flies were allowed to get accustomed to the new situation and mate for three to four days on regular fly food and subsequently transferred to apple juice agar vials.

2.4 Ectopic gene expression

2.4.1 Gal4/UAS system

The Gal4/UAS system provides a method for targeted gene expression, which allows the selective activation of any cloned gene in a tissue- and cell-specific pattern (Brand and Perrimon, 1993). This system comprises two components: the transcriptional activator Gal4 and its target sequence, the upstream activating sequence (UAS). Both components originate from the yeast *Saccharomyces cerevisiae*. The *gal4* gene is randomly inserted into the genome via a transposable element (P-element), which is then carried by transgenic flies, the so-called Gal4-line or driver line (Fig. 5). The second strain of transgenic flies carries a P-element in which the transgene of interest lies downstream of the UAS-Sequence. This target gene is silent in the absence of Gal4. Crossing female virgins of the Gal4-line to male flies of the UAS-strain leads to the expression of the target gene under the control of the Gal4 enhancer. In the progeny of this cross, the UAS-Gene X can then be expressed and observed. This method allows the analysis of a gene of interest in a specific tissue to a certain stage during development.

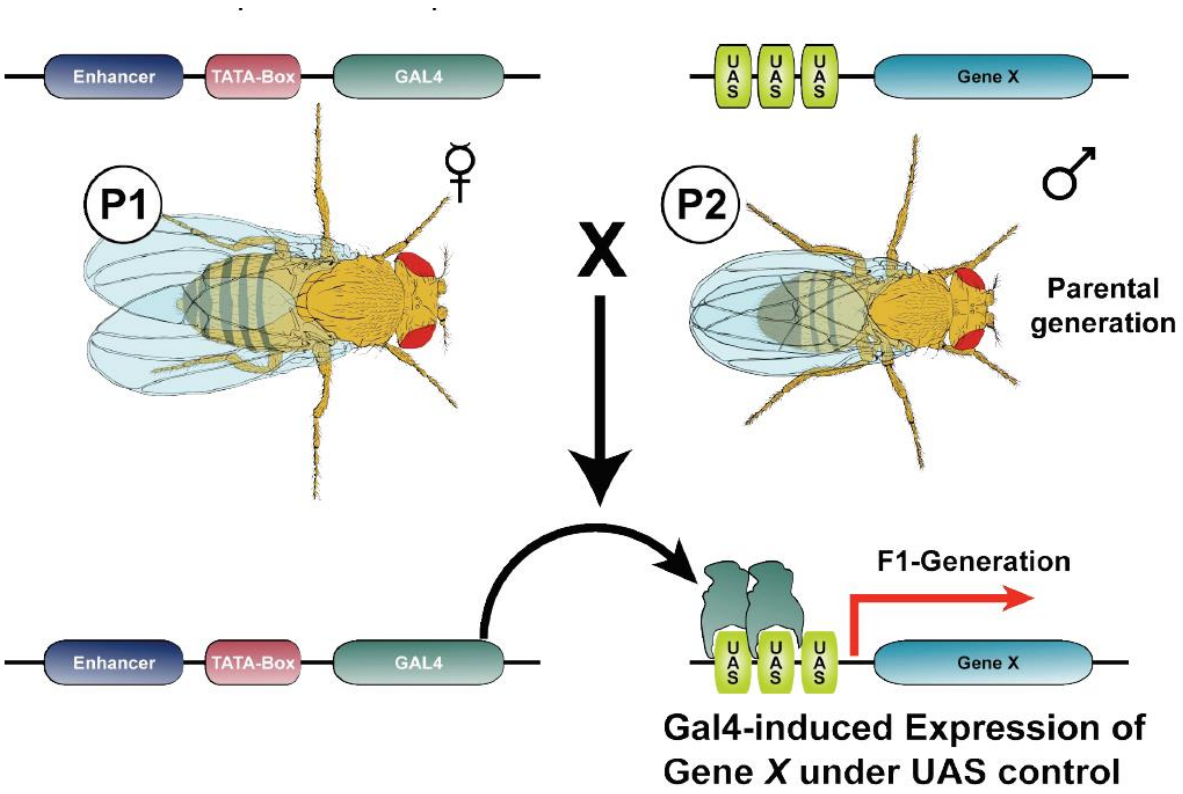


Fig. 5: The Gal4/UAS system
Virgin female flies of the Gal4-line (P1) carrying the transcriptional activator Gal4, and are mated to male flies of the UAS-line (P2). Those carry Gene X, which can be any gene of interest downstream of several UAS-sequences (light green). In the following generation (F1) the Gal4 protein binds to this UAS-sequences and allows the transcription of the gene of interest (kindly provided by C. Berger and C. Rickert)

2.4.2 Maternal-Gal4-shRNA System

In a developing *Drosophila* embryo, mRNAs have a maternal origin, a zygotic origin or both (Staller et al., 2013). The MTD-Gal4 Flystock contains a vector employing short hairpin RNAs (shRNAs), which when expressed in the female germline during oogenesis, effectively knock down the maternal transcript of a certain gene. With this method it's possible to reduce the transcription of a target gene already in the oocyte, leading in a stronger knockdown and thereby in a stronger phenotype (Fig. 6).

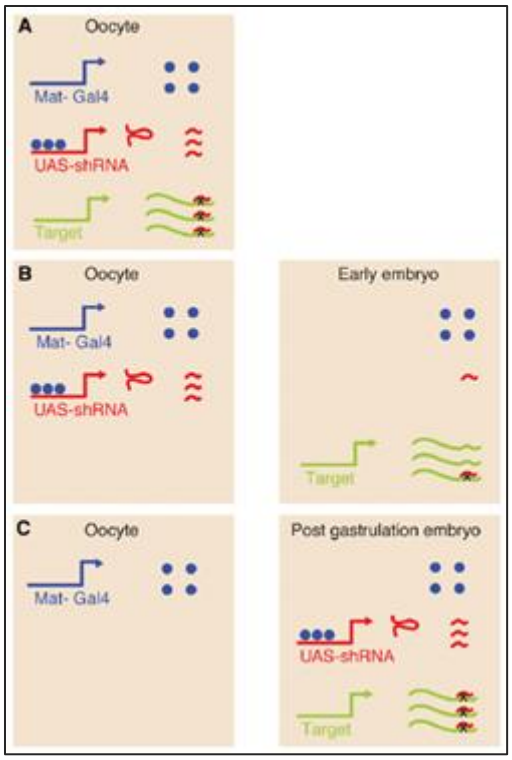


Fig. 6: Strategies for knockdown of maternal and zygotic transcripts
A) The maternal GAL4 driver activates shRNAs. They are then depleting target genes. B) Depletion of zygotic transcripts by loading the embryo with maternally derived shRNAs. C) Depletion of the zygotic transcript following zygotic activation of shRNAs by maternally loaded Gal4 protein. (Source: Staller et al., 2013).

To create stronger phenotypes, virgins of the F1 Generation were re-crossed with male flies from the RNAi fly strain and eggs of the F2 Generation were used for further investigations.

2.4.3 Dominant temperature-sensitive DTS system

The Dominant temperature sensitive (DTS) system was used to re-balance the mutant fly strains chic^{221} and tsrN^{121} with an ftz-lacZ balancer. DTS exhibits dominant lethality in triploid females. Therefore, virgins carrying the mutation were crossed with CyO/l (2) DTS911 males. The crossing was kept at 25°C during the egg and very early first instar stages of the development of the progeny and subsequently shifted to 29°C. At this temperature, all flies carrying the DTS allele die, whereas the surviving flies maintain the mutation over the lacZ reporter gene. All hatched flies were examined for escapers, which were, in the case of DTS91^{1} marked by *scutoid (sco)*, recognizable by lacking the anterior and posterior scutellar bristles. Such flies were discarded. The remaining flies were then transferred to a fresh medium vial generating a stable Fly strain.

2.4.4 RNA Interference

RNA Interference is a method in which specific mRNA sequences are destroyed, resulting in inhibited gene expression or suppression of the desired gene. Thereby Dicer, an RNaseIII-like enzyme, cleaves long double-stranded (ds) RNA into small interfering RNA RNA fragments (siRNAs). These fragments are then joining an RNase complex RNA-induced silencing complex (RISC), degrading the mRNA which enables the ability to form a functional protein (Agrawal et al., 2003).

2.4.5 Primer Design

A Primer is a short synthetic single-stranded oligonucleotide which mostly consists of 20-25 nucleotides. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature (T_m), which means the temperature at which half of the primer has annealed to the template. I tried to make the T_m of the primers between 55°C and 70°C, and without big differences to each other. To amplify the sequence of interest, in that case, the gene locus of *hb*, sequences were chosen, which cover the whole *hb*, *Kr*, *eve*, *pdm1/2*, *cas* gene locus, and also upstream and downstream regions of it. Since that region exceeds 20kb, I generated a probe which was divided in 10 consecutive DNA fragments, each covering 1150-1700 bp. The reverse primer sequence from one fragment was thereby the forward sequence for the following fragment to make sure that all fragments are included in the probe and no gaps occur.

2.5 Primers

Tab. 2: Primers used to prepare the DNA-probe for *hunchback* (*hb*)

Primer	Sequence	Tm° (in C)	Fragment-Size
Hb_US1n.fwd	CTAAGGGCTTCGACATGCCGG	71,6	1366 bp
Hb_US1n.rev	CGAACTGCAACCGGACTTAGCG	71,6	
Hb_US2n.fwd	CGCTAAGTCCGGTTGCAGTTCG	71,6	1385 bp
Hb_US2n.rev	CTCACTGCTCCTCACAACAGTG	65,1	
US3(-1)fwd	CTTGACATGGGCTTTTGGCCCG	58,6	1265 bp
US3(-1)rev	GGAAATTAAGGCCACTAAGTCGC	57,4	
US4(0)fwd	GGCGACTTAGTGGCCTTAATTTCC	57,4	1485 bp
US4(0)rev	GAAAAGTGCTCGCGGGTTACGC	58,6	
DS1fwd	CTCAAGCCAGAAGGATCTGC	53,8	1463 bp
DS1rev	GGATCGCCGCTGATAATTAA	49,7	
DS3.1fwd	GGAGAGTCCCAAATTCTGGAAGGG	59,1	1158 bp
DS3.1rev	GGGAGAAAGGGGGAAATAAAGTGGG	59,3	
DS3.2fwd	CCCACTTTATTTCCCCCTTTCTCCC	59,3	1659 bp
DS3.2rev	CCACGCCCCTCAAAAACTCTCC	58,6	
Hb_DS4n.fwd	GGAGAGTTTTTGAGGGGCGTGG	71,6	1443 bp
Hb_DS4n.rev	GTCAGCTCGAATTTAACGCCAATTGC	72,2	
Hb_DS5n.fwd	GCAATTGGCGTTAAATTCGAGCTGAC	72,2	1424 bp
Hb_DS5n.rev	GGGTACGATGTTCAGTAGTCGCC	67,6	
DS6.1fwd	GGCGACTACTGAACATCGTACCC	58,8	1225 bp
DS6.1rev	CGCCTAAAGTCGCGCAAAACATACG	59,3	
DS6.2fwd	CGTATGTTTTGCGCGACTTTAGGCG	59,3	1170 bp
DS6.2rev	CCCTGCACGGATTTGTCTCATGC	58,8	

Tab. 3: Primers used to prepare the DNA-probe for *Krüppel* (*Kr*)

Primer	Sequence	Tm° (in C)	Fragment-Size
US1 fwd	TGTAGCGAGCACATGGAGTC	53,8	1431 bp
US1 rev	TCTCTAGCCGCTGGTAGCTC	55,9	
US2 fwd	GGAGCTACCAGCGGCTAGAG	57,9	1755 bp
US2 rev	AGGACACCGAAAGTGAATGG	51,8	
US3 fwd	CCATTCACTTTCGGTGTCC	51,1	1205 bp
US3 rev	TTGGGAATCATGTGAGAACG	49,7	
US4 fwd	AACGTTCTCACATGATTCCC	49,7	1414 bp
US4 rev	CACCACCCACCATTGTCAG	53,2	
US5 fwd	AACTGACAATGGTGGGTGG	51,1	2749 bp
US5 rev	GACCGGTTTTAGAAAATCTTC	48,5	
DS1 fwd	AATTGTGGCTAGGGATGTGC	51,8	1276 bp
DS1 rev	GCTTTTGAGGGTGAGGATTG	51,8	
DS 2 fwd	CAATCCTCACCCTCAAAAGC	51,8	1295 bp
DS2 rev	GTAATTGCAGAAGGGCCAATC	52,4	
DS3 fwd	ATTGGCCCTTCTGCAATTAC	49,7	2831 bp
DS3 rev	GCCAACTAGGGACACAAATAC	52,4	
DS4 fwd	ATTTGTGTCCCTAGTTGGCG	51,8	1446 bp
DS4 rev	CTATTGTGAGGTACATGCCG	51,8	
DS5 fwd	CGGCATGTACCTCACAATAG	51,8	1711 bp
DS5 rev	ACATCCTTTCATTCCGGAGC	51,8	

Tab. 4: Primers used to prepare the DNA-probe for *even-skipped* (*eve*)

Primer	Sequence	Tm° (in C)
eve1_Fwd	GCCACTGTGCCAACTAACCG	62,5
eve 1 _Rev	GACACACTGACTTCGTCCTG	60,5
eve2_Fwd	CAGGACGAAGTCAGTGTGTC	63,2
eve2_Rev	CAACGACGACAGGACACAG	64,0
eve3_Fwd	CTGTGTCCTGTCGTCGTTG	63,5
eve3_Rev	CAAGGAAGGCAGCGAAATCG	64,2
eve4_Fwd	CGATTTCGCTGCCTTCCTTG	64,2
eve4_Rev	CACACAGCGAGAAACCAGGC	62,5
eve5_Fwd	GCCTGGTTTCTCGCTGTGTG	62,5
eve5_Rev	CTCGAGTGTGCTGCTCTCAG	61,6
eve6_Fwd	GGACCTGGCTCTCTGGATTAG	61,6
eve6_Rev	GCATGAAGTGGCACCCATTC	60,5
eve7_Fwd	GAATGGGTGCCACTTCATGC	60,5
eve7_Rev	GCCAGCCGTGTGAATCTTCG	61,5
eve8_Fwd	CGAAGATTCACACGGCTGGC	60,5
eve8Rev	GCTAAATTGATTGCCCTGCGG	61,2
eve9_Fwd	CCGCAGGGCAATCAATTTAGC	61,2
eve9_Rev	CATCTTATCGTTCTTGCCGCC	62,9
eve1O_Fwd	GGCGGCAAGAACGATAAGATG	62,9
eve1O_Rev	CTCCGGTCCGTTAATCAG	61,6

Tab. 5: Primers used to prepare the DNA-probe for *castor* (*cas*)

Primer	Sequence	Tm° (in C)
Cas1_Fwd	GAAAATGCTCTGGGAAAACCCAGC	62,9
Cas 1 _Rev	CCGAATCCGTAATGCGGAGC	62,5
Cas2_Fwd	GCTCCGCATTACGGATTCGG	62,5
Cas2_Rev	GCACAACAATGAGCGAGAATTGGC	65,2
Cas3_Fwd	GCCAATTCTCGCTCATTGTTGTGC	65,2
Cas3_Rev	GAAAATCACAAGGAAACCGGTTTGGAC	64,1
Cas4_Fwd	GTCCAAACCGGTTTCCTTGTGATTTTC	64,1
Cas4_Rev	GGAGATGTTCCTTACCTTCGGATTC	64,1
CasS_Fwd	CTTCTCACTATACATTGCCCATTCC	64,1
CasS_Rev	CTTTGGCAGCTGACCCCGTG	64,6
Cas6_Fwd	CACGGGGTCAGCTGCCAAAG	64,6
Cas6_Rev	GAACTAAATGGGAGGGTGCAC	61.2
Cas7_Fwd	GTGCACCCTCCCATTTAGTTC	61,2
Cas7_Rev	CCTTATCCTGAGCCAAATGACC	62.1
Cas8_Fwd	GGTCATTTGGCTCAGGATAAGG	62,1
CastRev	GGGCTGACTACAAGACTTCG	60,5
Cas9_Fwd	CGAAGTCTTGTAGTCAGCCC	60,5
Cas9_Rev	GAAATCCTCCTGAAACTCAAGGC	62,9
Cas1O_Fwd	GCCTTGAGTTTCAGGAGGATTTC	62,9
Cas1O_Rev	GTCGGGGAACTCGAATATCAC	61,2
Cas11_Fwd	GTGATATTCGAGTTCCCCGAC	61,2
Cas11_Rev	CCTCTAGGATCGAAACCTTCAC	62,1

Tab. 6: Primers used to prepare the DNA-probe for *nubbin* (*pdm)*

Primer	Sequence	Tm° (in C)
Pdm1_Fwd	ATTTAGCTGCCCTCAATTGACTCG	63,6
Pdm 1 _Rev	CAGCGCGCCATTGCATATGTC	63.2
Pdm2_Fwd	GACATATGCAATGGCGCGCTG	63,2
Pdm2_Rev	GTTTATTTTGTGACTTGATTGGGGC	64,1
Pdm3_Fwd	GCCCCAATCAAGTCACAAAATAAGC	64,1
Pdm3_Rev	CTCAAACGGCGGCGAGAAAAAC	64,2
Pdm4_Fwd	GTTTTCTCGCCGCCGTTTGAG	64,2
Pdm4_Rev	CCTCACGCCAATAAACGCGC	62,5
Pdm5_Fwd	GCGCGTTTATTGGCGTGAGG	62,5
Pdm5_Rev	GCAAGTCGCACCTTAGGGC	61,6
Pdm6_Fwd	GCCCTAAGGTGCGACTTGC	61.6
Pdm6_Rev	CACTACACACTCGCTGACAC	60.5
Pdm7_Fwd	GAGTTCGAGACCCCCAATTG	60,5
Pdm7_Rev	CACATGGTTATTGGGTTCTGG	59,5
Pdm8_Fwd	CCAGAACCCAATAACCATGTG	59,5
Pdm8Rev	GCTAATGTACGCGATTCTGGG	61,2
Pdm9_Fwd	CCCAGAATCGCGTACATTAGC	61,2
Pdm9_Rev	AGTTGGGCAGTTGGTTTAAAGGG	62,9
Pdm1O_Fwd	CCCTTTAAACCAACTGCCCAACT	62,9
Pdm1O_Rev	GCGGGCAGAGGTAGTAGAG	61,6
Pdm11_Fwd	CTCTACTACCTCTGCCCGC	61.9
Pdm11_Rev	GTTCCATTGTTAAAGCGTAGCG	60,1

Tab. 7: Primers used to prepare the RNA-probe and antisense-probe for HDAC-1

Primer	Sequence	Tm° (in C)	Fragment-Size
Fwd-HDAC1	GGAGGCGTTCTATACCACCG	65.5	409 bp
Rev T7 HDAC1	TATTACGACTCACTATAGGGAGACAGTGCAACGGAGGTCTCAT	78.7	
Kr_Intron Fwd	GACCAAATTAAAATATTCCCCAAG	58,3° C	400bp
Kr_Intron.Rev_T7	TGGAAATGTCTTAAGAGCAACGTAATACGACTCACTATAGGGAGA	57,2° C	

2.6 Used enzymes

Tab. 8: Enzymes used in this project

Enzyme	Reference	Usage
DNase 1	Invitrogen	Cleaving DNA to single strands during nick translation
DNA polymerase 1	Invitrogen	Incorporation of amine-modified dNTPs during nick translation
Taq Polymerase	Qiagen	Merging PCR fragments and filling gaps between nucleotides

2.7 Used antibodies

Tab. 9: Used primary and secondary antibodies

	Antibody	Species	Dilution
Primary antibody			
	α-Worniu	Mouse	1:50
	α-Deadpan	Guinea Pig	1:500
	α-Invected	Mouse	1:10
	α-Lamin	Rabbit	1:500
	α-Lamin	Mouse	1:500
	α-Engrailed	Rabbit	1:100
	α-Dig-AP	Sheep	1:1000
	α-Polycomb	Rabbit	1:100
	α-Hunchback	Guinea Pig	1:300
	α-Hb9+	Rabbit	1:1000
	α-A2103(Actin)	Rabbit	1:10000
	α-Histone 3	Rabbit	1:1000
	α-Tubulin	Mouse	1:500
	α-GFP	Rabbit	1:500
	α-Islet+	Rat	1:100
Secondary antibody			
	α-mouse Alexa 647	Donkey	1:500
	α-mouse Alexa 568	Goat	1:500
	α-rabbit Alexa 488	Donkey	1:500
	α-rat Alexa 647	Donkey	1:500
	α-Mouse-POD	Goat	1:10000
	α-Rabbit-POD	Donkey	1:10000
	α-Guinea pig Alexa 647	Goat	1:500

2.8 Preparation of genomic DNA

30-35 Larvae were collected and incubated at -80°C for 1h or in liquid nitrogen for 5 min. They were subsequently ground in Buffer A (see Tab. 14) and incubated at least for 30 minutes at 65°C. After the Incubation, 1ml of Buffer B (1 Vol. 5M KAc; 2,5 Vol. 6M LiCl) was added to the tissue and the ground larvae were kept for 15 min. on ice and 15min at -80°C. Afterward they were centrifuged for 15 min at 12000 rpm and the supernatant was transferred in a new spin column and diluted in 720 µl of Isopropanol. The supernatant was then removed and the DNA pellet at the bottom of the tube air-dried. To resuspend the Pellet, 100 µl EB-Buffer were added and the mixture incubated for one hour at 65°C.

2.9 Fixation and Decolonization of embryos

For embryo collections, flies were transferred to fresh medium sprinkled with dry yeast and shifted to 2,8% apple juice, where the flies were allowed to feed for 2-3 days. Eggs were collected once over the day at 25°C and in addition over night at 18°C. For Fixation, they were dechorionated with 6% sodium hypochloride for two minutes and rinsed into a mesh. Embryos were subsequently transferred in a prepared Eppendorf cap containing fixation buffer A (see table 11, p 25). They were then incubated in that solution for 25min at RT on a rotating wheel with 150rpm. After the fixation, the lower phase has been removed and 500µl methanol was added and the cap has been vortexed for one minute. During this time the surface tension between the two phases damages the vitelline membrane of the embryos which then sink to the bottom of the vial. The methanol has been replaced with a new aliquot and the devitellinization step was repeated two times. After the embryos sank to the bottom of the cap the supernatant, including all heptane and methanol was removed and 750µl ethanol was added.

2.10 Preparation of a DNA probe

To generate a directly labeled DNA probe, the Invitrogen FISH Tag^{TM} DNA Kit (Thermo Fisher Scientific) was used. This kit employs a two-step labeling technology which comprises

the nick translation to enzymatically incorporate an amine-modified nucleotide, followed by chemical labeling with Alexa Fluor® dyes. Since the *hb* gene locus region is very large in size, and the coverage of approximately 10-12kb of this region with the probe was desired, it was subdivided into ten DNA fragments. Therefore the purified PCR-fragments were incubated in 50µl of the Nick translation reaction batch (see table 12, p 25). The Incubation took place at 15°C for 2h. After adding 50µl nuclease-free water the reaction batch was vortexed and a binding buffer was added. After several washing steps according to the Invitrogen DNA-FISH Tag Kit™ manual, the sample was eluted in the 55µl elution buffer. 10µl of 3M sodium acetate (pH 5,2), 1µl glycogen, 39µl nuclease-free water and 250µl of 100% ethanol were added and the sample was stored at -20°C for 30min. The purified fluorescent dye-labeled DNA was used as a probe in the following In-Situ-Hybridization.

2.11 DNA Fluorescence in situ hybridization

The DNA Fish was made according to the protocol from Frédéric Bantignies, Institut de Genetique, Montpellier. For the Hybridization, a directly labeled DNA probe (see 2.9) was used in order to make the gene locus of *hb* visible in the NBs. Before hybridization, the embryos were transferred from MeOH into PBTween in five washing steps, lasting 5 min each:

Tab. 10: Transferring embryos from MeOH to PBTween

I	90% MeOH, 10% PBTween
II	70% MeOH, 30% PBTween
III	50% MeOH, 50% PBTween
IV	30% MeOH, 70% PBTween
V	100% PBTween

The tissue had then to be incubated for two hours at RT in PBT containing 1% RNase A, in order to eliminate all present RNA molecules. Afterward, the RNase A was removed and the tissue was incubated in 0,3% PBTween for one hour at RT. The tissue was then transferred into a pre-Hybridization Mixture (PHM). This occurred in four different washing steps, lasting 20min each:

Tab. 11: Transferring embryos from PBT to PHM

I	80% PBT, 20% pHM
II	50% PBT, 50% pHM
III	20% PBT, 80% pHM
IV	100% pHM

In order to denature existing DNA, the embryos were incubated in 100% PHM for 15min at 80°C in a thermomixer block. At the same time the probe was diluted in 30µl of FISH Hybridization Buffer (FHB) and denatured at 95°C for 10min. While the embryos were still at 80°C, as much as possible of the supernatant was removed and the denatured probe (now in a 5ng/µl concentration) was added without prior cooling. This mixture was then covered by a drop of mineral oil and incubated in a thermomixer with 37°C with 450rpm overnight. The post-hybridization comprised 8 washing steps, of which the first five steps took place in the thermomixer at 37°C with 850 rpm and the last three steps at RT on a rotating wheel at RT. These eight washing steps (table 9) aim to transfer the embryos from a high concentration of formamide to 100% PBT.

Tab. 12: Transferring embryos from FHB to PBT

I	50 % formamide; 2xSSC: 0.3% CHAPS
II (repeated I)	50 % formamide; 2xSSC: 0.3% CHAPS
III	40 % formamide; 2xSSC; 0.3 % CHAPS
IV	30 % formamide; 70% PBTween
V	20 % formamide; 80% PBTween
VI	10 % formamide; 90% PBTween
VII	100% PBTween
VIII	100% PBT (0,3%)

Embryos expressing balancer lacZ were first immunostained for the balancer gene and then selected under the fluorescence binocular. The usual DNA FISH and AB-staining were then performed on embryos, not showing lacZ expression.

2.12 Immunohistochemistry

The Immunostaining of the embryos was performed with primary antibodies for 2h at RT or overnight at 4°C on a rotation wheel with a moderate shake. The antibodies were diluted in PBT according to Table 6. After Incubation the antibody solution was removed and stored at -20°C. The embryos were rinsed three times- and subsequently washed three times for 10min in PBT. The Incubation with a secondary antibody, diluted in PBT, took place 2h at RT or overnight at 4°C in the dark and on a rotation wheel with a moderate shake. Afterward, the antibody-solution was removed and the embryos were washed 5 times with PBT, each washing step lasting 10 min, before they were washed with PBS for 5 min. The stained embryos were then stored in 70% Glycerol. Like this they can be stored at -20°C for several weeks.

α-Deadpan (Dpn) stains exclusively the NB nucleus, α-lamin (lam) marks the lamina, representing the nuclear envelope. The α-Engrailed (En) antibody was used to identify engrailed positive NBs.

2.13 RNA-FISH and Alkaline phosphatase staining

Alkaline phosphatase (AP) is a universal pluripotent marker for all types of pluripotent stem cells including embryonic stem cells, embryonic germ cells, and induced pluripotent stem cells). Fixation and Dechorionization of embryos were performed as for the "normal antibody staining". The prehybridization with the RNA probe took place for one hour in 500µl of hybridization buffer mixed with 5µl ssDNA at 55°C on a rotation wheel with 350rpm. The probe was denaturated at 95°C for 10 min together with the hybridization buffer and 5µl ssDNA (10mg/ml) ssDNA and added to the embryos with prior cooling on ice. The hybridization took place overnight at 55°C. Afterward, the probe was removed and stored at -20°C, and the embryos were washed with hybridization buffer for 30 min, subsequently washed with a mixture of hybridization buffer and PBTween-DEPC (1:1). 4 washing steps with PBTween occurred (20 min @65°C and 300rpm), afterward 1x 10min @RT. The embryos were incubated for 1,5 hrs @ RT in α-Dig-AP. After incubation, they were rinsed three times and washed 3 times with PBTween at RT and 2x 5 Min with AP detection buffer. For one reaction 3µl NBT and 1,5 µl, BCIP was added to 1ml AP-buffer and the solution was

added to the embryos. AP expression will result in a red/purple stain for Color Red and a blue stain for Color Blue, while the absence of AP expression will result in no stain. The staining was controlled under the binocular. After the embryos were stained in the desired intensity, they were rinsed 3 times in PBTween and afterward washed for 30 min with Methanol. This immediately stops the dyeing. After several washing steps with PBTween (3x 10 min) and PBS (1x 5min), the embryos were kept in 70% Glycerol

2.14 Filet preparation of early Drosophila embryos

For analysis of the staining, the embryos were transferred into a drop of SlowFade® Gold antifade reagent (Invitrogen) on a microscope object slide. This mounting medium prevents the staining to fade. Embryos with the developmental stage 9-12 were then prepared with two very thin and sharp needles. The first step was removing the head and then the embryos were sliced carefully along the germband. The in this manner opened embryos were then lined up, with the anterior on top. The stained fillet preparations were imaged using the confocal laser-scanning microscope (LSM SP5)

2.15 Measurement of gene locus position in the nucleus of NBs

Prepared DNA FISH embryos were examined and documented under the confocal laser-scanning microscope (LSM). The Analysis of the images was then performed using FIJI ImageJ. For analysis, the NB is mapped in three different perspectives, along with the x-/y-axis, the x-/z-axis and the y-/z-axis. Within this three sections, I chose the one with the shortest distance from the FISH signal to the nuclear envelope, this perspective was then used for the gene locus measurements. The NB was divided into three zones, with equal area sizes (Fig. 7).

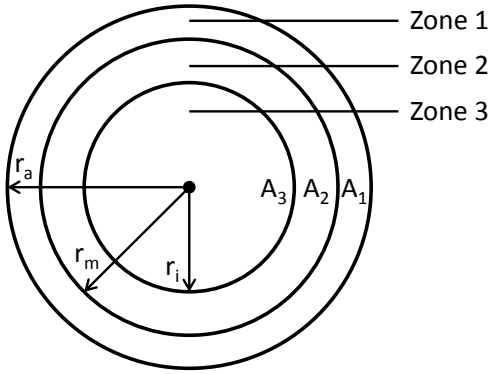


Fig. 1: The 3-Zone-model for NB Analysis
NBs are divided in three zones with the same area size A1-A3. The outer zone is referred to Zone 1, the intermediate zone to zone 2 and the inner zone to zone 3.

The zone borders are calculated under the premise of equal zone areas. A circle divided into three concentric zones with equal areas is used as a model of the NB. The border radii are calculated using the equation of circles $r^2\pi$. In analogy to Hediger et al. (2004) the most peripheral zone is zone 1, a ring with the width of 18.4% of the total nuclear radius (r_a). Between 18.4% and 42.3% of the nuclear radius (r_m) lies zone 2. In the center of the circle lies zone 3 with a radius of 57.7% of the total nuclear radius. The locus position is calculated by dividing the peripheral locus distance through the total radius of the NB. The outer zone is referred to as zone 1 in the following, the intermediate zone is zone 2 and the most inner zone is called zone 3. A specifically custom-made macro for FIJI (kindly provided by Philipp Seeger, M. Eng, and Hochschule Darmstadt) performed those calculations automatically. The NB outlines and the gene locus signal were selected and the program calculated NB diameter and the shortest distance from the FISH signal to the nuclear envelope. According to the calculations shown in Fig. 7, the gene locus FISH signal is then graduated in one of the three zones, giving information about the localization of the gene locus within the NB nucleus.

2.16 S15C and G14 R mutagenesis polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a microbiological method often used to amplify specific DNA sequences in vitro. First, specific oligonucleotides working as primers are required, which need to be complementary to both 3’ ends of each sense and antisense strand of the target DNA. Second, an enzyme needs to be present, which amplifies the DNA sequences starting at the primers, the DNA polymerase. As a template DNA, a pUAST-attB vector is used, which has integrated the fusion construct of nGFP: Act5C and the w+ gene. Finally, free deoxynucleotides (dNTPs) are necessary. The amplification consists of three steps; first denaturation, second annealing of the primers and third elongation of the nucleic acid. When the template DNA has denatured the primer can easily access the target sequence. Afterward

the primers hybridize to the template DNA, so-called annealing. During the last step, the elongation, the free dNTPs polymerize to strains. The annealing temperature has to be adjusted to the primers' nature and should be normally 5°C below their melting point. The so-produced DNA fragments proliferate exponentially during 25 to 35 circles. In our project, we use the PCR to create an exchange of base pairs causing an amino acid exchange. To induce the change of base pairs we create a mutagenesis primer. The reverse primer has the wild-type sequence of the act5C gene and the forward primer bears the mutation. To minimize the error ratio the circle's number is reduced to 16 and high fidelity DNA polymerase from KAPA is used. After a few attempts with several primers, it became apparent that an essentially important factor is the mutation’s location. The mutation must be located at the very beginning of the forward primer without any base, which is complementary to the wild-type sequence, in front of it.

PCR reaction

ddH2O	33,5µl
KAPA Hifi Puffer (5x;2mMMg)	10µl
dNTPs	1,5µl
Template DNA	1µl
Forward Primer	1,5µl
Reverse Primer	1,5µl
KAPA Hifi-Polymerase	1µl
Total volume :	50µl

Agarose gel electrophoresis

To examine the success of the PCR the gel electrophoresis is used, which is a bio-molecular method to separate macromolecules according to their size. The agarose molecules build many cross connections and shape a molecular sieve. The pores’ width, influenced by the agarose concentration of the gel, defines the velocity with which the DNA fragments can pass through it. The DNA fragments move according to their negative charge by applying an

electric field, to the opposing, positive site. Small molecules move faster through the gel than bigger ones because they can pass the pores more easily. To visualize the separated fragments later on under a gel documentation system with UV light, ethidium bromide is used. This is an intercalating and, in the UV-spectrum, light-absorbing molecule.

During this project, 0,7% agarose gels are used. Therefore 0,35 g agarose is solved and boiled twice in 50 ml 1xTAE buffer using a microwave. After cooling the solution under water to approximately 60°C, 10µl of ethidium bromide is added under the fume cupboard and mixed by gentle agitation. Subsequently, the solution is poured into a gel chamber with a gel slide and a gel comb. After hardening, the gel comb is removed and the gel on the gel slide can be transferred to an electrophoresis chamber filled with 1xTAE buffer. When the pockets have filled with the buffer, the probes can be added. Here for 1µl of 5xLoadingDye (Fermentas) for each probe is taken and mixed with 4µl of your probe using the pipette. According to the size of your fragments, different markers can be elected. Because our fragment is approximately 10, 3 kb big, the hype ladder (Bioline) is used as a molecular marker. The gel was running for 20 min. with an applied electric field of 100V. Finally, the gel is documented by using a gel documentation system as mentioned above.

0,7% agarose gel

Agarose	0,35g
1xTAE-buffer	50ml
Ethidiumbromide	10µl

Ethidium bromide (Working-solution)

1mg/µl	Ethidium bromide

Digest with DpnI

After the PCR product was detected via gel electrophoresis, a digestion with DpnI is necessary to remove the template strain. To maximize the efficiency FastDigest DpnI enzyme is used. The restriction reaction is incubated for 1h at 37°C and subsequently inactivated for 20 min at 80°C. The PCR products are subsequently purified after digestion by the Quiagen PCR Clean-Up-Kit.

Restriction reaction

PCR-Reaction	20µl
Fast Digest-Puffer10x (Thermo Scientific)	10µl
DpnI Fast Digest (Thermo Scientific)	2µl
ddH2O nucleasefree	68µl
total volume:	100µl

Ligation

After digestion and purification, the ligation of the linear fragment was carried out. During this step, the ends of the phosphorylated primers recombine with each other and the construct is now a circulated plasmid. The following reaction is incubated at least for 1h or overnight at RT. Afterward the ligase needs to be inactivated by incubation at 65°C for 10 min.

Linear DNA	20µl
T4-DNA-Ligase-Buffer 10x(Fermentas)	5µl
T4-DNA-Ligase 1U/µl(Fermentas)	2µl
ddH2O	23µl
total volume:	50µl

Transformation

For final analysis by sequencing, the recirculated construct needs to be amplified. Chemicompetent cells of the Escherichia coli strain DH5α are used to gain the necessary

amount of DNA. These cells are stored at -80°C and need to be defrosted for 2 min. on ice. Then 5µl of the ligation solution was added and it is incubated for 20 min on ice. Subsequently, a heat shock for 30 s at 42°C was given, which makes the membrane more permeable for the construct to penetrate. Immediately after that treatment 0,5ml of pre-heated SOB-medium (37°C) is added and the cell solution is pre-incubated for 1h at 37°C in an incubator or a thermomixer with gentle agitation. Afterward, the cell solution is plated on pre-warmed LB-amp-plates (75 mg ampicillin /l) using 200-250 µl for each plate and a sterile spatula. The plates are incubated overnight in an incubator at 37°C

Preparation of plasmid DNA

To isolate the plasmid DNA (G14R and S15C) afterward, colonies were picked for DNA extraction using Qiagen midiprep kit. The concentration of each probe was measured by the Nanodrop 1000/2000 (PeqLab) and then sequenced by Starseq. As our first sequencing primer the Actin sequencing primer rev. is used to check if the mutation at the beginning of the actin5C gene is there. Afterward the rest of the gene is examined by using the Actin sequencing primer forward to eliminate all doubt that there are no other mutations which cause an amino acid exchange or a frameshift mutation. Finally, our probes are screened by using the P1 HSP70 forward primer. This primes from the vector into our construct and the very beginning is examined. The resulting sequences are each compared with the wild-type situation.

Sequencing solution

DNA	400-600 ng
Sequencing primer	1 µl
ddH2O	x µl
total volume:	7µl

2.17 Initial Preparations for injection

Apple juice agar

Before starting the injection, middle size Petri dishes need to be filled with orange or apple juice agar. Therefore juice and water are mixed up and before adding the sugar beet syrup;

200 ml are reserved for solving the agarose in an extra pot. Once the initial solution had cooked, the dissolved agar is added and everything is mixed up. After it has boiled once more, it should be immediately poured into the middle size Petri dishes. They either can be closed immediately or dried for several minutes covered by a towel. They can be stored at 4°C for approximately two weeks.

For 1litre

Water	500 ml
Apple juice	450 ml
Sugar beet syrup	50 ml
Agar	28 g

Needles

The good quality of the injection needles is critical for the success of the injection. Needles are pulled by the Flaming/Braun Micropipette Puller with programm66, which has the following parameters: heat 565°C; pull= 108; velocity= 80; time= 200. Once all the parameters are set, the capillary need to be fixed in the right jig. Subsequently, it is slide to the middle and gently pushed through the heating filament. After fixing the capillary with the left jig, it's worth checking both jigs again. Then the machinery lid can be closed the lid of the machinery and the button PULL needs to be pressed. Finally, both parts are removed by opening the jigs, but only the right part is suitable for injection because the other one is to short.

Furthermore, capillaries are opened by whetting. This is another critical step during this procedure. Needles which are too elongated will bend and break when they impale the embryo. Needles that are too blunt, on the other hand, won't break, but they will damage the embryo quite severe, which in turn will minimize the survival rate drastically. The needles used during the project are sharpened with a grindstone, at an angle of 35° and under visual control by a microscope.

Preparation of coverslips with heptane glue

During the injection procedure it's necessary for the embryos to stick on the coverslip, otherwise, the embryos would move too much and a piercing into the embryo wouldn't be possible. Therefore the coverslips need to be prepared with heptane glue, but the amount is decisive. The hatching larvae will stick to the coverslip by taking too much glue and it is impossible to pick them. If it isn't enough glue the desired effect won't occur. After transferring some glue onto a coverslip by using a pipette tip, the coverslip is turned up site down and spread out over a tissue. Thus an even spreading over the whole coverslip is achieved. Now they can be stored in a big size petri dish for several weeks.

Blue agar

Besides the orange juice agar, also blue agar is necessary for lining up the embryos for injection later on. The agar is accordingly dissolved and boiled in the water. Then Patent blue (Patent blue V sodium salt of Sigma, 21606, 10g) is added until the solution is dark blue because the color's intensity will fade during agar's hardening. Finally, the solution is poured into a flat container, e.g. a middle size petri dish and after hardening it can be closed and stored at 4°C. It should be rapped with parafilm to maximize the durability.

For 2,8 % agar

Water	100ml
Agar	2,8g
Patent Blue	x

Total volume: 100ml

Injection solution

After different versions of injection-solutions, first undiluted, secondly diluted with injection buffer and water, the injection buffer was omitted and if necessary the solution was diluted with distilled water. For injection plasmid DNA, extracted and purified using midiprep was used. The elution buffer, which belongs to the kits, is replaced by distilled water. The injection solution needs to be stained with PATENT BLUE, to track DNA under the microscope. Therefore some patent blue is added to the injection solution and DNA is centrifuged for 30 min. Afterwards the supernatant is carefully transferred into a fresh tube and the lower part is discarded. To ensure that the plasmid DNA stays unharmed, it is kept on ice during the whole injection process, and it is made fresh for every injection day.

2.18 Injection procedure

First of all the microinjection machine needs to be started, because the establishment of the pressure takes several minutes. During the whole procedure, the flies are kept in egg-laying caps, on orange juice agar with yeast mixed with apple juice, in an incubator at 25°C. The embryos are collected at an interval of 45 min, by changing the agar plate. For decelerating the aging process of the embryos at a room temperature of 20-22°C is favorable. 6% bleach is added on top of the agar plates for 2 min. to dechorionate the embryos. The solution is subsequently poured into a tube with mesh and to collect all the embryos the agar plates are rinsed several times with water, pouring the liquid into the tube. After placing a piece of the blue agar, which should have the size of a coverslip, on an object slide and the embryos can be transferred to the blue agar by using a scalpel or a brush. Once the slide is located under a binocular and the lining up of the embryos is started by using a needle. The posterior pole of the embryos should point in one direction, e.g. towards the right side. It is possible to have up to 5 rows, with 25-30 embryos, on one piece of blue agar. After the last row, a coverslip prepped with heptane glue is placed on top with the glue side facing down. Now it is pressed down carefully to fix the embryos on the coverslip without mashing them. Then the turned back coverslip, with the embryos on top, is placed on a fresh object slide and fixed t with a drop of water. For injection, the rows of embryos need to lay vertical on the slide. The embryos are dried in a desiccator for approximately 8 min. to minimize the inner pressure. Thus injecting the embryos without leaching out is much easier. Meanwhile, the needles are filled with 1µl injection solution, and after putting it in the holder and screw containing the rubber is tightened gently but firmly. Subsequently, the slide is taken out of the desiccator, the eggs are covered with 10S oil and the slide is transferred onto the microscope stage. The posterior pole needs to point towards the direction of the needle. When the microscope is focused on the embryos, the needle is moved forwards until its tip is in the cone of the microscope's light. Under visual control through the ocular, the needle is lowered until its tip penetrates the surface of the oil. The needle's tip needs to be in the focal plane with the embryos. After pressing CLEAN, the pressure and injection time can be set, such that a small droplet is released. If no solution comes out, the tip can be opened by taping at an embryo or on the sharp edges of a broken coverslip by pressing clean. Afterward the injection is started by moving the embryos towards the tip and impaling them gently. The needle’s tip shouldn’t enter more than one-fifth the length of the embryo. After pressing INJECT once, a small

droplet of the blue solution diffuses within the embryo. If that's not the case, it can either be injected again or the pressure can be increased as well. It sometimes can be difficult to inject enough solution without exploding the embryos, then the needle or the time in the desiccator can be changed. After successful injection, the embryos are moved off the needle in a quick motion. The speed limits cytoplasmic leaking out of the embryo, but a slight leaking doesn't harm the embryo. Only the embryos up to stage three should be injected. Thus they don't have any pole cells at their posterior end. The other embryos need to be poked because they won't integrate the injected DNA.

After injection, the coverslip is transferred into a weighing dish and gets covered plentifully with 3S oil. The weighing dish is placed in a humidity chamber and it is incubated in incubator at 25°C for 24h after injection.

2.19 Gathering and storage of the injected flies

20-26 h after the injection the embryos need to be checked regularly every 45 -60 min. Hatched larvae are gently removed from the coverslip by using a preparation needle, they then swim on the surface of the oil. From there they can be picked up easily and transferred into a small vial with blue food and some inactivated yeast. Mite-proof plugs are used to close the vial and the number of the collected larvae is noted. This vial is maintained in the incubator until all flies are hatched. From time to time the blue food needs to be checked and moisten again if necessary.

2.20 Crossings

Before all crossings throughout this project virgins of one stock need to be collected. For this the flies are kept at 25°C during the day, freshly hatched females are collected every 3-4h and kept in a separate vial at 18°C. This period of time is required for the hardening of the female's exoskeleton, which, in turn, is necessary for the mating process. Overnight they maintain at 18°C to decelerate development and hence hatchings. To distinguish virgins from older females take the light body color as well as the black spot on the abdomen as an indication. For all crossing males and females are taken at a ratio of 1 to 3.

2.21 Cell Fractionation and Western Blot using *Drosophila* embryos

Collect the dechorionated embryos from O/N egg laying (or specific stages if required) on nylon mesh and dry them thoroughly if to be flashed freeze using liquid nitrogen. Alternatively proceed directly for dancing the embryos in PBS. Dounce the embryos in 1X PBS 10 times with loose pellet, further centrifuge the lysate for 10 mins, 4°C 50g. Collect the supernatant and centrifuge at 4°C 360g for 10 minutes. Discard the supernatant. Re-suspend the cell pellet in the 500ul swelling buffer and keep on ice for 5 minutes. Centrifuge at 4°C 360g for 10 mins, discard supernatant. Re-suspend cell pellet in 100ul Swelling buffer/lysis buffer supplemented with 0.5% Triton X-100.Centrifuge at 4°C 5000g for 10 minutes. Collect the pellet that is nuclear and supernatant contains a cytoplasmic fraction. Repeat step 5 with 1000ul lysis buffer and 6 again for nuclear fraction. Re-suspend the nuclear pellet in 1000ul HB Buffer and centrifuge at 4°C 5000g for 10 minutes. Discard supernatant and collect the nuclear pellet. Resuspend the nuclear pellet in 100ul RIPA buffer and sonicate at high settings 5X.Spin nuclear extract and cytoplasmic extract @ 14000rpm 4°C 10 mins. Take the supernatant from both the extracts and quantify the amount of protein by Bradford for Western blot analysis. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker. Load 20–30 μg of total protein from cell lysate or tissue homogenate, or 10–100 ng of purified protein. Run the gel for 1–2 h at 100 V. Further, proceed with transferring the gel on the membrane. The membrane can be either nitrocellulose or PVDF. Activate PVDF with methanol for 1 min and rinse with transfer buffer before preparing the stack. The time and voltage of transfer may require some optimization. Transfer of proteins to the membrane can be checked using Ponceau S staining before the blocking step. Block the membrane for 1 h at room temperature or overnight at 4°C using blocking buffer. Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer. Overnight incubation at 4°C; other conditions can be optimized. Wash the membrane in three washes of TBST, 5 min each. Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 h. Wash the membrane in three washes of TBST, 5 min each. For signal development, follow the kit manufacturer's recommendations. Remove excess reagent and cover the membrane in a transparent plastic wrap. Acquire image using darkroom development techniques for chemiluminescence, or normal image scanning methods for colorimetric detection.

2.22 Chemicals and solutions

Tab. 13: 20x PBS

20x PBS	
NaCl	151,94g/l
Na2HPO4x H2O	19,88 g/1 l
NaH2PO4x H2O	8,28 g/1 l

Tab. 44: Fixation Buffer A

Fixation Buffer A	
ddH_2O	8,3 ml
KCl (1M Stock)	600 µl
NaCl (5M)	30 µl
Spermine (0,15M, 1000X)	10 µl
Spermidine (0,5M, 1000X)	10 µl
EDTA(0,5M, 250X) pH: 8,0	40 µl
EGTA(0,5M, 1000X) pH: 8,0	10 µl
PIPES (150mM) pH: 7,4	1 ml
Paraformaldehyde	0,4 g

Tab. 15: Nick translation reaction batch for the DNA-probe preparation

Nick translation reaction batch:	
10x Nick translation Buffer	5 µl

DTT (0,1M)	5 µl
10x DNA nucleotide mix (5M)	5 µl
Polymerase I	1,7 µl
DNAse I working solution (1:10 dilution)	6 µl

Tab. 56: Buffer A for the Preparation of genomic DNA

Buffer A (genomic DNA):

100mM Tris-HCl (pH7,5)

100mM EDTA

100mM NaCl

0,5% SDS

Tab. 17: PBTween

PBTween

20x PBS	2,5 ml
H_20	47,5 ml
Tween 20	50 µl

PBT (0.3% Triton)
0,3% Triton-X100 (v/v) in 1x PBS

RNAse A
Stock solution
(Sigma-Aldrich)

Tab. 18: Pre Hybridization Mixture for DNA FISH

Pre Hybridization Mixture (PHM)

Formamide	25 ml
20x SSC	10 ml
NaH_2PO_4 (pH 7; 100mM)	5 ml
ddH_20	10 ml
Tween 20	50 µl

Tab. 69: FISH Hybridization Buffer (FHB) for DNA FISH

Formamide	2,5 ml
20x SSC	500 µl
ssDNA (1mg/µl)	250 µl
ddH_20	321 µl
Dextran sulfate	142 µl

Tab.20: Detection Buffer for Alkaline Phosphatase (RNA-FISH)

AP Detection Buffer

5M NaCl	1 ml
1M $MgCl_2$	2,5 ml
1M Tris (pH 9,5)	5 ml
Tween 20	50 µl
ddH_20	41,2 µl

Solutions and reagents

Lysis buffers

These buffers may be stored at 4°C for several weeks or aliquoted and stored at -20°C for up to a year.

NP-40 buffer

– 150 mM NaCl

– 1.0% NP-40 (possible to substitute with 0.1% Triton X-100)

– 50 mM Tris-HCl, pH 8.0

– Protease inhibitors

RIPA buffer (radioimmunoprecipitation assay buffer)

– 150 mM NaCl

– 1.0% NP-40 or 0.1% Triton X-100

– 0.5% sodium deoxycholate

– 0.1% SDS (sodium dodecyl sulphate)

– 50 mM Tris-HCl, pH 8.0

– Protease inhibitors

Tris-HCl

– 20 mM Tris-HCl

– Protease inhibitors

Running, transfer and blocking buffers

Laemmli 2X buffer/loading buffer

– 4% SDS

– 10% 2-mercaptoethanol

– 20% glycerol

– 0.004% bromophenol blue

– 0.125 M Tris-HCl

Check the pH and adjust to 6.8

Running buffer (Tris-Glycine/SDS)

– 25 mM Tris base

– 190 mM glycine

– 0.1% SDS

Check the pH and adjust to 8.3

Transfer buffer (wet)

– 25 mM Tris base

– 190 mM glycine

– 20% methanol

– Check the pH and adjust to 8.3

For proteins larger than 80 kDa, we recommend that SDS is included at a final concentration of 0.1%.

Transfer buffer (semi-dry)

– 48 mM Tris

– 39 mM glycine

– 20% methanol

– 0.04% SDS

Blocking buffer

3–5% milk or BSA (bovine serum albumin)

Add to TBST buffer. Mix well and filter. Failure to filter can lead to spotting, where tiny dark grains will contaminate the blot during color development.

2.23 Equipment and software

Tab. 7: Laboratory Equipment

Name / Modell	Manufacturer
Stereo microscope M80	Leica
Fluorescence microscope	Olympus
Confocal Laser scanning Microscope TCS SP5	Leica
Electrophoresis gel chamber HE33	Hoefer, Holliston, USA
Gel documentation system E-Box vX2	PeqLab
Microwave	Techno Star
Magnetic mixer	Heidolph
pH meter CG840	Schott
NanoDrop 2000	Thermo Scientific, Waltham, USA
Shaker Rotamax 120	Heidolph
Scale AM40	Mettler
UV transilluminators	MS Laborgeräte, Wiesloch
Centrifuge 5417R5 / 424R	Eppendorf, Hamburg
Table centrifuge, Spectrafuge	neoLab
Heating block TB1	Biometra
Thermocycler T-Gradient/T-Personal	Biometra
Thermomixer (Compact)	Eppendorf
Western blot System	Biorad
Vortexer VF2	Jahnke & Kunkel

Tab. 22: Used Kits and Buffers

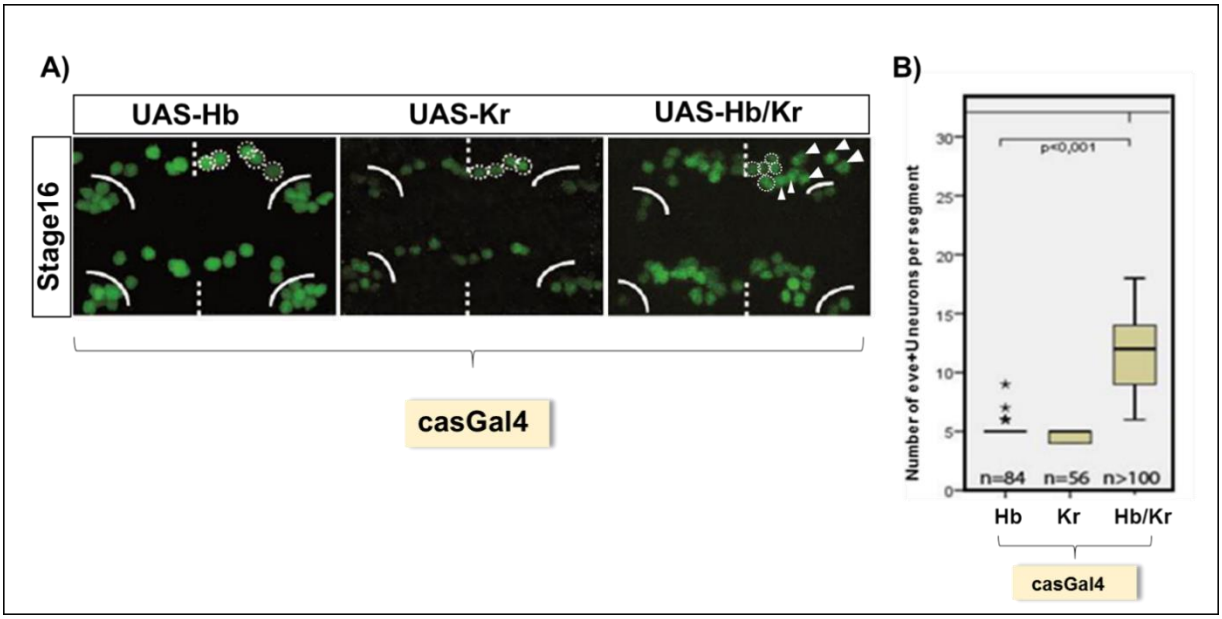
Name / Modell	Manufacturer
GenElute TM PCR Clean-up Kit	Sigma-Aldrich
Taq PCR Kit	Qiagen
FISH Tag TM DNA Kit	Thermo Fisher Scientific
6x DNA Loading Dye	Fermentas
SlowFade® Gold antifade	Thermo Fisher Scientific

3. Results

3.1 Co-expression of Kr enables Hb to induce ectopic Eve+ U motoneurons in NB 7-1 outside early competence window

NB7-1 produces the Even-skipped (Eve)-positive U1-U5 motoneurons during its first five cell divisions followed by a variable number of Eve-negative interneurons (Fig 2). Hb loss of function mutants lack early-born U1/U2 neurons, and prolonged Hb expression within this NB will generate many additional Eve-positive U1-like neurons until end of embryogenesis (Isshiki et al., 2001; Novotny et al., 2002). Also, continuous *hb* expression leads to prolongation of Kr protein expression it is assumed that the *Kr* gene gets a positive regulatory input from Hb on the transcriptional level (Isshiki et al., 2001). Although Kr specifies the cell types of the second temporal window it is been co-expressed with *hb* in WT neuroblasts (Isshiki et al., 2001). On the first glimpse, this suggests that *hb*-dependent fates should be dependent on the combined activity of Hb and Kr. However, in Kr-lof mutants *hb*-dependent fates are still present and only fates solely dependent on Kr are missing (Isshiki et al, 2001). Importantly, re-expression of Hb after the NBs fifth division has no effect on NB competence (Pearson and Doe, 2003). This shows that NB7-1 loses its competence to generate Eve-positive motoneurons in response to Hb activity latest after five divisions and this is referred as 'neuroblast early competence window' (Kohwi et al., 2013). Intriguingly, in *Drosophila* we found that Kr regains the ability of Hb to induce ectopic U neurons after its late re-expression (casGal4xUAS-Hb/Kr) whereas single Hb (casGal4xUAS-*hb*) and Kr (casGal4xUAS-Kr) late re-expression failed to induce additional U motoneurons (Fig 8A). In NBs, *cas* expression peaks in stage 12, subsequent to its third mitotic division (Grosskortenhaus et al., 2005) therefore combined expression of *hb* and Kr was driven using casGal4 (casGal4xUAS-Hb/Kr). Next to test whether endogenous Kr is active during co-expression with *hb* we performed RNA FISH against *Kr* intron in embryonic stages 12-13 (Fig 8C). As expected, *Kr* is active during prolonged *hb* expression in early competence (*insc*Gal4xUAS-*hb*) window as seen by intron signals (green dots in NBs) however late *hb* re-expression by using casGal4 which drives earliest after the 4th division of NB 7-1 could not activate *Kr* anymore (Fig 8C). Interestingly, co-expression of Hb and Kr by casGal4 later in development resulted in activation of endogenous *Kr* transcript in NBs (Fig 8C). These results suggest that indeed shut down of competence window is due to silencing of *Kr* locus and the combined activity of Hb

and Kr maintains the competence for the production of Eve-positive U neurons in NB 7-1 and ectopic co-expression of these factors outside of the competence window regains competence.



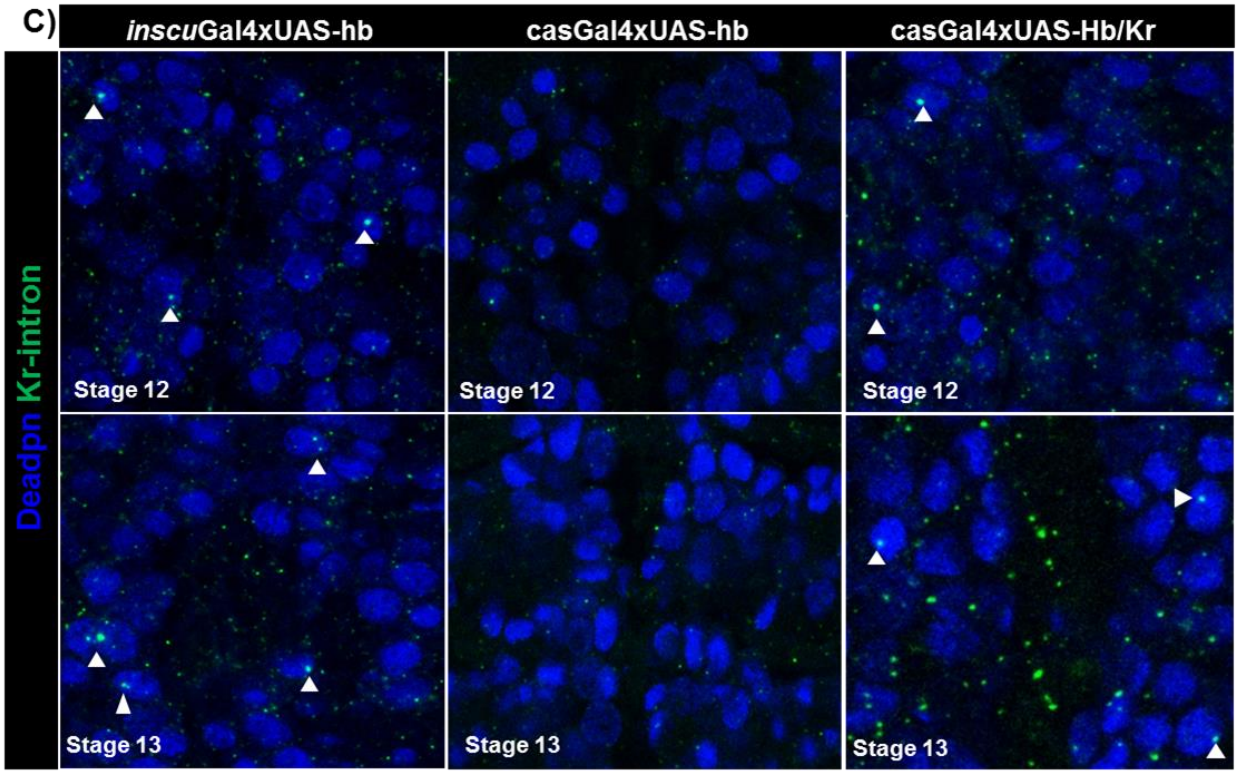
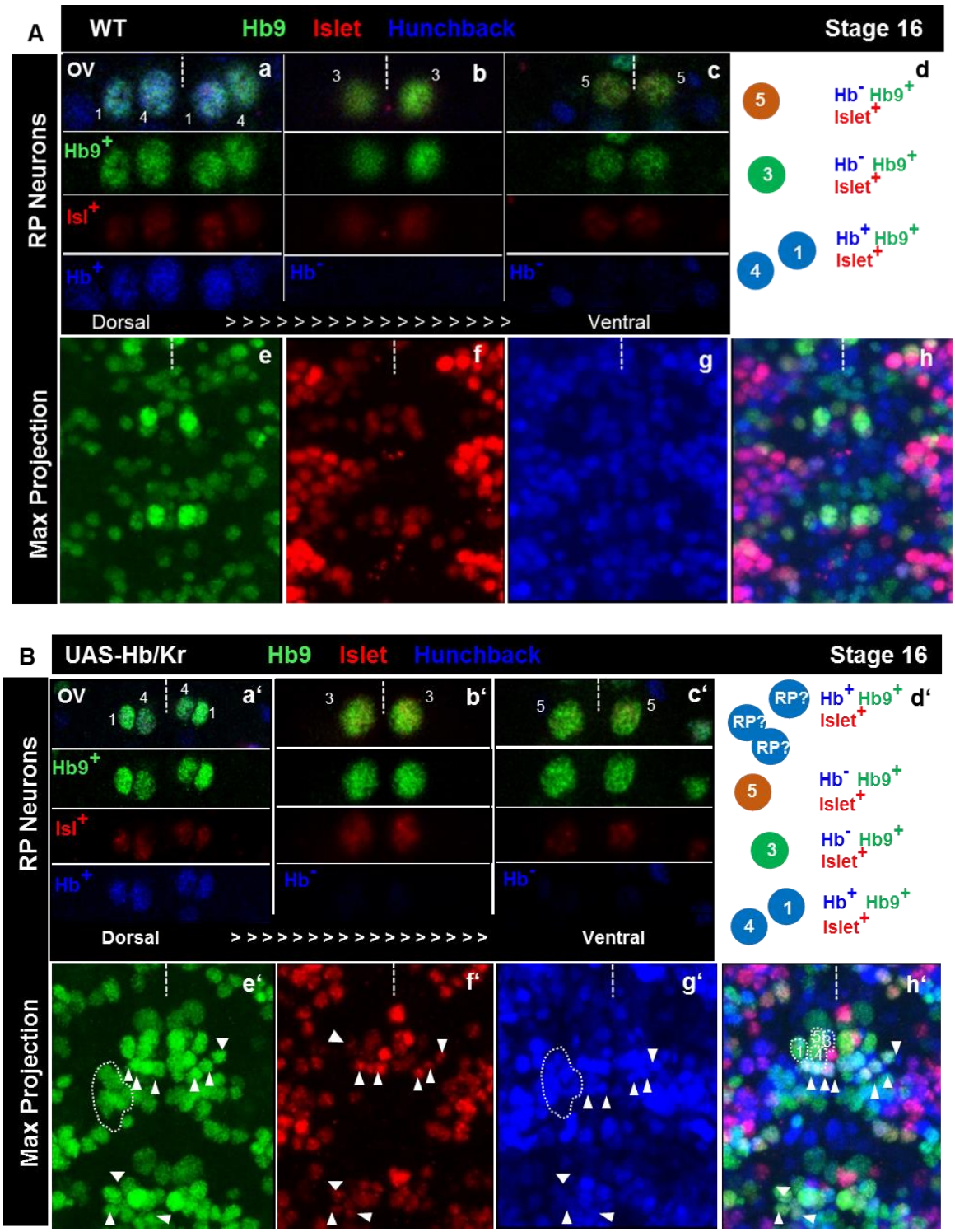


Fig 8. Co-expression of Kr enables Hb to induce ectopic Eve+ U motoneurons in NB 7-1 outside early competence window. A) Single ectopic late expression of Hb and Kr using casGal4 does not produce ectopic U neurons shown in first two insets. The third inset shows casGal4 driven Hb/Kr co-expression which leads to a mean of 7 ectopic Eve-positive U neurons in 100% of hemisegments n=134 (stage 16) (highlighted by arrowheads) B) Box plot quantification showing number of U motoneurons per segment, single ectopic expression of Hb and Kr generates 5 U motoneurons whereas in case of combine expression of Hb/Kr there are 13 U motoneurons produce per segment n=number of segments analyzed. (Student T-test showing statistical significance where $p<0.001$) (Results A and B acquired from Sebastian Jansen, Institute for Genetics C) RNA FISH against Kr transcript was performed in embryonic stages 12-13, Kr is active during prolonged hb expression in early competence window *(insc*Gal4xUAS-hb). However late hb re-expression by using casGal4 could not activate Kr anymore. Interestingly, co-expression of hb and Kr by casGal4 later in development resulted in activation of endogenous Kr transcript in NBs (Kr intron signals shown by arrowheads)

3.2 Hb and Kr maintains competence beyond the early window of RP neurons generation derived from NB 3-1

To test whether the role of Hb and Kr regarding the competence window is more general we sought to analyze at least one other lineage NB3-1. NB3-1 is known to generate the well-characterized RP1, RP4, RP3 and RP5 motoneurons (Bossing et al., 1996; Landgraf et al., 1997; Schmid et al., 1999). Hb and Kr also specify early temporal identity in NB 3-1 lineage, extending their role as multi-lineage temporal identity factors to a different spatial domain of the CNS (Tran and Doe, 2008). RP1/4 are Hb^{+} Kr^{+}, RP3 is Hb^{-} Kr^{+}, and RP5 is Hb^{-} Kr^{-} and these motoneurons additionally are also positive for $Hb9^{+}$ and $Islet^{+}$ markers (Tran and Doe, 2008). Further, it was shown that continuous *hb* expression in early time point of development led to the production of additional RP1/4 motoneurons (Tran and Doe, 2008). This proves that Hb is necessary and sufficient to specify early-born RP1/RP4 temporal identity within the NB3-1 lineage, resembling its role in specifying the first temporal identity in the NB7-1 and NB7-3 lineages (Isshiki et al., 2001; Novotny et al., 2002). Here we extend our observation similar to NB 7-1 lineage by ectopically expressing Hb and Kr together late in competence window using casGal4 driver line (casGal4xUAS-Hb/Kr). We observed formation of additional Hb^{+}, $Hb9^{+,}$ and $Islet^{+}$ neurons (average of 7 neurons per hemisegment), resembling early born cell fates by presence of these markers in hemisegments (Stage16 = 5 embryos, 72 hemisegments), as compared to WT (Stage 16 = 4 embryos, 65 hemisegments), furthermore we also could observe additional cells positive for Hb9+ and Hb but negative for islet (Fig 9A

and B). There were no additional cells seen in single ectopic Hb and Kr expression late in development (Fig 9C). These results show that the ability of Hb and Kr to regain the competence is not specific for NB 7-1 but similar mechanism also exist in NB 3-1 lineage. However, the detailed characterization of these additional neurons remains a challenge due to a limited number of markers expressed in early-born cell fates.



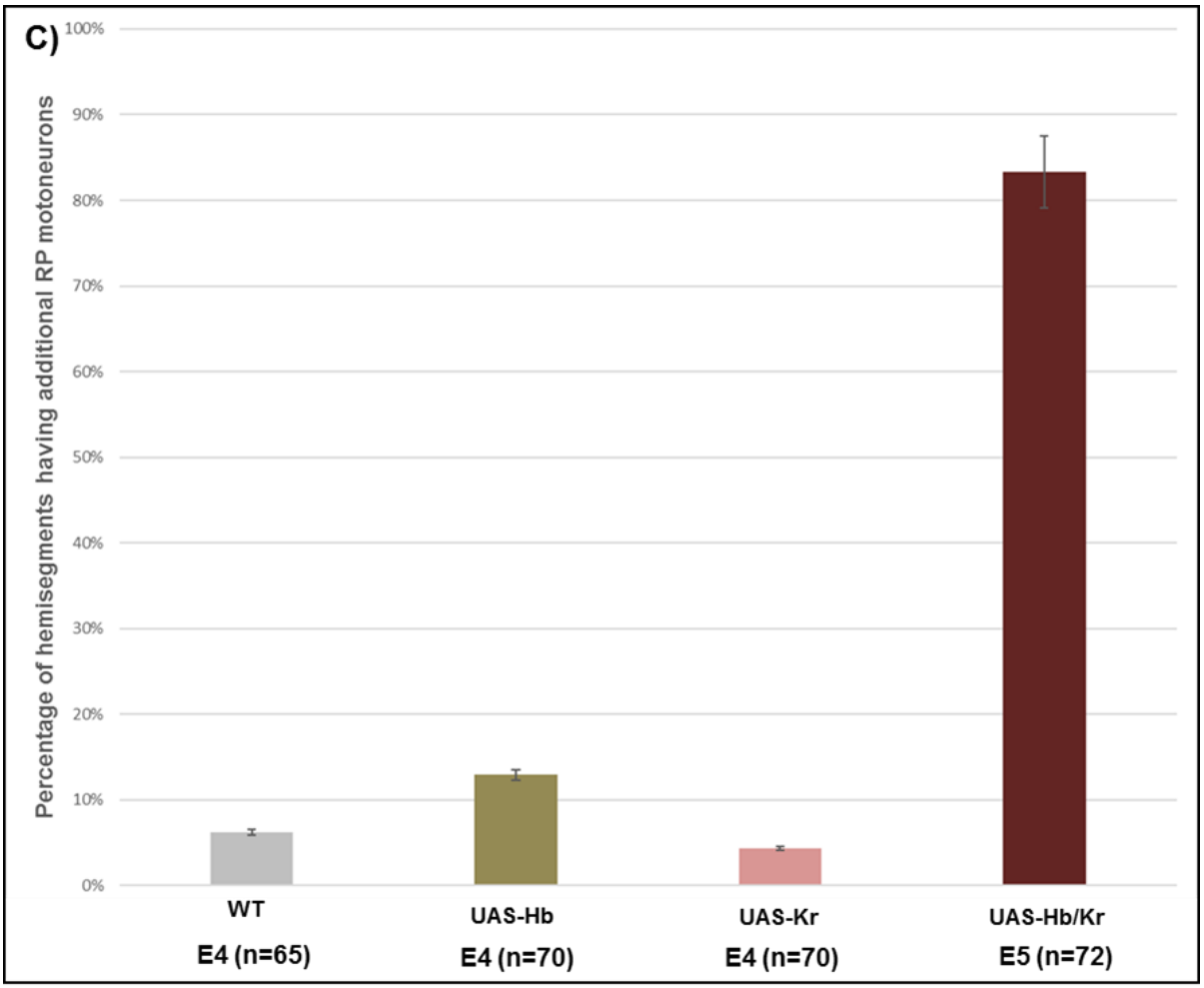


Fig 9. Co-expression of Kr enables Hb to induce ectopic neurons in NB 3-1 lineage. A) NB3-1 is known to generate the well-characterized RP1, RP4, RP3 and RP5 motoneurons. RP1/4 which are more dorsal are positive for Hb, Hb9 and Islet as shown in overlay version in first inset 'a' and cartoon d, RP3 and RP5 which are more ventral are negative for Hb but positive for Hb9 and islet markers shown in insets b and c. Insets e, f, g shows an individual maximum projection of Hb9 in green, Hb in blue and Islet in red of two segments while 'h' shows overlay max projection of all three channels in WT. B) In casGal4xUAS-Hb/Kr we observed the formation of additional Hb+, Hb9+ and Islet+ neurons marked by arrowheads in insets e', f', g' and h', resembling early born cell fates by the presence of these markers. Furthermore, we also could observe additional cells positive for Hb9+ and Hb but negative for islet mark dotted lines C) Graphical analysis showing the percentage of hemisegments having additional neurons positive for Hb9, Hb, and Islet. Combine expression of Hb/Kr driven by casGal4 have 80% of hemisegments consisting of additional neurons as compared to single ectopic Hb and Kr expression driven by casGal4 and WT where very few hemisegments possessed such additional neurons suggesting that Hb and Kr when co-expressed has the ability to regain the competence in NB 3-1 lineage.

3.3 Analysis of gene locus position using a "3 zone model"

Here we show that shut down of competence window is due to silencing of *Kr* locus and the combined activity of Hb and Kr maintains the competence ectopic co-expression of these factors outside of the competence window regains competence. Therefore my next aim was to check if this silencing of *Kr* locus is linked to a nuclear re-organization of the chromatin. Therefore to measure the intra-nuclear locus position at different stages I opted the 3 zone model system published by (Hediger et al., 2004) where, the distance from the middle of the spot to the middle of the envelope signal (a), and the nuclear diameter (b), are measured. By dividing 'a' by a/2 (p ¼ 2a/b), we can classify the spot position into three concentric zones of equal surface. The outermost zone I contains peripheral spots ($p \leq 0.092$). Zone II regroups intermediate positioned spots ($0.092 < p \leq 0.212$). Zone III contains internal spots ($p > 0.212 \leq 0.5$) (Fig 7, Methods and Materials section 2.21). Change in nuclear localization of *hb* locus from interior towards periphery (nuclear lamina) was shown by (Kohwi et al., 2013), where they observed strong correlation of silencing of *hb* locus with termination of the early competence window. The *hb* locus positions overlap with the nuclear lamina after the end of the early competence window in stage 12. Thus it was concluded that neural progenitors undergo a re-organization of genome architecture whilst aging, therefore changing the set of genes which is available to specify the progeny. These findings also reflects gene silencing mechanism within NB population with alteration in gene locus position and how this can have influence on activity of that gene. In order to confirm that results, found by Kohwi et al., 2013, are reproducible in our hands using zone model system, a proof of principle experiment was conducted, where we analyzed nuclear repositioning of *hb* locus in WT and in overexpression of Hb driven by *engrailed*-Gal4. The visualization and investigation of the *hb* genomic region in all NBs reveals an *hb* locus repositioning from the inner nuclear region in stage 9/10 as almost 60% of measured alleles are in zone 3 and it moves towards nuclear periphery as seen in the stage 11, intermediate stage 42% of measured alleles are in zone 2 and then found at nuclear periphery at stage 12 which marks the end of competence phase (Fig 10A). These results obtained with our measurement method using zone model system thus reflects and supports the results published by Kohwi et al 2013. Next, we analyzed repositioning of *hb* locus when Hb itself is overexpressed using *en*-Gal4 driver line. We found that in *en*-Gal4 xUAS-Hb2x, *hb* locus does re-localize similar to WT in Engrailed positive NBs i.e. it is initially interior zone 3 during stage 10 and moves towards periphery and stays

there at stage 12 (Fig 10B), suggesting that Hb can specify early-born neuronal identity but cannot extend the competence window for postmitotic *hb* expression, thus reflecting again similar results published by Kowhi et al 2013.

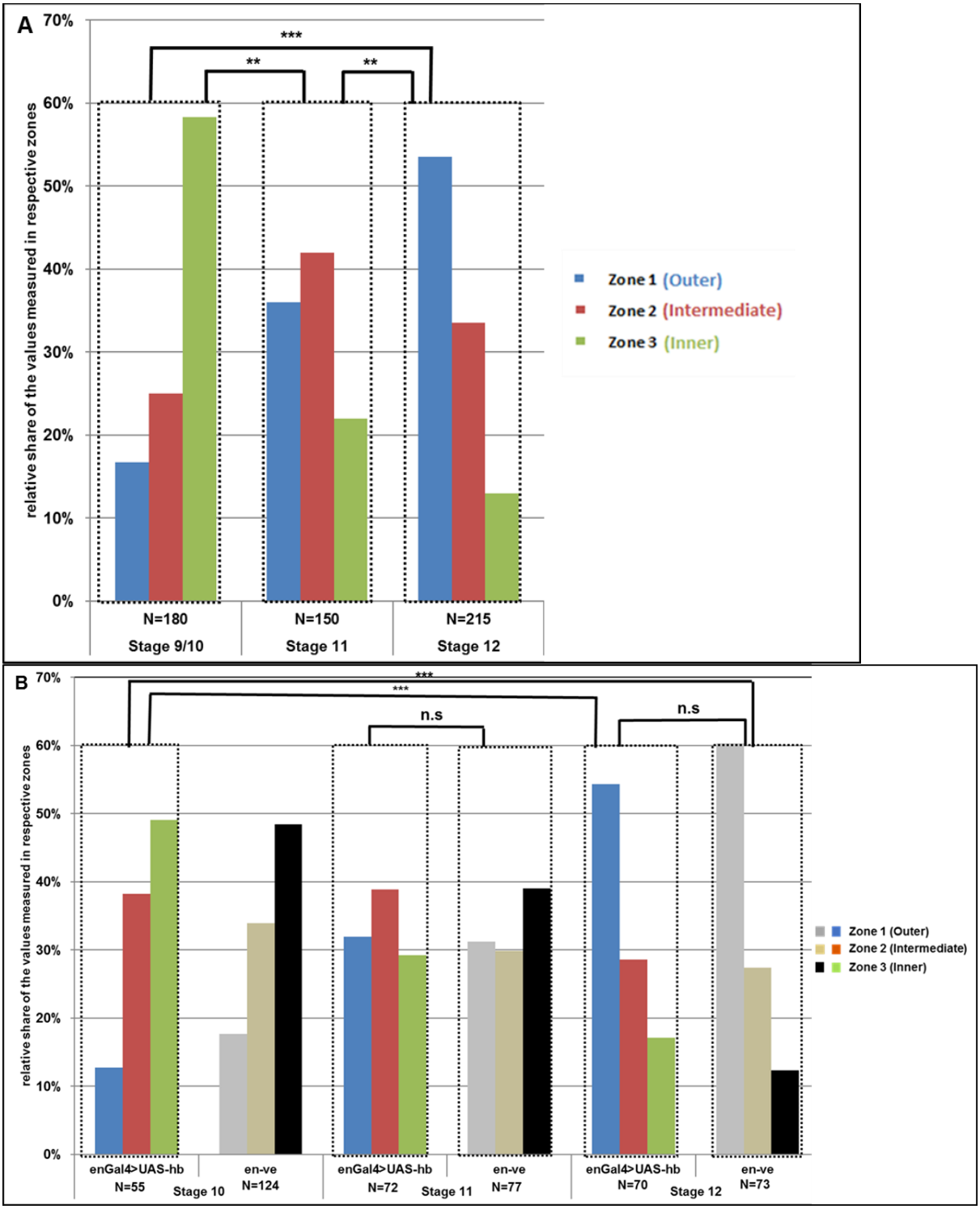
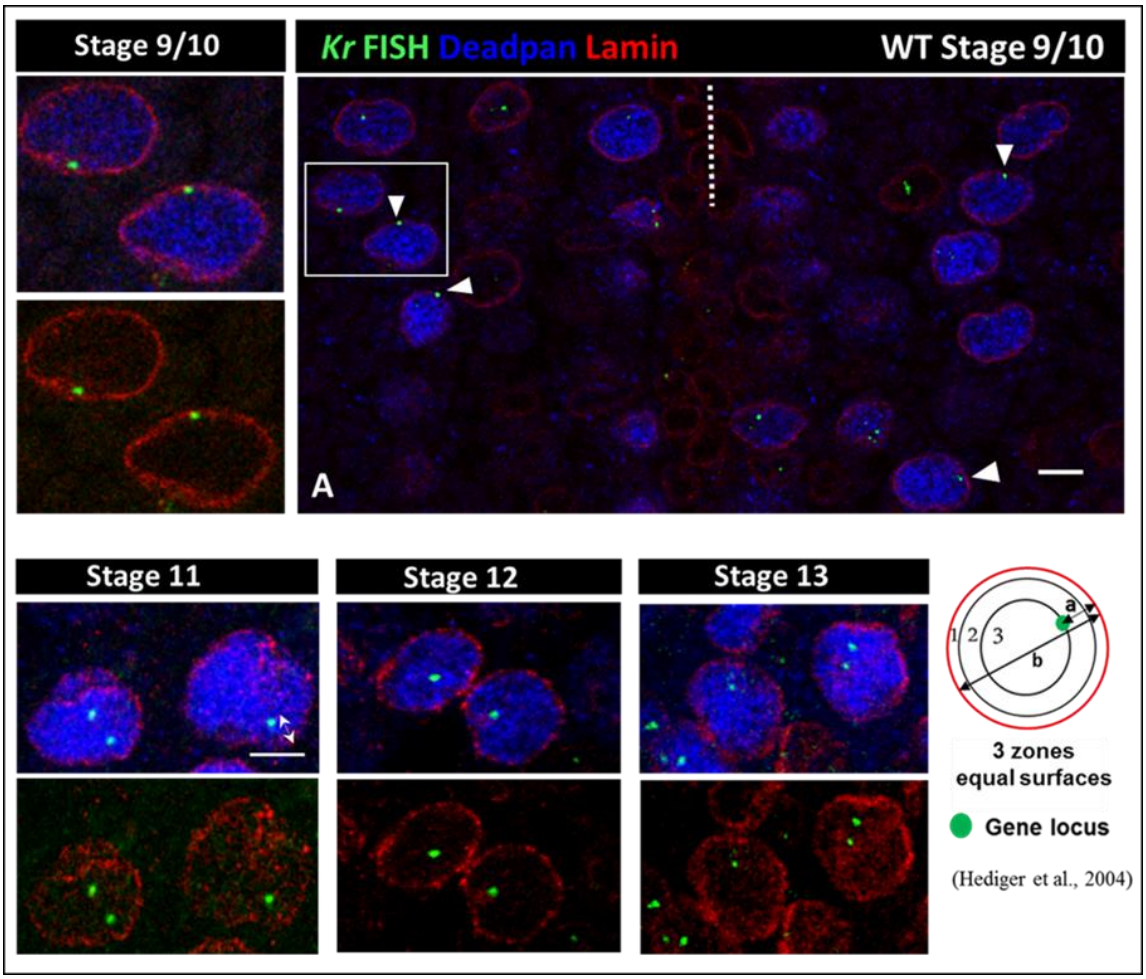


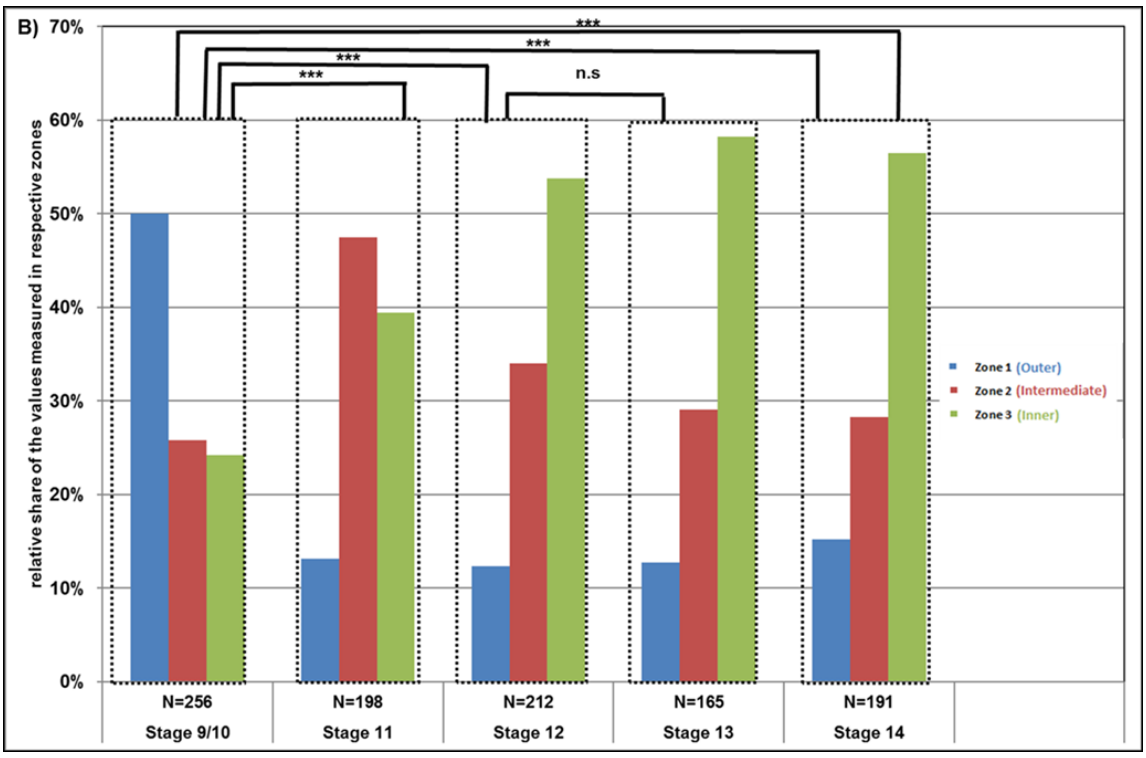
Fig.10 A. Graphical analysis using zone model system depicting re-localization of *hb*-locus from inner nuclear region to periphery between stage 10, stage 11 and stage 12 in WT embryos (N= number of alleles measured). *hb* locus repositions from the inner nuclear region in stage 9/10 as almost 60% of measured alleles are in zone 3 (green bar) and it moves towards nuclear periphery as seen in the

stage 11, intermediate stage 42% of measured alleles are in zone 2 (red bar) and then found at nuclear periphery i.e. zone 1 (blue bar) at stage 12 which marks the end of competence phase. **B.** Graph represents comparison between distributions of *hb*-locus in Hb overexpression in Engrailed positive NBs compare to internal control Engrailed negative NBs in zones 1, 2 and 3 in stage 10, stage 11 and stage 12.It can be seen *hb* locus re-localizes from nuclear interior to periphery in case of Hb overexpression and this pattern is similar to WT, suggesting that that Hb can specify early-born neuronal identity but cannot extend the competence window (Kohwi et.al 2013)**.** Chi2 test was carried out to check statistical significance ($p<0,001$).

3.3 *Kr* locus re-localizes to inner nuclear region in all NBs in WT at the end of competence window

In *Drosophila* embryo one can image CNS where NBs can also be stained using markers like Deadpan or Worniu as they progress through their cell lineages during development. Here, we followed the sub-nuclear position of the *Kr* locus within the population of wild-type NBs at different developmental stages in vivo (Stage9/10-Stage14). Immunostaining was done to detect NB and DNA fluorescence in situ hybridization (DNA FISH; Bantignies et al., 2011) to detect the *Kr* genomic locus within these cells in intact embryos. Our goal was to determine whether the *Kr* gene re-localizes to silencing compartment close to lamin parallel with loss of NB competence. To address this we produced a fluorescently labeled DNA probe spanning approximately 12 kb within the *Kr* coding region. After insitu hybridization, embryos were stained with antibodies against NB marker Deadpan and nuclear envelope marker Lamin (Fig 11A). For analysis of re-localization in NB we followed zone model system. We found interesting observation that *Kr* locus indeed re-localizes within the relevant time window but in the opposite direction: initially in embryos stage 9/10 it is detected at the heterochromatic region i.e. nuclear periphery (zone 1) as 50% of measured alleles are in zone 1 and then moves towards nuclear interior as soon as competence window is terminated at stage 12 as nearly 53% of measured alleles are in zone 3 (interior) and maintains this position even until stage 14 of embryogenesis in most of the NB population (Fig 11B). Additionally, we could also detect up to two *Kr* gene foci in some of NB nucleus at all stages of development (Fig 11A stage 11 and stage 13 NBs). In contrast to NBs, *Kr*-locus re-localization does not occur in epithelial cells. From stage 9 to stage 12, the *Kr* genomic region is observed to remain in one particular sub-nuclear compartment i.e. nuclear interior (Fig 11C).


Stage 9/10
Kr FISH Deadpan Lamin
WT Stage 9/10
A
Stage 11
Stage 12
Stage 13
1
2
3
a
b
3 zones
equal surfaces
Gene locus
(Hediger et al., 2004)


B)
70%
60%
50%
40%
30%
20%
10%
0%
relative share of the values measured in respective zones
***
***
***
***
n.s
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)
N=256
Stage 9/10
N=198
Stage 11
N=212
Stage 12
N=165
Stage 13
N=191
Stage 14

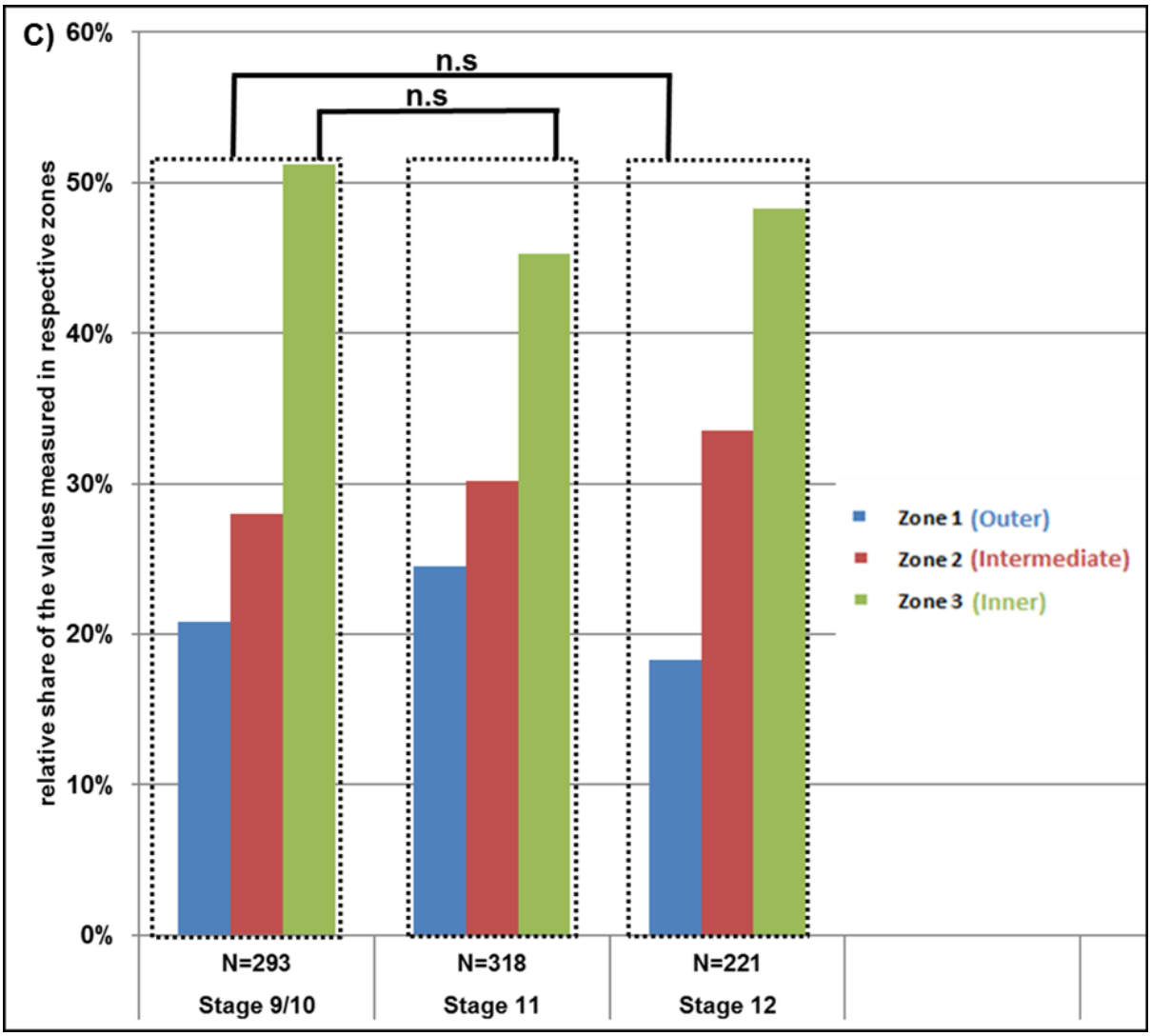
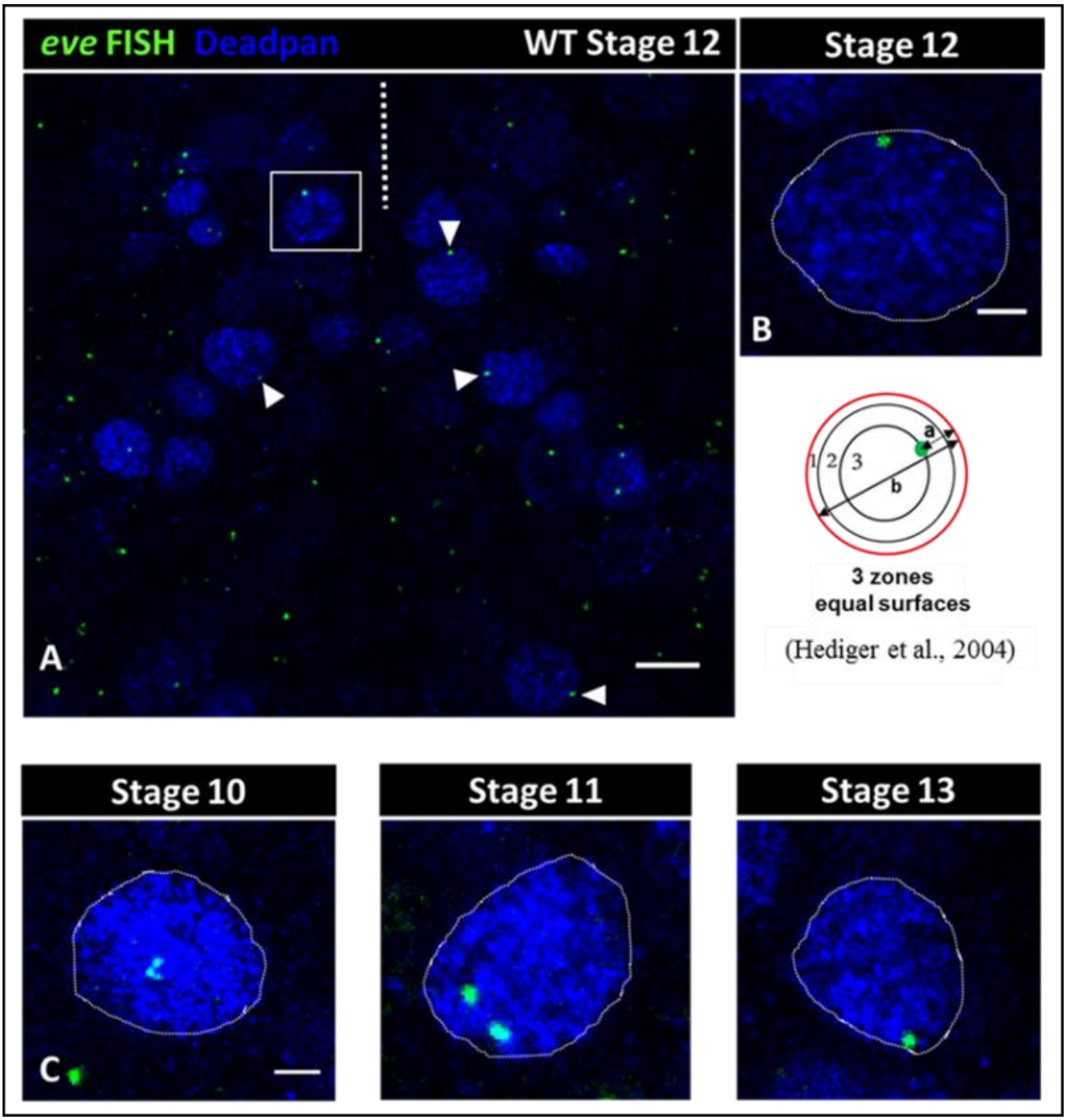
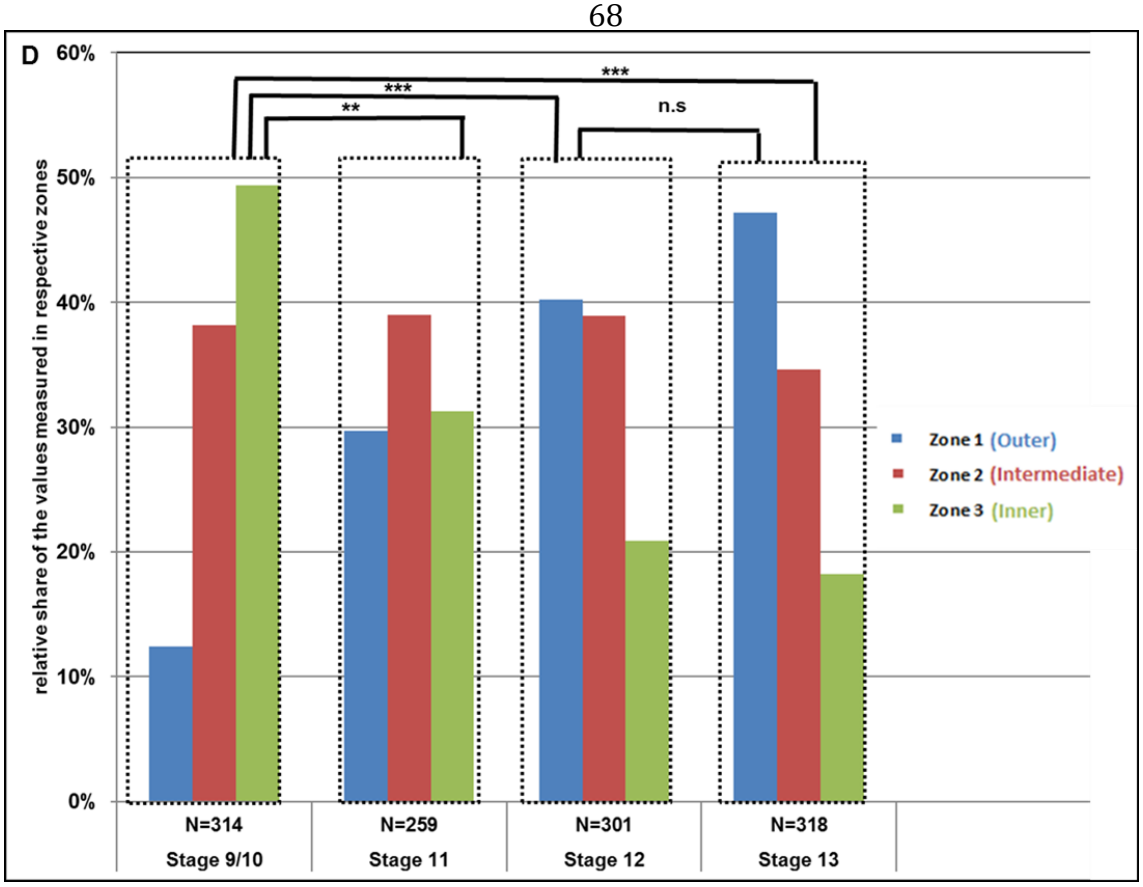


Fig 11 A. Re-localization of *Kr*-locus (green) from periphery (marked by arrow heads in stage 9/10) to inner nuclear region between stage 9/10, stage 11, stage 12, stage 13. Deadpan (NB marker) blue, Lamin (nuclear envelope) red. Cartoon represents the measurement method for gene locus (green dot) in NB nuclei where shortest distance ´´a´´ is measured from center of FISH signal to periphery and this distance is divided by diameter of cell nucleus ´´b´´ indicating the position of locus in zone 1 (outer), zone 2 (intermediate) and zone 3 (inner) irrespective of shape of nuclei. **B.** Graph represents the analysis of the distribution of *Kr*-locus in these zones in WT embryos NBs in stage 9/10, 11, 12, 13, 14 and it can be seen that gene locus is close to periphery in stage 9/10 and re-localizes into inner nuclear region at stage12 and this position is maintained until stage 14 **C.** Graph represents the analysis of the distribution of *Kr*-locus in epithelial cells in stage 9/10, 11, 12 and it can be seen that gene locus is staying constantly in nuclear interior in all three stages i.e. it's not re-localizing in epithelial cells. (N= number of alleles analyzed). Scale bar 50µm. Chi2 test was carried out to check statistical significance ($p<0,001$).

3.4 *eve* locus re-localizes to nuclear periphery in all NBs in WT at the end of competence window

Eve (Even skipped) is transcription factor expressed in subset of post-mitotic motoneurons born during early competence window of certain NBs. For instance, during the first five divisions, NB 7-1 (competence window) generates five GMCs (GMC-1 to GMC-5) that further give rise to five Eve positive U-motoneuron (U1-U5). Here we analyzed re-localization of *eve* gene locus during competence window phase. Our goal was to determine whether the *eve* gene re-localizes similar to *Kr* and *hb* parallel with loss of NB competence. Indeed, we observed *eve* locus re-localizes within the relevant time window similar to *hb* locus (Kohwi et al., 2013): initially in embryos stage 9/10 it is detected at the interior of nucleus and then moves towards nuclear periphery as soon as competence window is terminated at stage 12 in all NB population (Fig 12A, B, C and D). Re-localization of *eve* was absent in epithelial cells similar to *hb* and *Kr* (Fig 12E). Like in the case of *hb* the *eve* locus stays at a more peripheral position. Thus, we conclude that the competence ends with a potential silencing of *eve* locus similar to *Kr* (our data) and *hb* locus (Kohwi et al., 2013).


eve FISH Deadpan
WT Stage 12
A
Stage 12
B
1
2
3
a
b
3 zones
equal surfaces
(Hediger et al., 2004)
Stage 10
C
Stage 11
Stage 13


D
60%
50%
40%
30%
20%
10%
0%
relative share of the values measured in respective zones
***
***
**
n.s
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)
N=314
Stage 9/10
N=259
Stage 11
N=301
Stage 12
N=318
Stage 13

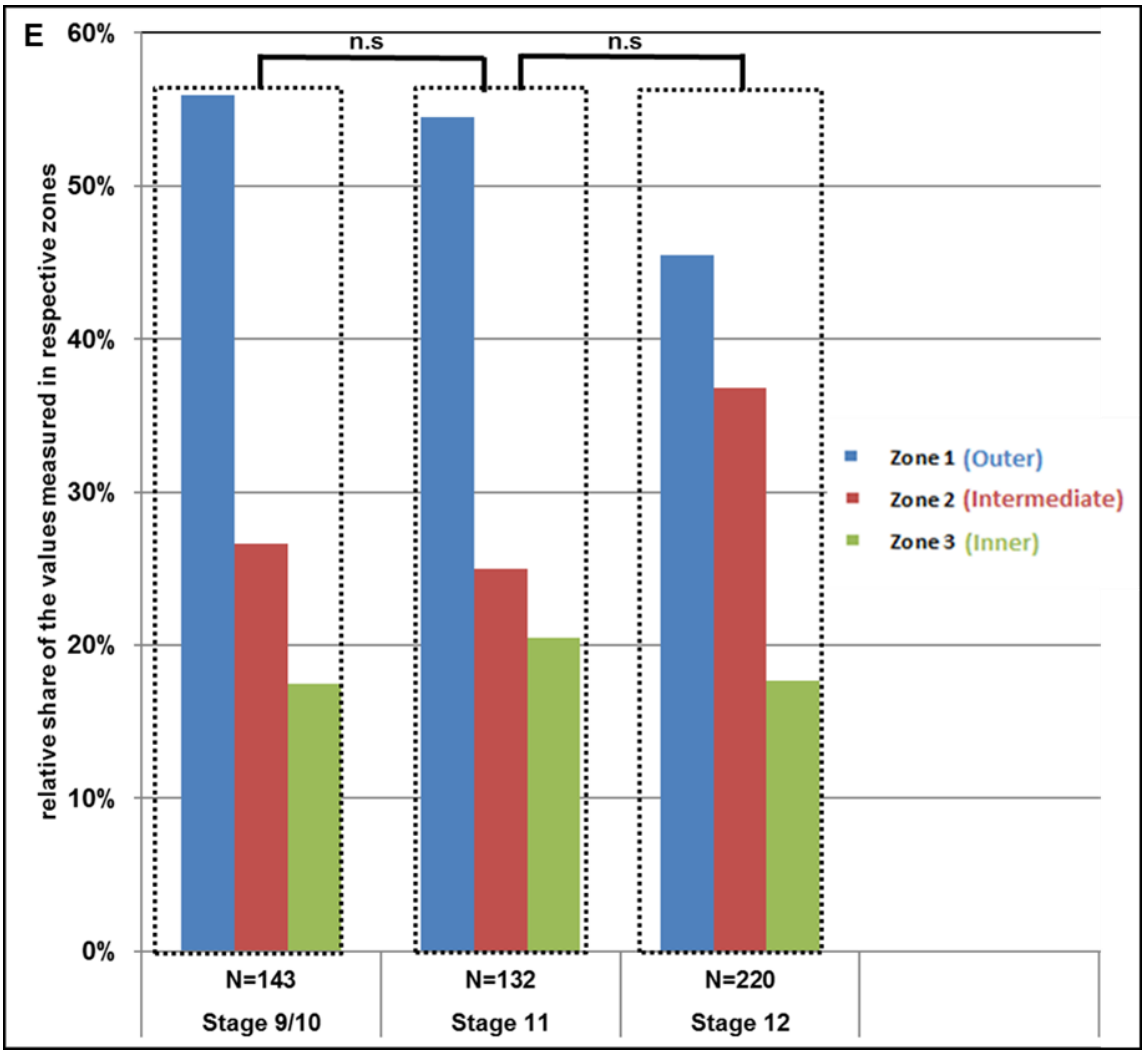
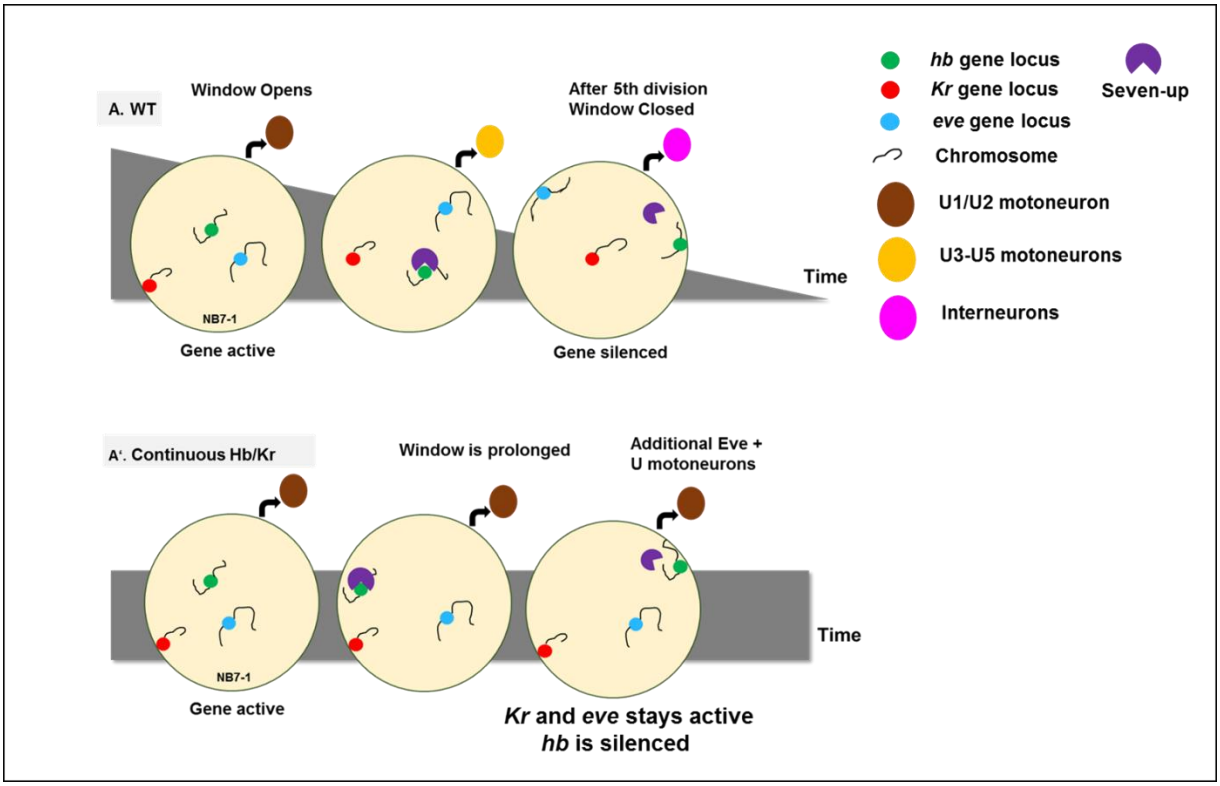

E
60%
50%
40%
30%
20%
10%
0%
relative share of the values measured in respective zones
n.s
n.s
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)
N=143
Stage 9/10
N=132
Stage 11
N=220
Stage 12

Fig 12 A, B and C. Re-localization of *eve*-locus (green) from nuclear interior to periphery (marked by arrow heads in stage 12 NBs in Fig A) between stage 9/10, stage 11, stage 12, and stage 13. Deadpan (NB marker) blue, Cartoon represents the measurement method for gene locus (green dot) in NB nuclei where shortest distance ´´a´´ is measured from center of FISH signal to periphery and this distance is divided by diameter of cell nucleus ´´b´´ indicating the position of locus in zone 1 (outer), zone 2 (intermediate) and zone 3 (inner) irrespective of shape of nuclei. **D.** Graph represents the analysis of the distribution of *eve*-locus in these zones in WT embryos NBs in stage 9/10, 11, 12, 13 and it can be seen that gene locus is into inner nuclear region in stage 9/10 and re-localizes to periphery at stage12 and this position is maintained until stage 13 **E.** Graph represents the analysis of the distribution of *eve*-locus in epithelial cells in stage 9/10, 11, 12 and it can be seen that gene locus is staying constantly in nuclear periphery region in all three stages i.e. it's not re-localizing in epithelial cells. (N= number of alleles analyzed). Scale bar 50µm. Chi2 test was carried out to check statistical significance ($p<0,001$).

3.5 Silencing/re-localization of *Kr* and *eve* can be blocked by continuous Hb expression in early competence window

The competence to generate Eve+ postmitotic neurons is extended in NB7-1 when Hb or Kr is continuously expressed in a high dosage (Grosskortenhaus et al. 2006; Isshiki et al., 2001). Additionally, Eve-expression is able to be reactivated by a combined re-expression of Hb and Kr at later stages (casGal4xUAS-Hb/Kr), while a sole late re-expression of either Hb or Kr is not sufficient to reactivate eve (Sebastian Jansen, unpublished data; Institute for Genetics, University of Mainz). These observations suggest a competence window in NB7-1 keeping the eve-locus open for transcription. The length of this window seems to be dependent on a combined activity of both Hb and Kr. A continuous expression of Hb is therefore able to extend the competence for NB7-1 to generate Eve+ progeny, indicating that a potential transcriptional silencing for the *Kr* and *eve* gene might be deferred. If the observed *Kr* and *eve* locus re-localization indeed has the objective to transcriptionally silence these genes, a repositioning would expect to be decelerated or even blocked in embryos continuously expressing Hb in high concentrations. Here we analyzed *Kr* locus re-localization in embryos with two copies of UAS-*hb* driven by *en*-Gal4, where expression is only in engrailed positive NBs. Likewise we expressed *hb* driven by *insc*-Gal4, where expression is in all NBs and analyzed *eve* locus re-localization. We found that re-localization is indeed blocked or slowed down for both the loci. In case of *Kr* locus we found that in engrailed negative NBs the re-localization was wild-typic showing that this effect is cell autonomous (Fig 13B). In engrailed positive NBs *Kr* locus position is maintained closed to periphery from stage 11 as 52%

measure alleles are in zone 1 as compared to engrailed negative cells where it is positioned mostly intermediate and moving towards interior i.e. zone 3 and even in late stage 12 the position of *Kr* locus is maintained towards periphery in *hb* overexpression 50% of measured alleles are in zone 1 as compared to internal control where it is already in to interior of nucleus (Fig 13B). Similarly, *eve* locus was found to be in the interior of nucleus in stage 11 and stage late 12 driven by *insc*-Gal4 as seen in graphical analysis 53% measured alleles are in zone 3 in stage 11 and this position is maintained further as 55% measured alleles are found in zone 3 in stage 12 as compared to WT, where locus is moving more towards periphery i.e. zone 1 (Fig 13C). It was shown that in *en*-Gal4 xUAS-Hb2x, *hb* locus does re-localize similar to WT i.e. it is initially interior zone 3 during stage 10 and moves towards periphery and stays there at stage 12.These results propose the assumption that continuous *hb* expression maintains *Kr* and *eve* transcriptionally active and once *hb* is turned off, *Kr* might re-localized towards a certain nuclear compartment into inner nuclear region and eve towards periphery in order to be silenced permanently.



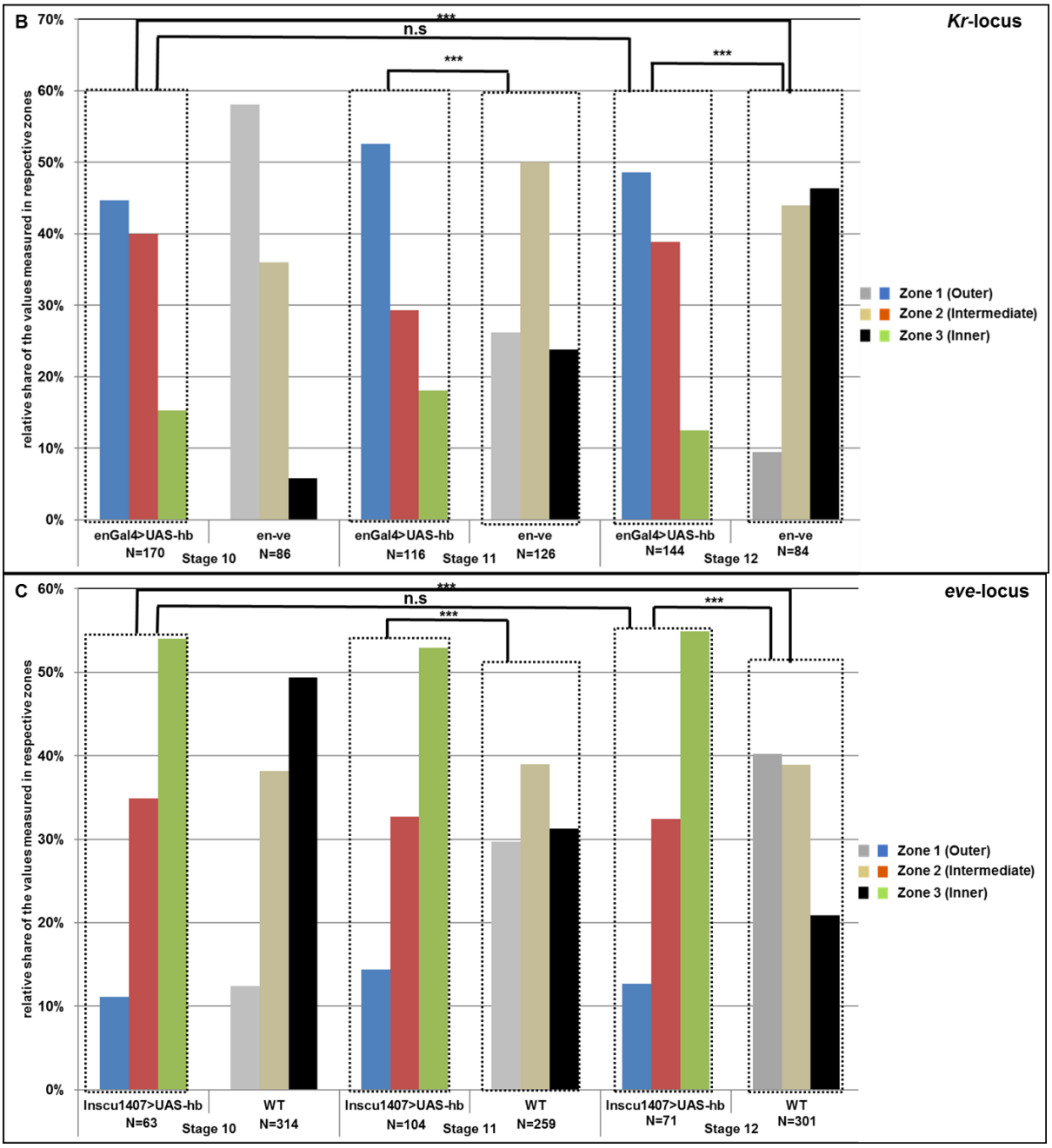


Fig 13 A, B and C. Re-localization of *Kr* and *eve* loci is halted in Hb overexpression early in competence window. **A.** Comparison of gene locus re-localization between WT and Hb overexpression. In WT, *hb* (Green spot) and *eve* (Cyan spot) loci re-localizes from interior to periphery within the nucleus whereas *Kr* (Red spot) re-localizes from periphery to nuclear interior at end of competence. On the other hand it can be seen that continuous *hb* expression driven by *en*-Gal4 extends competence window and maintains *Kr* re-localization peripheral throughout and *eve* into nuclear interior i.e. they are both active, whereas *hb* itself re-localizes and get silenced similar to WT, this could be because Svp might initiate silencing process for *hb* in both the cases. **B.** Graphical analysis of *Kr* locus in case of Hb overexpression. At stage 11 and stage 12, in engrailed positive NBs *Kr* locus position is maintained closed to periphery as compared to engrailed negative cells where it is positioned mostly intermediate and moving towards interior i.e. zone 3. There is no significant change in *Kr* re-localization between stage 10 and stage 12 in Hb overexpression. **C.** Similarly, *eve* locus was

found to be in the interior of nucleus in stage 11 and stage late 12 driven by *inscu*-Gal4 as compared to WT where it is moving towards periphery within these stages seen in graphical analysis. (N= number of alleles analyzed). Chi2 test was carried out to check statistical significance ($p<0,001$)

3.6 Re-localization of *Kr* and *eve* is accelerated in *hb*^{15} mutant

Hb is expressed not only during NB development, but also during early stages of embryogenesis (Lehmann & Nüsslein-Volhard, 1987). *hb* null mutation results in embryos with a disrupted morphology. Therefore, to overcome this situation *hb*^{15} mutants which harbor a gap rescue construct in order to eliminate Hb expression exclusively in the CNS were used, In this mutant the postmitotic neurons U1 and U2 were missing which could at least partially dependent on a shortening of the early competence window in NB 7-1 (Isshiki et al., 2001; Novotny et al., 2002). Since we know that continuous Hb expression resulted in slow down of *Kr* and *eve* locus re-localization, here we examine the possible effect on re-localization in *hb* mutants. We could see that in absence of *hb*, *Kr* locus re-localization was faster in early stage 9/10, as seen in graphical analysis 40% of measured *Kr* alleles in *hb* mutants are already in zone 3 as compared to WT where almost 55% of measured alleles are in zone 1. Also, in stage 11 it could be seen that in WT the position of *Kr* locus is intermediate 12% in zone 1, 48% zone 2 and very few in in zone 3 whereas in *hb* loss of function the locus is in zone 3 similar to stage 12 where in both the cases the *Kr* locus is seen interior of nucleus. This suggests that in absence of *hb* the re-localization of *Kr* is significantly faster and this co-relates with shortened competence window (Fig 14A). Next we analyzed if *eve* locus also tends to move faster and indeed we could see similar effect on *eve* re-localization as 43% of measured alleles were already in zone 1 i.e. peripheral at stage 9/10 as compared to WT where it is still in zone 3. Also for stage 11 the locus persistently stayed in zone 1 in *hb* mutants as 42% of measured alleles are in zone 1 as compared to WT where very few 29% of measured alleles are in zone 1 and most of them are intermediately positioned, no significant change could be seen in stage 12 locus position in both the genotypes (Fig 14B).Taken together, these results demonstrate accelerated *eve* localization at the nuclear envelope when Hb activity is absent. These results confirms our correlation of gene locus re-localization and competence, as continuous Hb extends the window and slows the re-localization of both *Kr* and *eve* loci while absence of *hb* makes window shorter and faster re-localization of both *Kr* and *eve* loci.

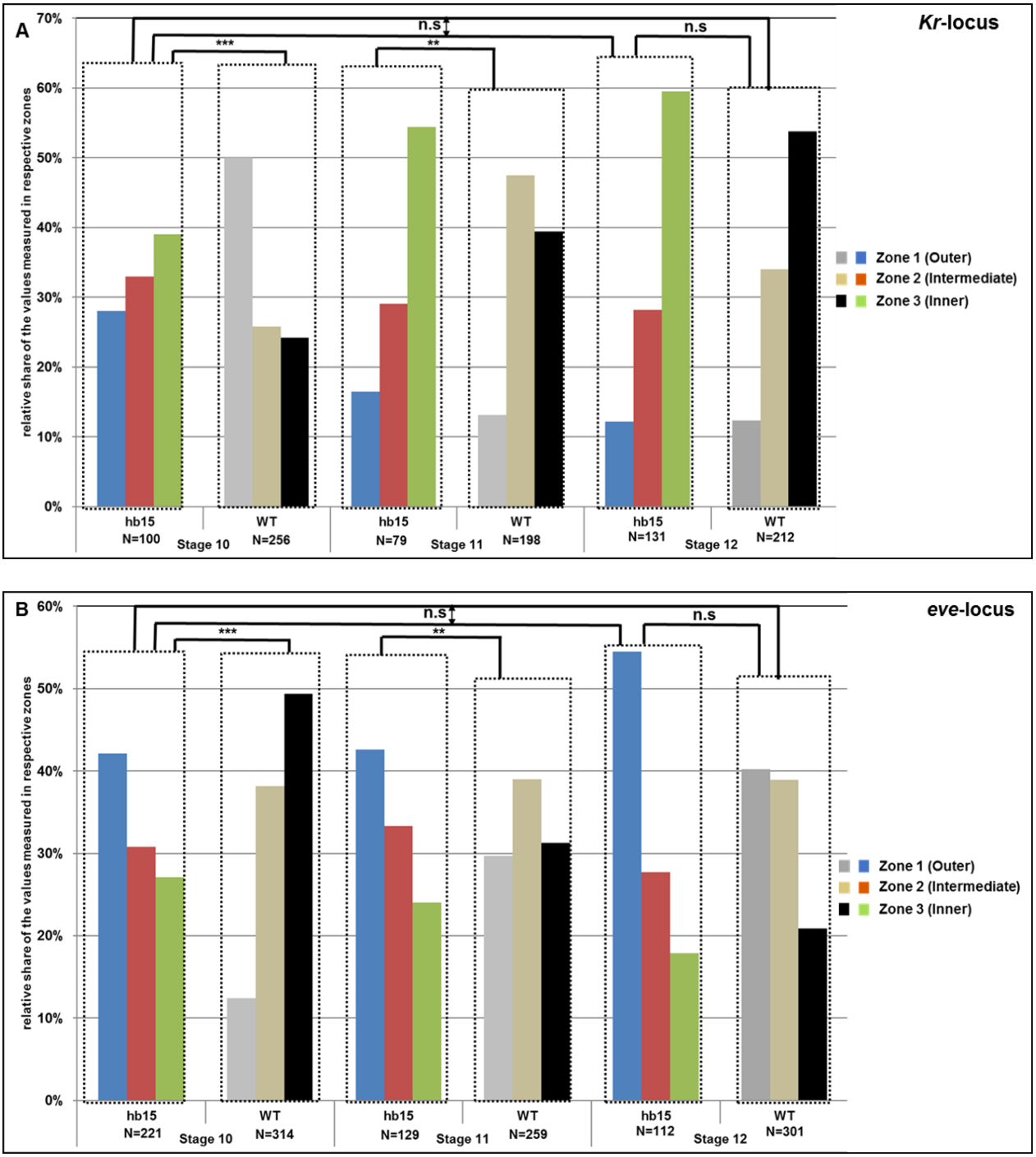
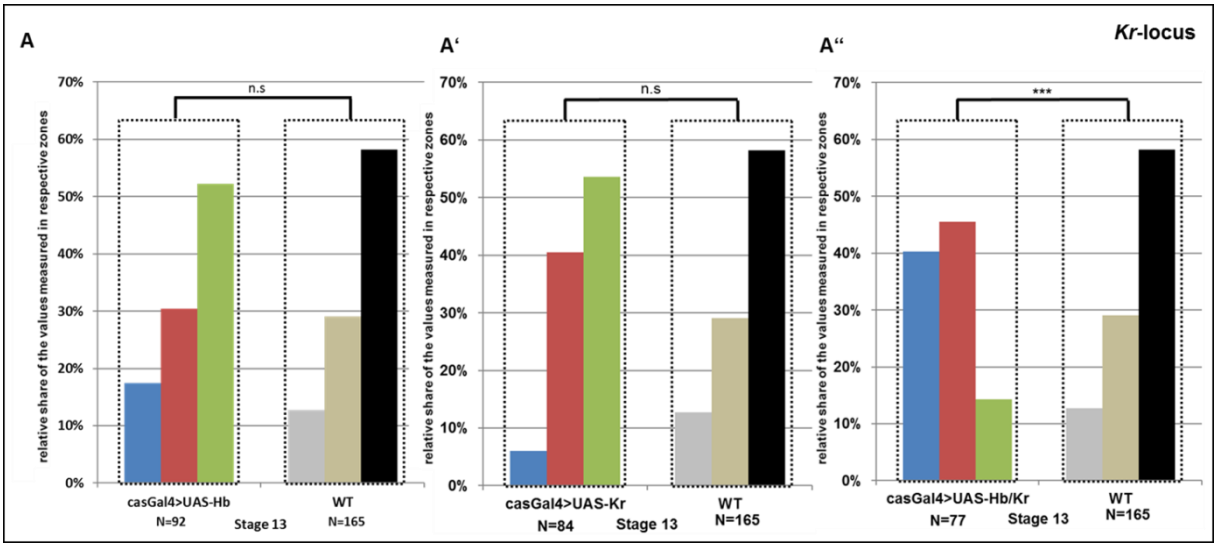


Fig 14 A. Graphical analysis represents comparison of the distribution of *Kr*-locus in *hb*15 mutant compared to WT in zones 1, 2 and 3 in stage 10, 11 and 12. It shows significant precocious re-localization of the *Kr*-locus away from the nuclear periphery at stage 10 and stage 11 into inner nuclear region when Hb activity is lacking. **B.** Graphical analysis represents comparison of the distribution of *ever*-locus in *hb*15 mutant compared to WT in zones 1, 2 and 3 in stage 10, 11 and 12. It shows the accelerated re-localization of the *eve* locus towards the nuclear periphery at stage 10 and stage 11 as compared to WT where locus is interior at these stages and then moves towards

periphery at stage 12. These results confirms our correlation of gene locus re-localization and competence, where absence of *hb* makes window shorter and faster re-localization of both *Kr* and *eve* loci. (N= number of alleles analyzed). Chi2 test was carried out to check statistical significance ($p<0,001$).

3.7 Re-localization of *Kr* and *eve* can be reverted by combined Hb and Kr expression outside of competence window

Since a combined re-expression of Hb and Kr was observed to re-activate *eve*, resulting in ectopic Eve+ postmitotic U-neurons, I speculated whether the nuclear *Kr* and *eve* locus positions do re-localize accordingly. With this experiment we could see that indeed *Kr* locus and *eve* locus re-localization was reverted. At stage 13 *Kr* locus was found towards periphery while in WT it is found interior supporting previous results that endogenous *Kr* is transcriptionally active (Fig 15A'') and *eve* was re-localized to interior of nucleus in all NBs whereas in WT it is found at periphery (Fig 15B''). Interestingly, single re-expression of Hb (casGal4xUAS-Hb) and Kr (casGal4xUAS-Kr) showed no impact on re-localization of *Kr* and *eve* loci (Fig 15A and A'; 15B and B'). These results shows re-localization of *Kr* and *eve* loci can be reversed after combine re-expression of Hb and Kr late and this correlates to our initial observation that co-expression of these factors together re-activates the competence window.



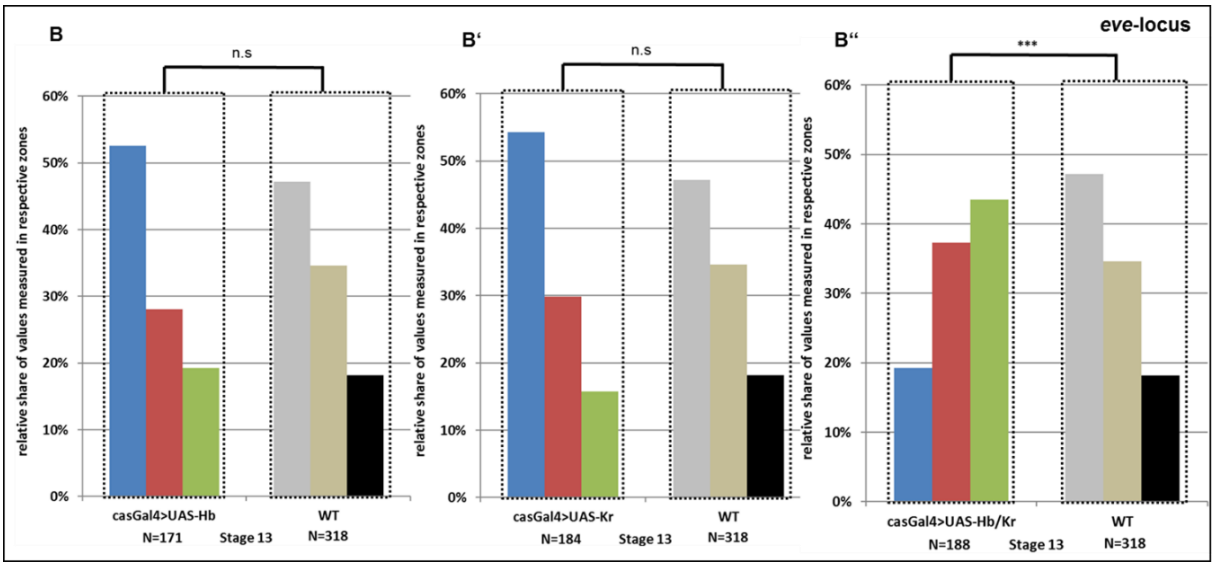
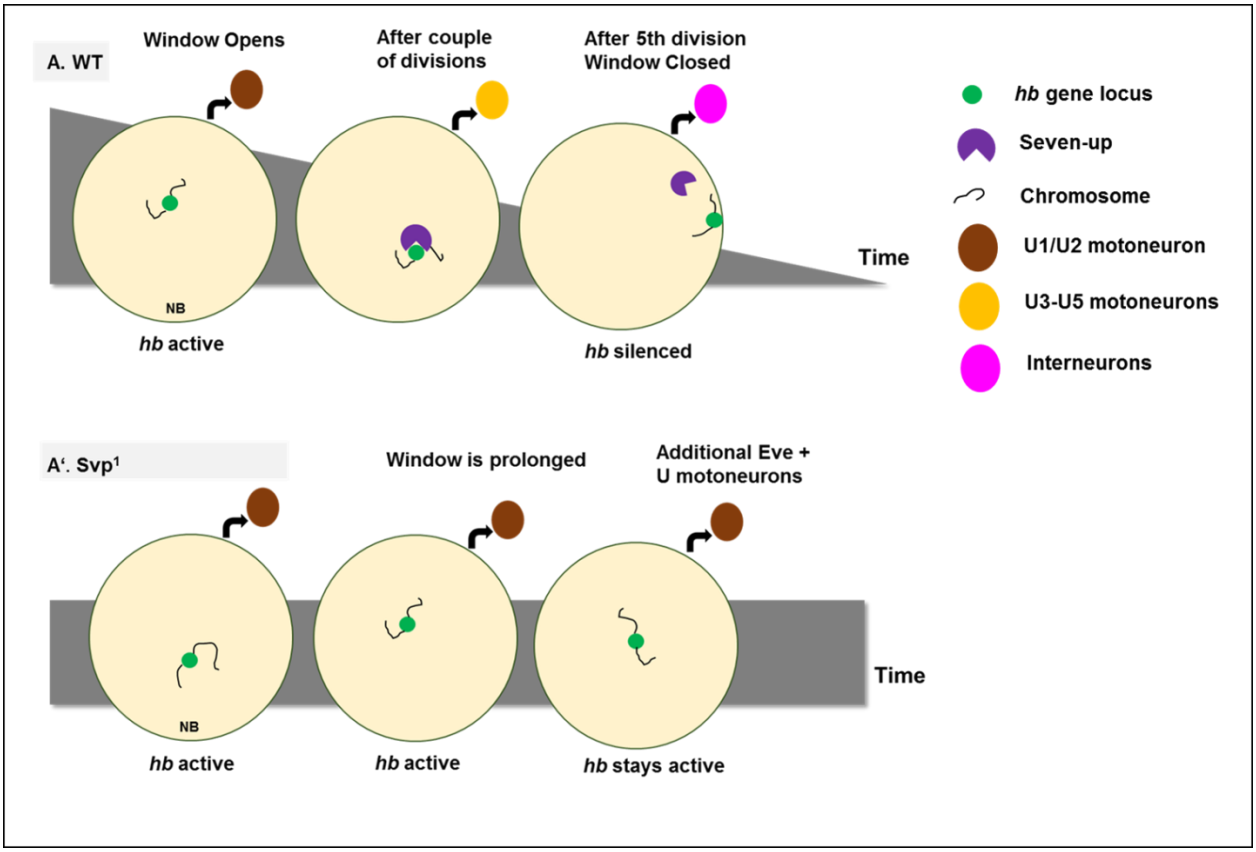


Fig 15 A, A’ and A’’ Graphical analysis represents comparison of the distribution of *Kr*-locus after single overexpression of Hb, Kr and combined Hb/Kr driven by casGal4 compared to WT in zone 1, 2 and 3 in stage 13. It can be seen that there is no change in *Kr*-gene locus re-localization in both situations where single Hb and Kr are ectopically expressed. However, combined ectopic expression of Hb/Kr resulted in reversion of *Kr* locus re-localization towards periphery as 40% of alleles are in zone 1 as compared to WT where locus is already into nuclear interior. Similarly **B, B’ and B’’** Graphical analysis represents comparison of the distribution of eve-locus after single overexpression of Hb, Kr and combined Hb/Kr driven by casGal4 compared to WT in zone 1, 2 and 3 in stage 13. Here we observed similar phenotype as like Kr locus, where combined ectopic expression of Hb/Kr resulted in reversion of *ever* locus re-localization towards nuclear interior at stage 13 as compared to WT where it’s re-localized to periphery. No significant change was seen in single ectopic Hb and Kr expression. (N= number of alleles analyzed). Chi2 test was carried out to check statistical significance ($p<0,001$).

3.8 Re-localization of *hb* is slowed down in Seven up (Svp) loss of function mutant

Previous studies demonstrated that Svp a homolog of mammalian COUP-TF is expressed in a brief pulse in the majority of early embryonic neuroblasts, where it suppress the activity of Hb, thereby allowing for the switch to the next stage of temporal competence. Loss of Svp function increased the number of early born cell types and it was also shown that Hb expression was maintained in many lineages (Kanai et al., 2005; Mettler et al., 2006). Here we analyzed *hb* gene locus re-localization in NBs in *svp* mutants and we see that re-positioning of locus is significantly halted or slowed down. Its position is maintained into interior throughout especially depicted in stage 11 and stage 12 where most of measured

alleles are in zone 3 as compared to WT where it is peripheral in these stages (Fig 16 A, A' and B). These results point us to the direction that Svp might act as an initiator or trigger of silencing process and absence of it results in maintaining activation of *hb* gene locus.



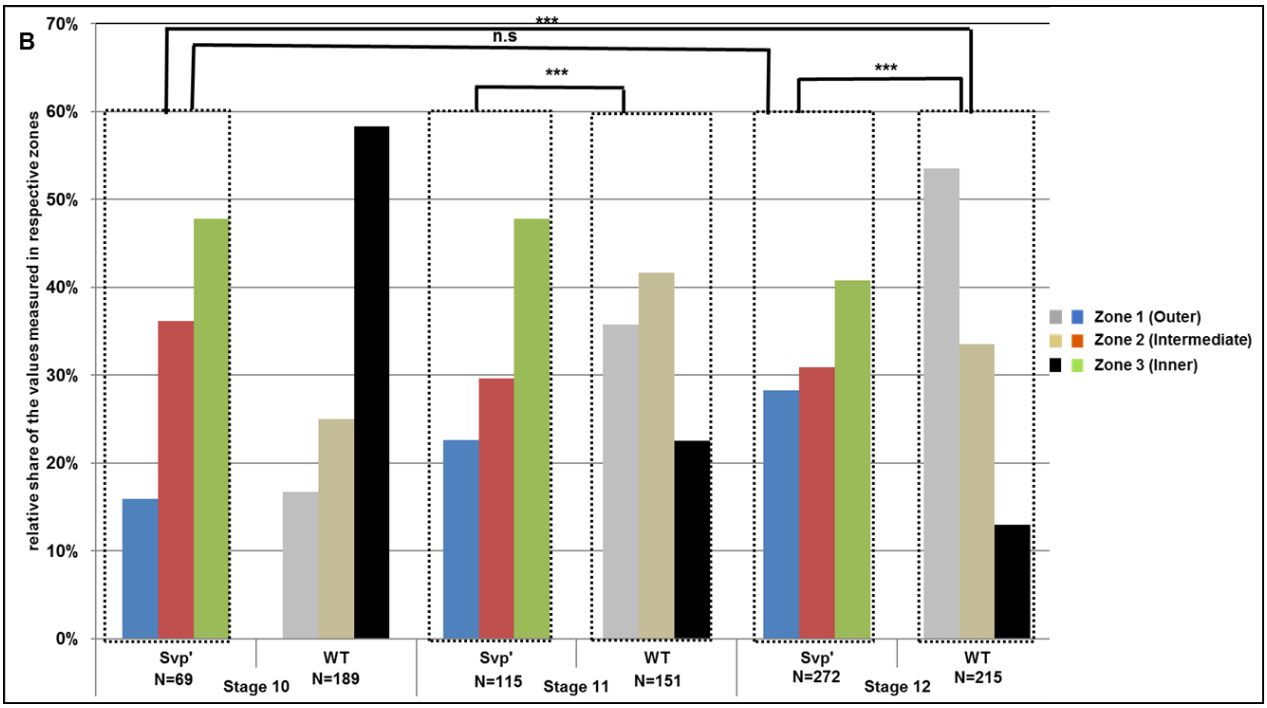


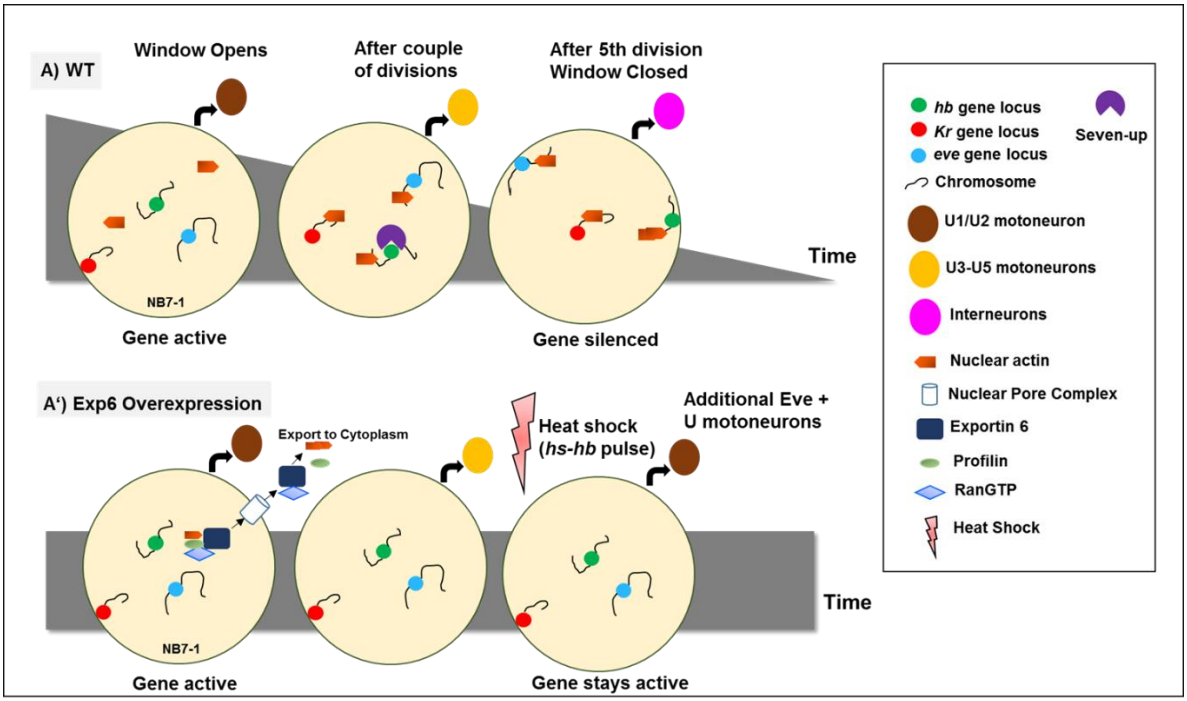
Fig 16 A. Comparison of gene locus re-localization between WT and Svp mutant. In WT, *hb* re-localizes from interior to periphery within the nucleus at end of competence. On the other hand it can be seen that Svp[1] mutants extends competence window by generating additional U motoneurons and maintains *hb* re-localization into nuclear interior i.e. it stays active. **B.** Graphical analysis where comparison of the distribution of *hb* locus within WT and Svp mutants. It can be clearly seen that *hb* re-localization is indeed slowed down in Svp mutants as in stage 12 its found in nuclear interior i.e. zone 3 as compared to WT where its peripheral and silenced. This phenomenon could be because Svp might initiate silencing process, thus acting as initiator of silencing process for *hb* in WT and when absent the silencing is lost and re-localization is halted. (N= number of alleles analyzed). Chi2 test was carried out to check statistical significance (p<0,001).

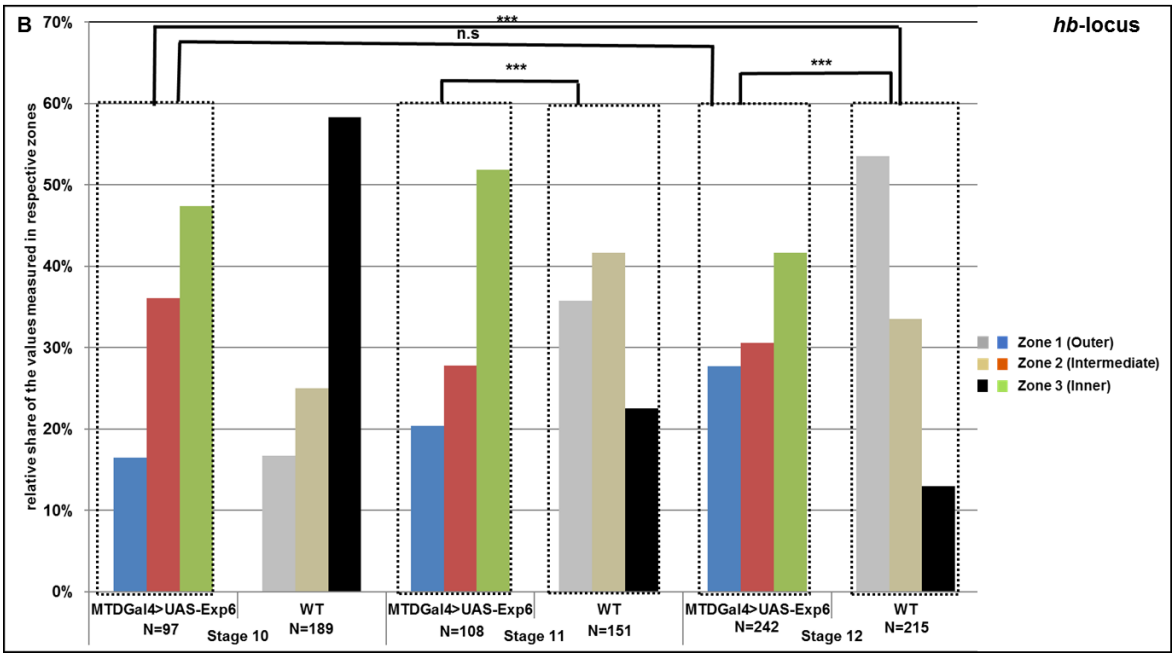
3.9 Re-localization of *hb*, *Kr* and *eve* is slowed down by continuous Exportin 6 expression

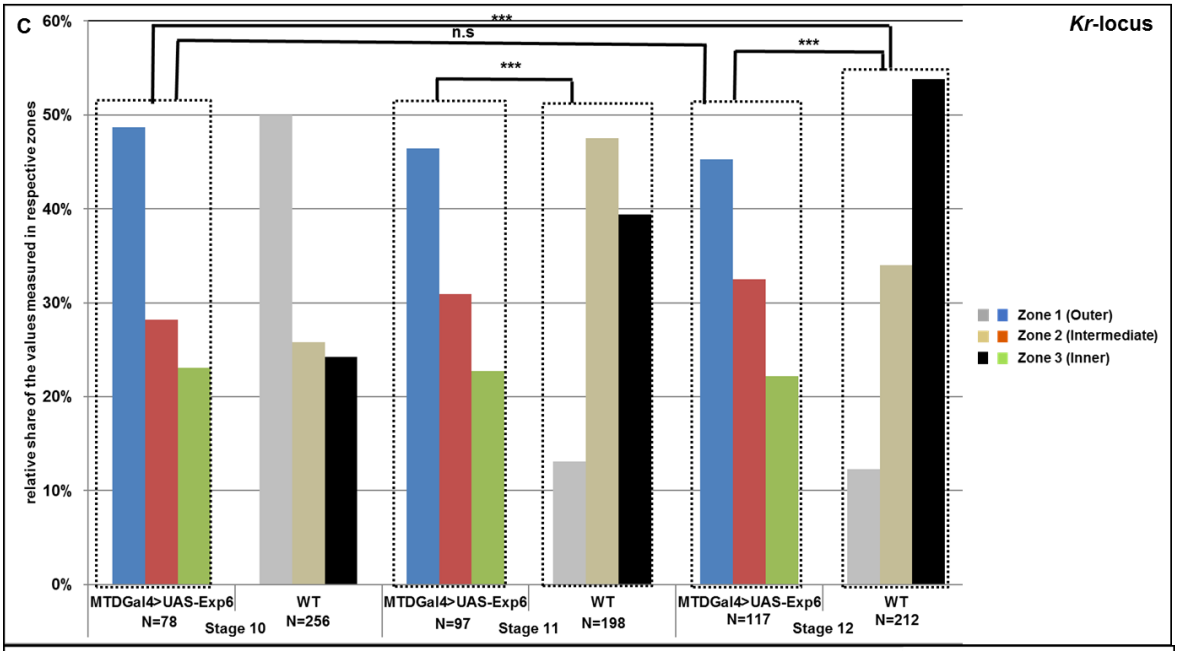
Until now we could show strong correlation between end of competence window accompanied by re-localization of gene loci. However less is known about possible mechanisms behind such chromosomal reorganization within the nucleus. There has been some research showing possible role of nuclear actin in such events. E.g. in vertebrates it has been deduced from overexpression experiments using dominant negative non-oligomerizing NLS-GFP-actin which resulted in deceleration of viral protein VP16 locus re-localizations whereas gain of function NLS-GFP-actin where actin is accumulated in nucleus showed acceleration (Chuang et al., 2007). Transport of such macromolecules like actin between the

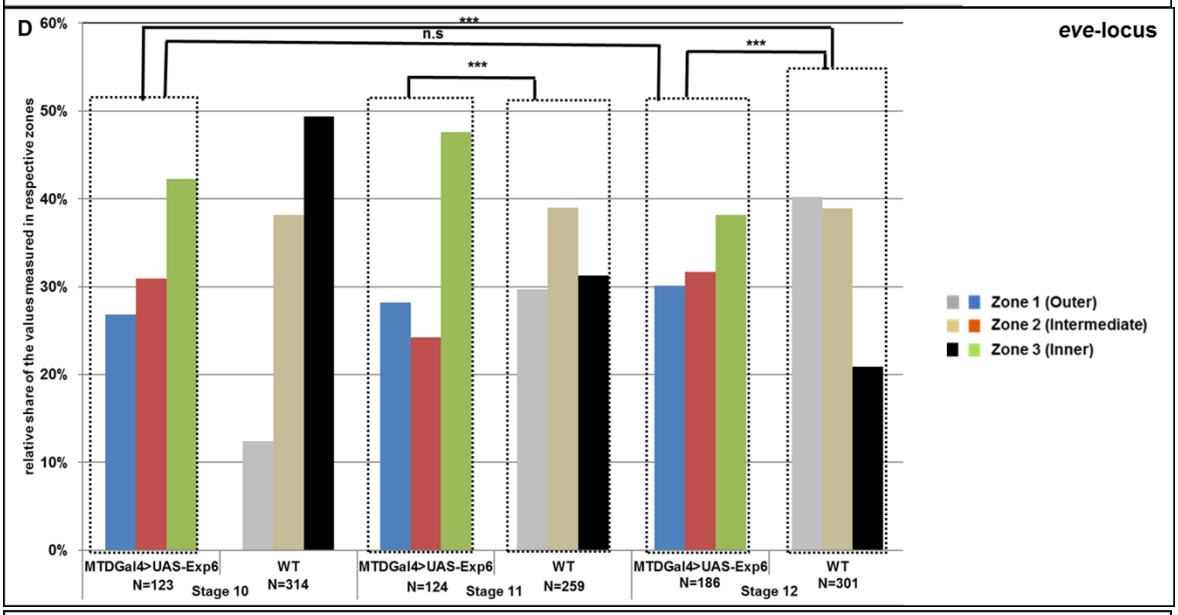
nucleus and the cytoplasm require nuclear pore complexes such as transport receptors. One such essential nuclear export receptor is Exportin 6 (Exp6) or Ellipsoid Body (Ebo) in Drosophila, which mediates nuclear export of profilin actin complex (Stüven et al., 2003). Knocking down Exp6 results in accumulation of actin in the nucleus (Thran et al, 2013; Dopie et al., 2012) in contrast overexpression of *exp6* driven by MTD-Gal4 (MTD-Gal4xUAS-*exp6*) enhanced the nuclear actin export that resulted in significant decrease of nuclear actin molecules. To verify whether this is also true in our hands we analyzed fractionated cells separating nuclear and cytoplasmic content by using western blot analysis (Refer Methods section 2.21). It can be seen that after staining with anti-Actin 2103 (Actin band at 42KD) antibody there is indeed loss of nuclear actin molecules in Exp6 overexpression as compared to WT whereas in Exp6 loss of function actin is accumulated into nucleus. For nuclear fractions Histone3 (H3) protein was used as control which is size of 18KD, and for cytoplasmic fractions Tubulin was used as control size of 55KD. The staining's of control indeed proved the purity of fractionation. Quantification was done by graphical analysis where relative quantification of nuclear actin fraction was carried out using Image J software (Fig 17E). In this graphical analysis it can be seen that in Exp6 overexpression the relative fraction of nuclear actin is 10% compared to WT. In loss of Exp6 mutant this value is 160% compared to WT (Fig 17E'). The MTD-Gal4 Flystock contains a vector employing short hairpin RNAs (shRNAs), which when expressed in the female germline during oogenesis; effectively knock down the maternal transcript of a certain gene. With this method it's possible to reduce the transcription of a target gene already in the oocyte, leading in a stronger knockdown and thereby in a stronger phenotype. In this experiment our goal was to achieve stronger expression of *exp6* right from oogenesis therefore we opted MTD-Gal4 as a driver line for our analysis. To investigate whether the manipulation of nuclear actin concentration affects the observed gene locus re-localizations we analyzed the positions of *hb, Kr and eve* loci after overexpression of *exp6*. The goal of experiment was to check whether reduced actin levels in nucleus could result in deceleration or slowdown of movement for observed gene loci. Indeed it was observed that repositioning of *hb, Kr and eve* loci were significantly halted or slowed down. *hb* and *eve* were found within the interior of nuclei at stages 11 and 12 when Exp6 is overexpressed as compared to control WT where both the loci *hb* and *eve* have more peripheral positions. No difference in re-localization was seen in stage 10 NB's (Fig 17B and D). In case of *Kr* gene locus at stages 11 and 12 it was found more peripheral as compared to WT control (Fig 17C). Together these results hints at that nuclear actin might play a role in

chromosomal reorganization within the nucleus where decrease in concentration of nuclear actin resulted in deceleration of movement for all three loci.






C
Kr-locus
relative share of the values measured in respective zones
60%
50%
40%
30%
20%
10%
0%
***
n.s
***
***
MTDGal4>UAS-Exp6
N=78
Stage 10
WT
N=256
MTDGal4>UAS-Exp6
N=97
Stage 11
WT
N=198
MTDGal4>UAS-Exp6
N=117
Stage 12
WT
N=212
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)


D
eve-locus
relative share of the values measured in respective zones
60%
50%
40%
30%
20%
10%
0%
***
n.s
***
***
MTDGal4>UAS-Exp6
N=123
Stage 10
WT
N=314
MTDGal4>UAS-Exp6
N=124
Stage 11
WT
N=259
MTDGal4>UAS-Exp6
N=186
Stage 12
WT
N=301
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)

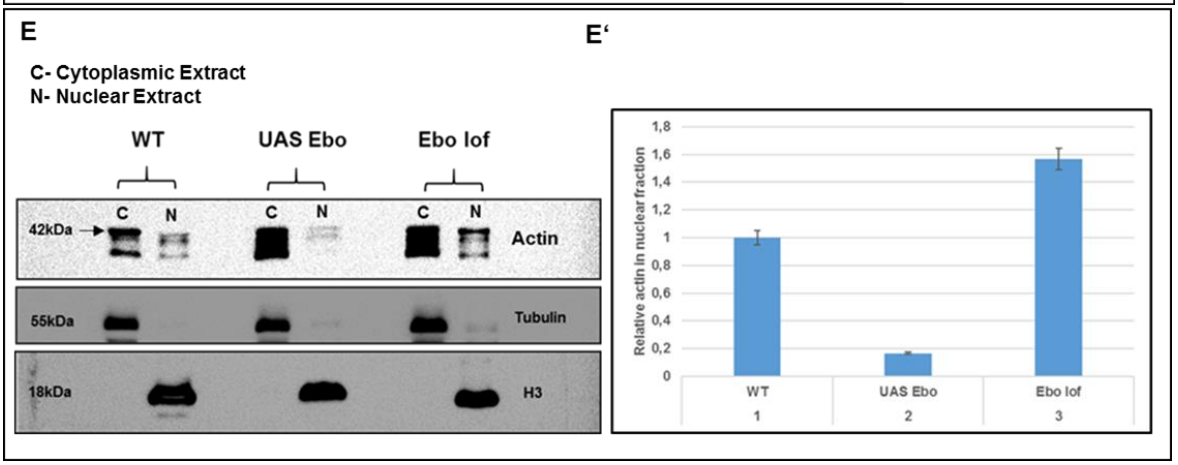
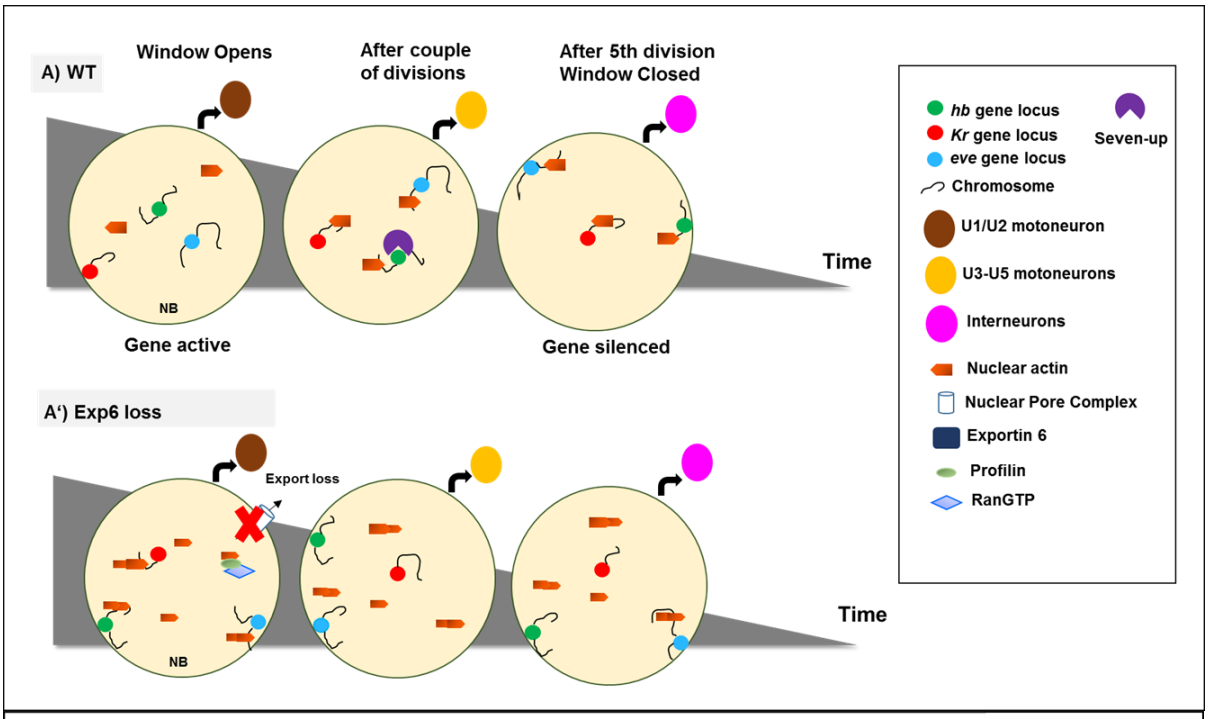
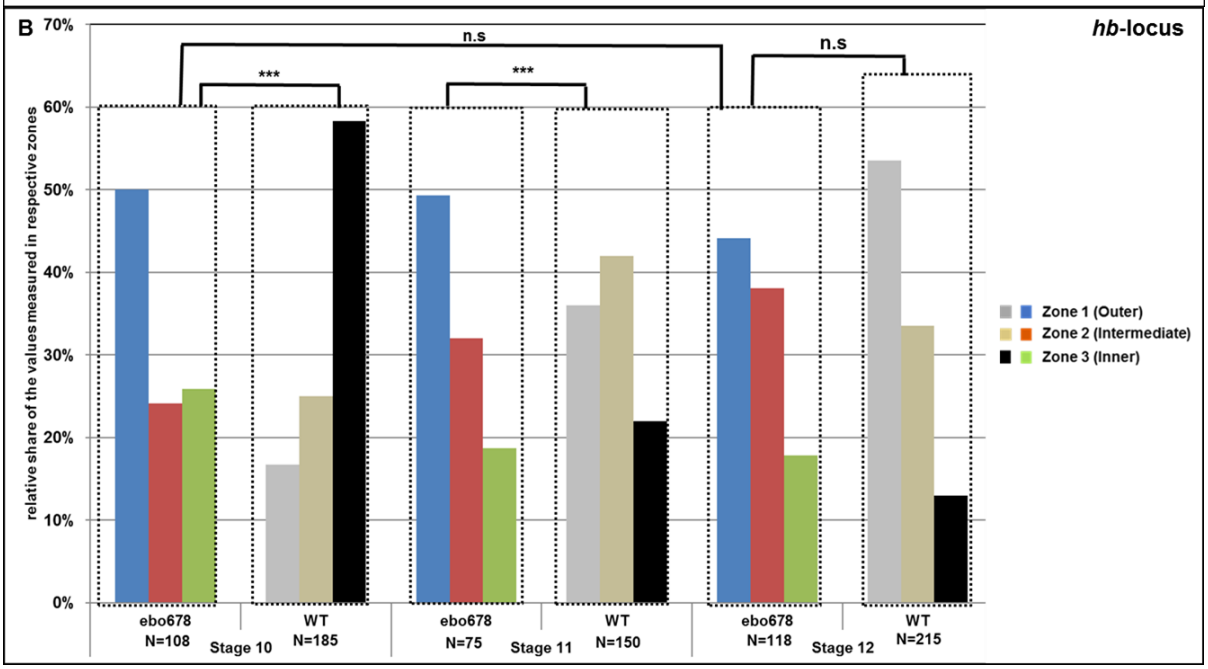

E
C- Cytoplasmic Extract
N- Nuclear Extract
WT
UAS Ebo
Ebo lof
C
N
42kDa
Actin
55kDa
Tubulin
18kDa
H3
E‘
Relative actin in nuclear fraction
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
WT
1
UAS Ebo
2
Ebo lof
3

Fig 17 A. Comparison of *hb*, *Kr* and *eve* gene loci re-localization between WT and Exp6 overexpression. In WT, *hb* (Green spot) and *eve* (Cyan spot) re-localizes from interior to periphery whereas *Kr* (Red spot) re-localizes opposite i.e. from periphery to nuclear interior within the nucleus at end of competence. On the other hand it can be seen that Exp6 overexpression maintains *hb* and *eve* position into nuclear interior whereas *Kr* position is also maintained to periphery throughout until stage 12 i.e. all genes stays active. This halting of loci re-localization might be due to the fact that overexpression of Exp6 results in pushing most of nuclear actin molecules out of nucleus resulting loss of movement. **B, C and D** Graphical analysis where comparison of the distribution of *hb*, *Kr* and *eve* locus within WT and Exp6 Overexpression. It can be clearly seen that re-localization for all three loci is indeed significantly slowed down in overexpression of Exp6 as in stage 11 and stage 12 *hb* and *eve* are found in nuclear interior i.e. zone 3 as compared to WT where its peripheral and silenced whereas *Kr* is found at periphery as compared to WT where it is already into nuclear interior. (N= number of alleles analyzed). Chi2 test was carried out to check statistical significance ($p<0,001$). **E and E'** Western blot analysis and quantification of nuclear actin fraction in WT, Overexpression of Exp6 and Exp6 loss of function. It can be seen that there is less nuclear actin molecules in Exp6 overexpression and accumulation of them in Exp6 loss as compared to WT. This quantification was done using two biological replicates.

3.10 Re-localization of *hb*, *Kr* and *eve* is accelerated in ellipsoid body mutant (*ebo*^{678})

Here we extend the hypothesis of an actin dependent locus re-localization in *ebo*^{678} mutant NBs, where significant increase in nuclear actin concentration was observed (Fig 17E and E'). The goal of this experiment was to check if nuclear actin accumulation results in acceleration or faster gene locus re-localization, since our previous data showed that less nuclear actin results slower re-positioning. As expected there was significant acceleration in re-localization for *hb*, *Kr* and *eve* loci observed within all NBs right from early embryonic stage 9/10. *hb* and *eve* are precociously seen more peripheral at stage9/10 in Ebo loss of function mutant as compared to WT where their position is within the nuclear interior. No difference was observed in re-localization at stage 12 (Fig 18 B and D). Similarly, *Kr* gene locus was already found within the nuclear interior at stage 9/10 in Ebo loss of function mutant in contrast to WT where it is found more at the peripheral region Again no significant difference was observed at stage 12 in both the genotypes (Fig 18C). Together these results show that loss of Ebo function causing high nuclear actin concentration leads to accelerated movements for *hb*, *eve* and *Kr* loci and strongly supports the idea that nuclear actin is involved in such gene locus re-localization events.


A) WT
Window Opens
After couple of divisions
After 5th division Window Closed
NB
Gene active
Gene silenced
Time
A') Exp6 loss
Export loss
NB
Time
hb gene locus
Kr gene locus
eve gene locus
Chromosome
Seven-up
U1/U2 motoneuron
U3-U5 motoneurons
Interneurons
Nuclear actin
Nuclear Pore Complex
Exportin 6
Profilin
RanGTP


B
hb-locus
relative share of the values measured in respective zones
70%
60%
50%
40%
30%
20%
10%
0%
n.s
***
***
n.s
ebo678 N=108
WT N=185
Stage 10
ebo678 N=75
WT N=150
Stage 11
ebo678 N=118
WT N=215
Stage 12
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)

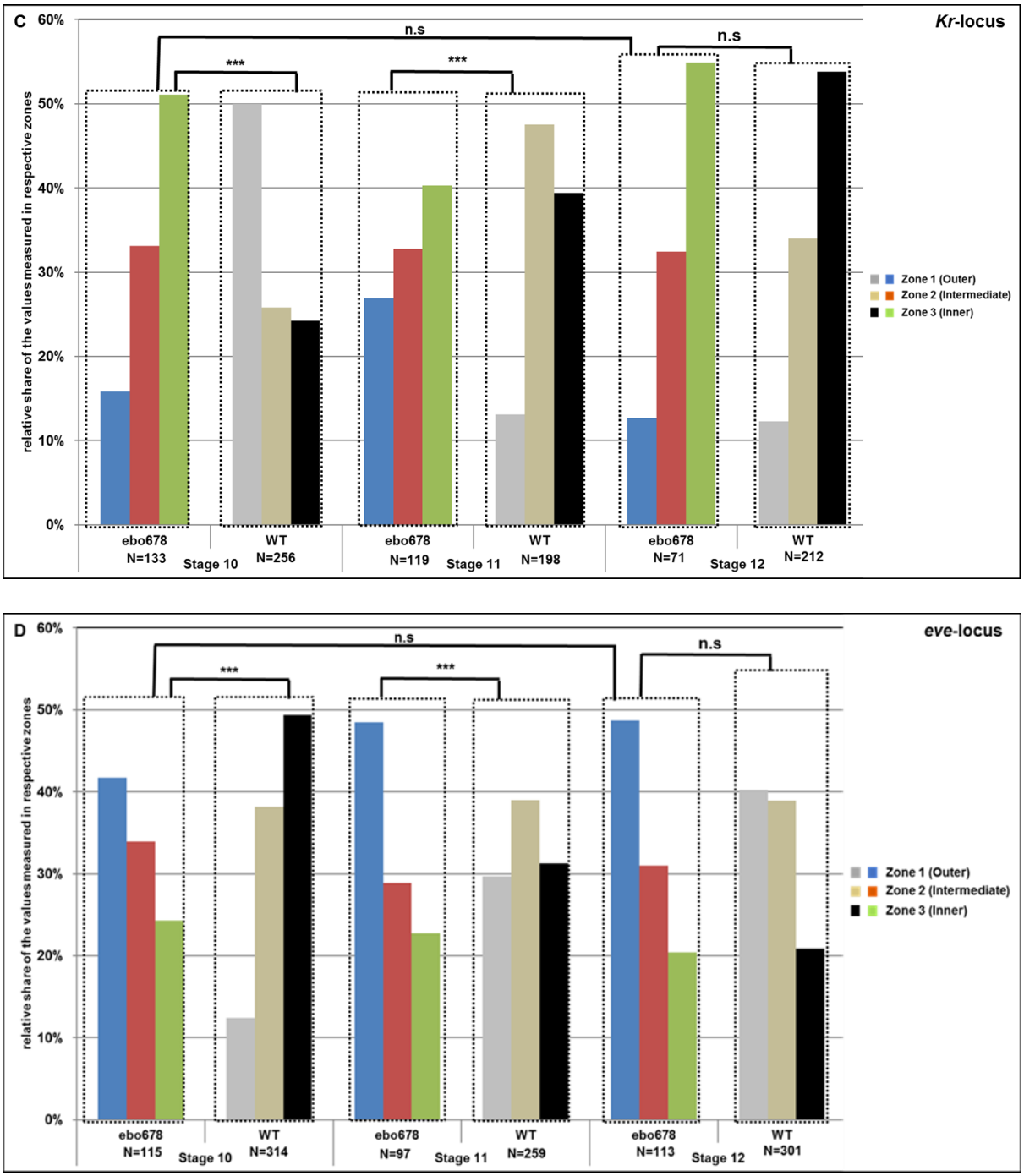
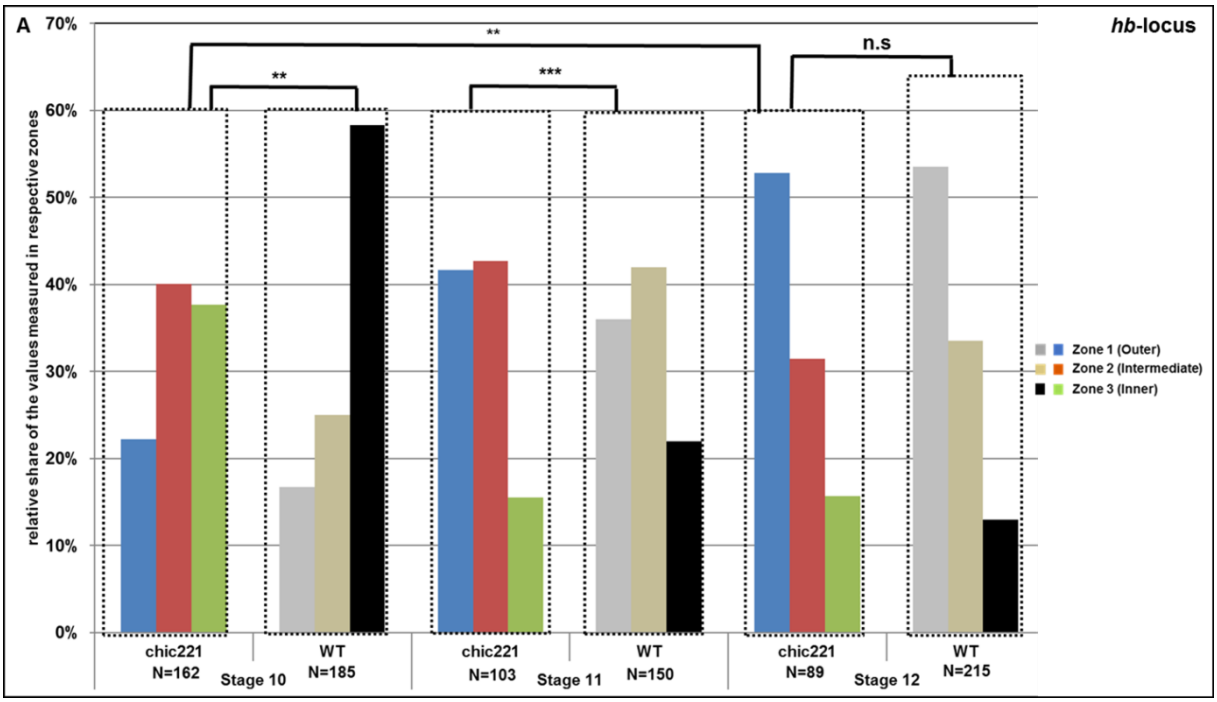


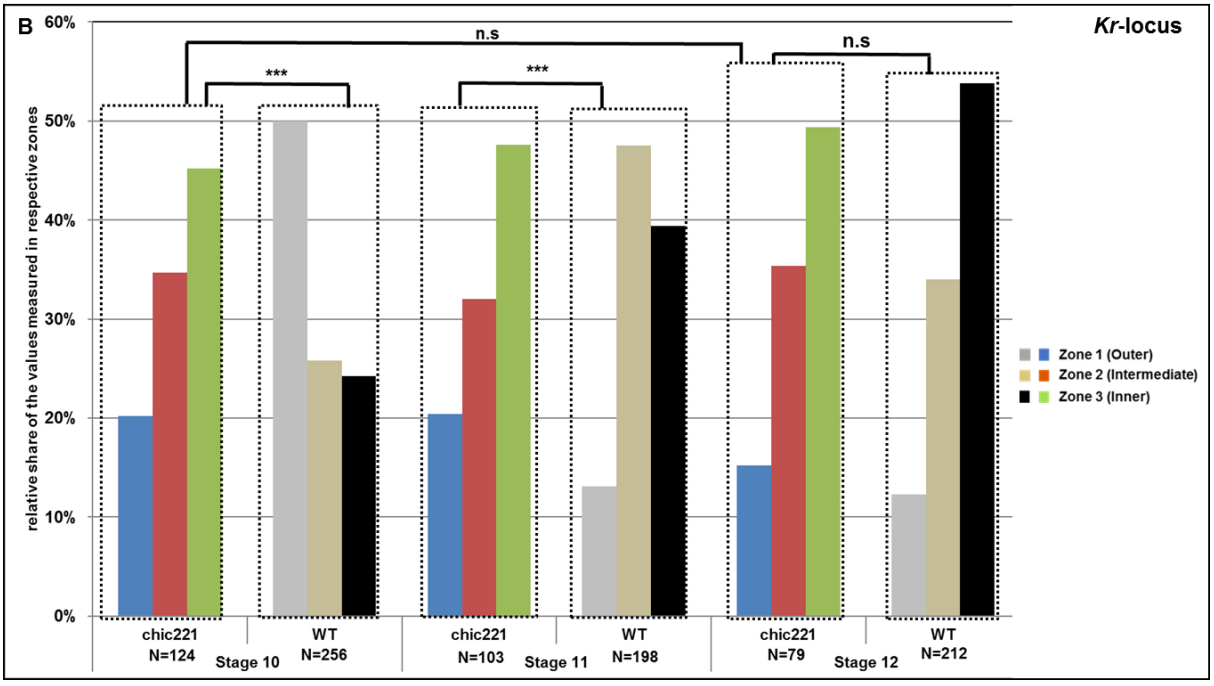
Fig 18 A. Comparison of *hb*, *Kr* and *eve* gene loci re-localization between WT and Exp6 loss of function. In WT, *hb* (Green spot) and *eve* (Cyan spot) re-localizes from interior to periphery whereas *Kr* (Red spot) re-localizes opposite i.e. from periphery to nuclear interior within the nucleus at end of competence. On the other hand it can be seen that Exp6 loss of function accelerates *hb* and *eve* position to nuclear periphery whereas *Kr* position is also accelerated to nuclear interior right from stage 9/10 i.e. all genes are precociously targeted to their silence zones right from beginning of competence. This acceleration of loci re-localization might be due to the fact that Exp6 loss of function results in accumulation of nuclear actin molecules into nucleus resulting faster movement. **B, C and D** Graphical analysis where comparison of the distribution of *hb*, *Kr* and *eve* locus within WT and Exp6 loss of function. It can be clearly seen that re-localization for all three loci is indeed significantly faster

in Exp6 loss of function as in stage 10 and stage 11 *hb* and *eve* are found towards periphery i.e. zone 1 as compared to WT where its nuclear interior or intermediate and active whereas *Kr* is found at nuclear interior as compared to WT where it is initially at periphery and active and then moves to interior at stage 12 to get silenced. (N= number of alleles analyzed). Chi2 test was carried out to check statistical significance ($p<0,001$).

3.11 Re-localization of *hb*, *Kr* and *eve* is accelerated in Chickadee (Profilin) mutant

The *Drosophila* homologue of Profilin, Chickadee (Chic) is a small actin binding protein that binds to actin monomers to form profiling-actin dimer and regulates filament polymerization within the cytoplasm (Stossel et al., 1985). In the nucleus the *profilin-actin* complex is exported from the nucleus into the cytoplasm by Exportin 6 (Stüven et al., 2003; Dopie et al., 2012). Thus, decreased Profilin levels in the NB should result in the accumulation of actin in the nucleus, again in an accelerated locus repositioning. To test this we analyzed all three gene loci (*hb, Kr* and *eve*) in *chic221* mutant background. For the *hb* locus I found accelerated re-localization towards the periphery of the NB nucleus. Almost 22% of the measured alleles in the early stages 9 and 10 were already located in zone 1, the outer region of the nucleus and less than 40% of all alleles were found in the most inner zone as compared to WT where 59% of loci are in Zone 3 and 18% are in zone 1(Fig.19A). No difference was observed for *hb* locus in stage 12 in WT and mutant embryos. Similarly, *eve* locus re-localization was also observed to be significantly faster. E.g. at early stage 9/10 already 42% of measured alleles were found in zone 1 (WT: 12%). No significant difference was seen in stage 12 measurements in both the genotypes (Fig 19C). Likewise, in early stage 9/10 embryos *Kr* gene locus was found more often in the inner nuclear zone in *chic* mutant embryos as compared to WT (51% vs 23%). This is complementary to the position in outer zone 1 where it is located less often in chic mutants as compared to WT (16% vs 50%) (Fig 19B). No difference was observed in stage 12 embryos for both the genotypes. Together these results provide us another hint that accumulation of actin in nucleus might result into acceleration of gene locus re-localization.


A
hb-locus
relative share of the values measured in respective zones
70%
60%
50%
40%
30%
20%
10%
0%
**
**
***
n.s
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)
chic221 N=162
WT N=185
Stage 10
chic221 N=103
WT N=150
Stage 11
chic221 N=89
WT N=215
Stage 12


B
Kr-locus
relative share of the values measured in respective zones
60%
50%
40%
30%
20%
10%
0%
***
***
n.s
n.s
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)
chic221 N=124
WT N=256
Stage 10
chic221 N=103
WT N=198
Stage 11
chic221 N=79
WT N=212
Stage 12

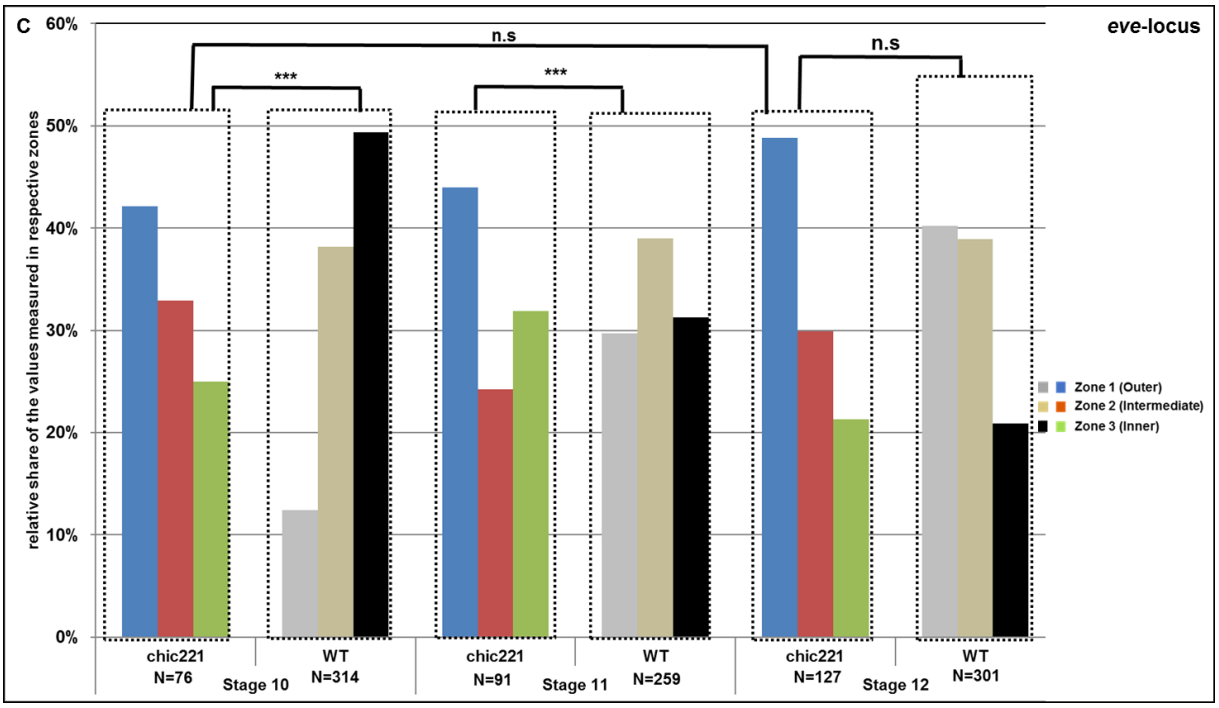


Fig 19 A. Graphical analysis represents comparison of the distribution of *hb*-locus in *chikadee* mutant compared to WT in zones 1, 2 and 3 in stage 10, 11 and 12. It shows significant precocious re-localization of the *hb*-locus towards the nuclear periphery at stage 10 and stage 11 in absence of Chikadee. **B.** Graphical analysis represents comparison of the distribution of *Kr*-locus in *chci221* mutant compared to WT in zones 1, 2 and 3 in stage 10, 11 and 12. It shows the accelerated re-localization of the *Kr* locus towards the nuclear interior at stage 10 and stage 11 as compared to WT where locus is peripheral at these stages and then moves towards interior at stage 12. **C.** Graphical analysis represents comparison of the distribution of *eve*-locus in *chic221* mutant compared to WT in zones 1, 2 and 3 in stage 10, 11 and 12. It shows significant precocious re-localization of the *eve*-locus towards the nuclear periphery at stage 10 and stage 11 in absence of Chikadee. These results confirm our previous hypothesis that accumulation of actin in nucleus might result faster re-localization of gene loci since loss of *chikadee* might result in poor nuclear actin transport into cytoplasm. (N= number of alleles analyzed). Chi2 test was carried out to check statistical significance (*** p<0,001 and ** p<0.01).

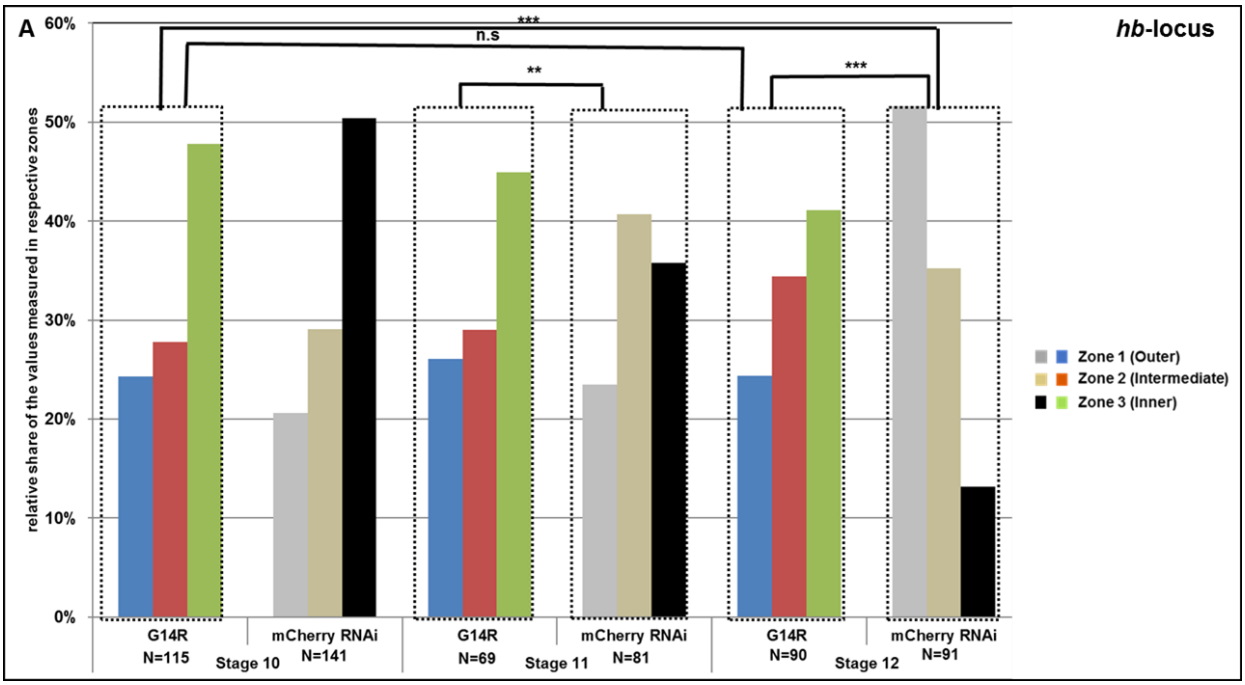
3.13 Re-localization kinetics of all investigated loci depend on oligomerizing actin

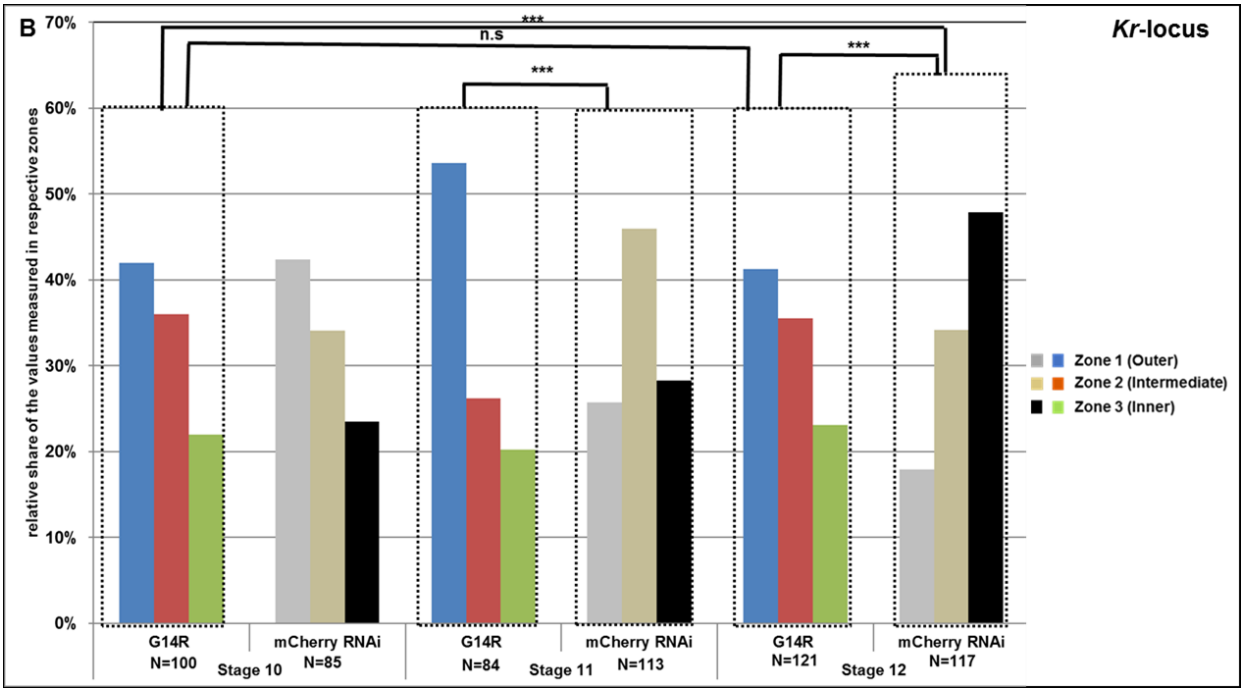
In the former chapters, we have shown that modifying actin concentration in the nucleus by manipulating the nuclear export pathway for nuclear actin affected the re-localization of the *hb*, *Kr,* and *eve*-loci during development. In order to test whether this role of nuclear actin depends on its oligomerization, we generated flies, which have a dominant-negative (DN) or a gain of function (GoF) form of ß-actin under the control of a Gal4-dependent UAS-sequence. Due to the strong conservation of ß-actin, I exchanged the glycine on position 14 by an

arginine to create a dominant-negative mutation of *Drosophila* actin5C (G14R). Likewise, for the gain of function version of actin 5C the amino acid serine in position 15 was changed into cysteine (S15C) causing stabilization of ß-actin oligomerization (similar to vertebrate system as mentioned in Result section 3.9). Both actin mutants were additionally fused with GFP and a nuclear localization sequence (NLS). After the establishment of the transgenic flies, we tested for changes in the re-localization behavior of the three gene loci after overexpressing these mutant versions of actin 5C.

3.13.1 *hb*, *Kr* and *eve* re-localization is slowed down in non-oligomerizing nuclear actin mutant G14R

As mentioned above we generated nuclear expressing non-oligomerizing actin mutant tagged with GFP (UAS-NLS-GFP-G14Ractin5C) and analyzed its effect on gene locus re-localization. Therefore we compared NLS-GFP-G14Ractin5C overexpression driven by MTD-Gal4 (referred as G14R hereafter) with mCherry-RNAi driven by MTD-Gal4 referred as a control. At stage 12 more than 40 % of measured *hb* alleles are still found within the interior of NB nuclei (zone 3) in G14R overexpression as compared to control NBs (13% in zone 3) and only 24% is found in the peripheral zone as compared to 53% in the control. No significant difference was seen when comparing the locus distribution between stage 10 and stage 12 in NBs of G14R embryos (Fig 20A). In case of *eve* locus re-localization significant change was observed in position where 45% percent of alleles were in zone 3 and only 18% are in zone 1 in G14R NBs at stage 12 as compared to control where only 20% of measured alleles are in zone 3 and 46% are in zone 1 i.e. peripheral, again no change was observed in stage 10 in both the cases (Fig 20C). Also, it was seen that re-localization of *Kr* locus was indeed slowed down at stage 12 where 42% of measured alleles are at zone 1 (peripheral) and fewer i.e. 25% are in zone 3 in G14R NBs as compared to control where only 18% of alleles are in zone 1 and almost 50% are in zone 3, no change was seen in stage 10 (Fig 20B). Together we can conclude from these results that non-oligomerizing of actin results in a slowdown of gene locus re-localization.


A
hb-locus
relative share of the values measured in respective zones
60%
50%
40%
30%
20%
10%
0%
***
n.s
**
***
G14R N=115
mCherry RNAi N=141
Stage 10
G14R N=69
mCherry RNAi N=81
Stage 11
G14R N=90
mCherry RNAi N=91
Stage 12
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)


B
Kr-locus
relative share of the values measured in respective zones
70%
60%
50%
40%
30%
20%
10%
0%
***
n.s
***
***
G14R N=100
mCherry RNAi N=85
Stage 10
G14R N=84
mCherry RNAi N=113
Stage 11
G14R N=121
mCherry RNAi N=117
Stage 12
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)

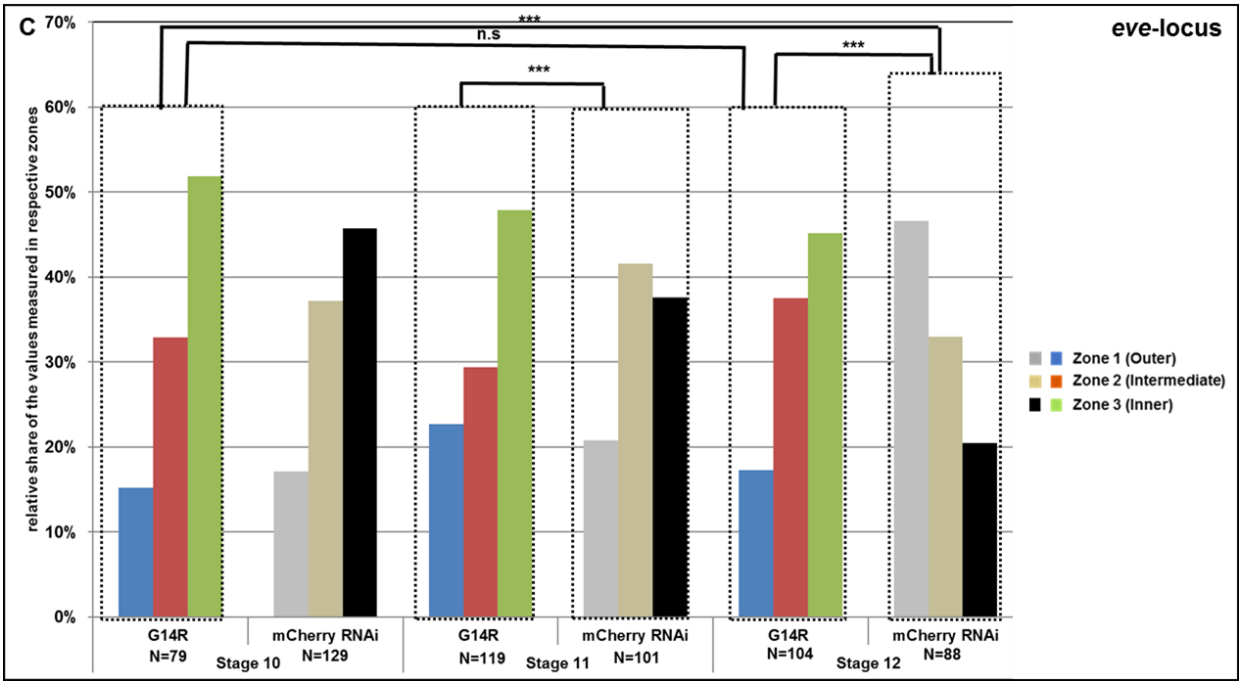
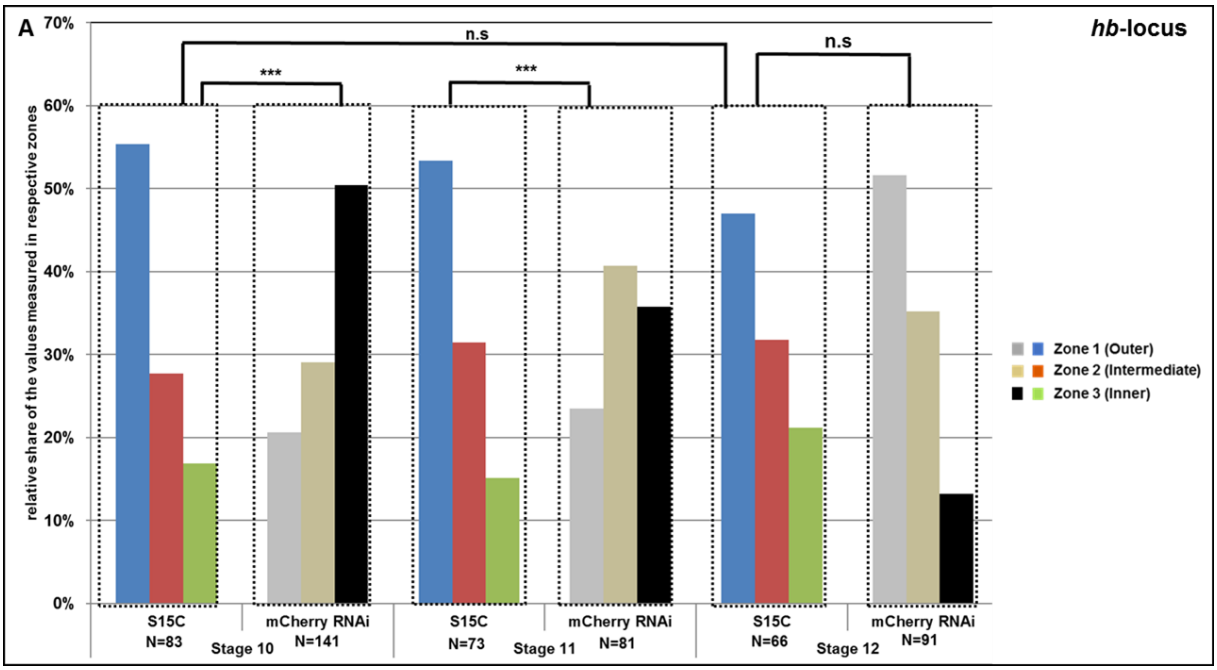


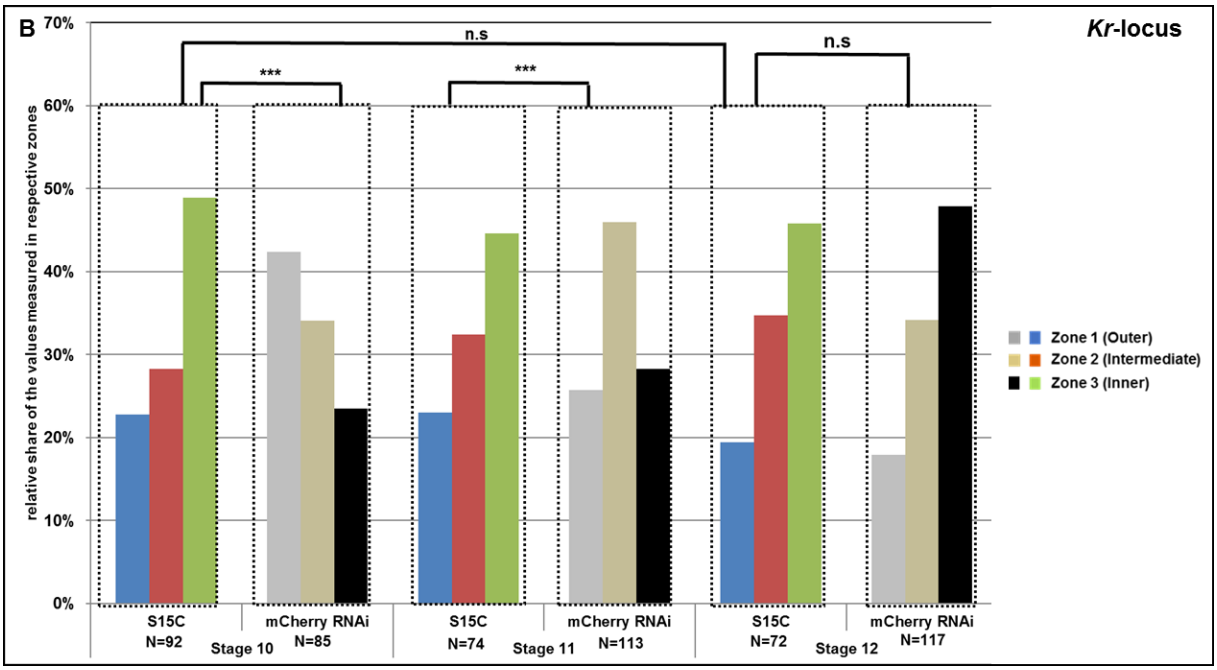
Fig 20 A, B and C Graphical analysis showing comparison of the distribution of *hb*-locus, *Kr*-locus and *eve*-locus after MTDGal4-driven overexpression of G14R actin mutant with nuclear localization signal (NLS) and fused to GFP to control mCherry RNAi driven by MTD-Gal4 in different zones 1, 2 and 3 in stage 10, stage 11 and stage 12. It can be clearly seen that re-localization for all three loci is indeed significantly slowed down in overexpression of G14R where oligomerization of nuclear actin is lost as in stage 11 and stage 12 *hb* and *eve* are found in nuclear interior i.e. zone 3 as compared to control where its peripheral and silenced whereas *Kr* is found at periphery as compared to control where it is already into nuclear interior. (N= number of alleles analyzed). The chi2 test was carried out to check statistical significance ($p<0,001$). The chi2 test was carried out to check significance ($p<0,001$).

3.13.2 *hb*, *Kr* and *eve* re-localization is accelerated in strongly oligomerizing nuclear actin mutant S15C

Next, we tested the possible effect of the UAS-NLS-GFP-S15C construct which enabled nuclear overexpression of a strongly oligomerizing actin version on gene locus re-localization. Indeed, complementary to the former experiments the re-localization of all three loci (*hb*, *Kr* and *eve*) was significantly faster as compared to control. In case of *hb* locus, 55% of a measured allele in stage 9/10 embryos of MTD-Gal4xUAS-NLS-GFP-S15C was found within periphery strongly oligomerizing actin mutant on gene locus re-localization and we could see that indeed the movement for all three loci *hb*, *Kr* and *eve* was significantly faster as compared to control. In case of *hb* locus 55% of a measured allele in stage 9/10 embryos of MTD-Gal4xUAS-NLS-GFP-S15C was found towards periphery zone 1 and 14% in zone 3 as compared to control mCherry RNAi where 20% are in zone 1 whereas 51% are in zone 3.

Also at stage 11, there was a significant change in locus re-positioning as 52% cases are at periphery whereas in case of control 38% of measured alleles are in zone 1. No change was noted in stage 12 re-localization in both the genotypes (Fig 21A). Similarly, for *eve* locus the movement was significantly faster as 50% of measured alleles in stage 9/10 were already in zone 1 in S15C NBs as compared to control where most of the cases it is still in zone 3 or interior of the nucleus and very few in are in zone 1. Also, in stage 11 it was evident that locus has already shifted close to the periphery in S15C as 50% of measured alleles are in zone 1 as compared to WT where most of the cases they are intermediately localized i.e. zone 2 and very few are in zone 1. No significant difference was observed in stage 12 in both the genotypes (Fig 21C). In case of *Kr* locus again it was seen that in case of S15C NBs close to 50% measured alleles in early stage 9/10 are already in the interior of nucleus i.e. zone 3 as compared to control where only 22% of measured alleles are in zone 3. Additionally, in stage 11 statistically significant differences can be seen as 45% measured *Kr* alleles are already in zone 1 in case of S15C mutant as compared to control where they are mostly in zone 2 or intermediate, no difference was seen re-localization of locus between S15C and control NBs (Fig 21B). Together, from this we conclude that expression and nuclear targeting of the F-actin stabilizing actin construct causes acceleration in *hb*, *Kr* and *eve* loci suggesting that nuclear actin could be one of the important factor involved in mechanism behind such re-localization events and strong oligomerization of nuclear actin indeed accelerated locus movement which was also shown in vertebrate system (Chuang at al., 2006).


A
hb-locus
relative share of the values measured in respective zones
70%
60%
50%
40%
30%
20%
10%
0%
n.s
n.s
***
***
S15C N=83
mCherry RNAi N=141
Stage 10
S15C N=73
mCherry RNAi N=81
Stage 11
S15C N=66
mCherry RNAi N=91
Stage 12
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)


B
Kr-locus
relative share of the values measured in respective zones
70%
60%
50%
40%
30%
20%
10%
0%
n.s
n.s
***
***
S15C N=92
mCherry RNAi N=85
Stage 10
S15C N=74
mCherry RNAi N=113
Stage 11
S15C N=72
mCherry RNAi N=117
Stage 12
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)

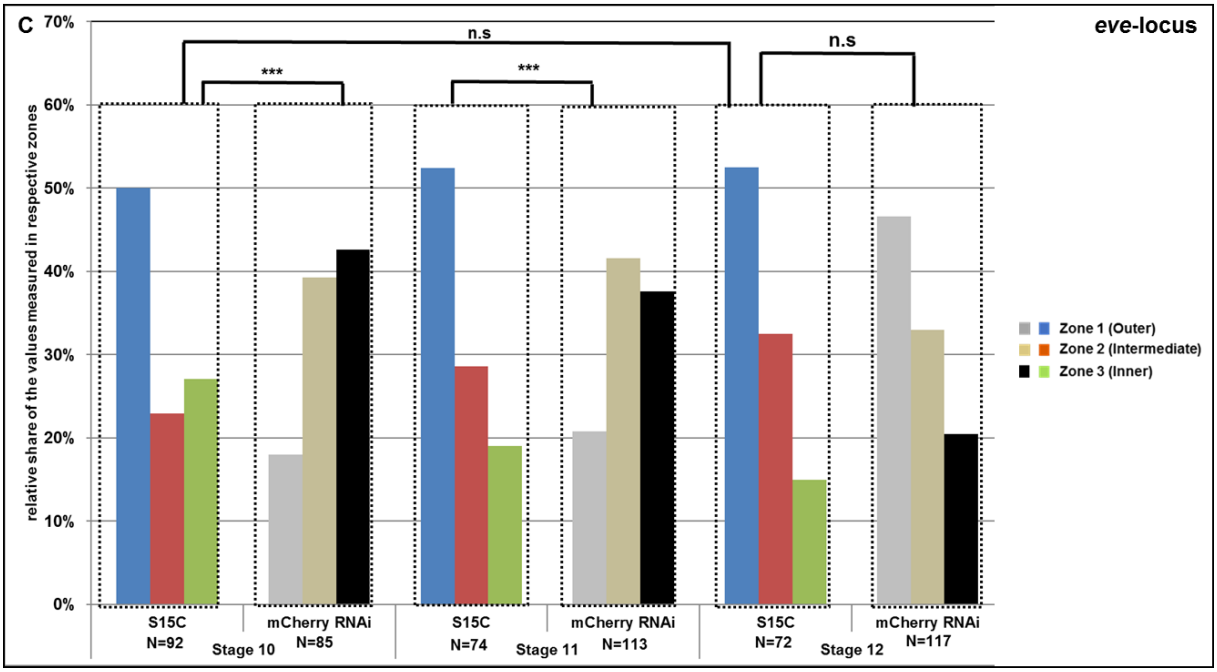


Fig 21. A, B and C Graphical analysis showing comparison of the distribution of *hb*-locus, *Kr*-locus and *eve*-locus after MTDGal4-driven overexpression of S15C actin mutant with nuclear localization signal (NLS) and fused to GFP to control mCherry RNAi driven by MTD-Gal4 in different zones 1, 2 and 3 in stage 10, stage 11 and stage 12. It can be clearly seen that re-localization for all three loci is indeed significantly accelerated in overexpression of S15C where oligomerization of nuclear actin is strong as in stage 11 and stage 12 *hb* and *eve* are found in nuclear periphery i.e. zone 1 as compared to control where its nuclear interior and active whereas *Kr* is found at nuclear interior as compared to control where it is at nuclear periphery. (N= number of alleles analyzed). The chi2 test was carried out to check statistical significance ($p<0,001$). The chi2 test was carried out to check significance ($p<0,001$).

3.14 Re-localization is a pre-requisite for locus silencing and determines the size of competence window

Our data and the data published earlier by others (Kohwi et al., 2013) suggested that neuroblasts lose competence to specify early-born fate by undergoing a developmentally-regulated re-structuring of the genome relocating the *hb* and *eve* gene loci to the nuclear lamina and *Kr* into interior of the nucleus (Kohwi et al., 2013; this work), The finding that reducing nuclear actin concentration inhibits re-localization of all three gene loci investigated prompted me to ask whether this results in a prolonged competence window as expected. For this, we tested whether additional U motoneurons can be generated in response to transient heat shock-mediated pulse of ectopic Hb protein late in NB7-1 competence window (stage

late 11/early 12) in the presence of Exp6 overexpression where re-localization is slowed down for *hb*, *Kr* and *eve* loci due to reduced nuclear actin levels and compared this with a similar heat shock-mediated pulse in WT background (Fig 22A). Indeed, after a heat shock at stage 11 we could see in embryos of enGal4xUAS-*exp6*;*hs-hb* that 40% of NB7-1 lineages generate ectopic Eve+ U neurons as compared to 10% in similarly treated control embryos., Likewise, after a *hb* expression pulse given at stage 12 20% of segments showed additional U motoneurons as compared to control where less than 10% were observed (Fig 22B and D). Additionally, when examined for *Kr* transcript by RNA FISH using an intron probe we could see endogenous *Kr* expression in *en*Gal4xUAS-*exp6*;*hs-hb* while this is not seen in the control (Fig 22C). We thus conclude the ectopic Hb pulse in reduced nuclear actin level induces early cell fates and enables Hb-dependent activation of the *Kr*-locus supporting causal relationship between nuclear actin activity and NB competence.

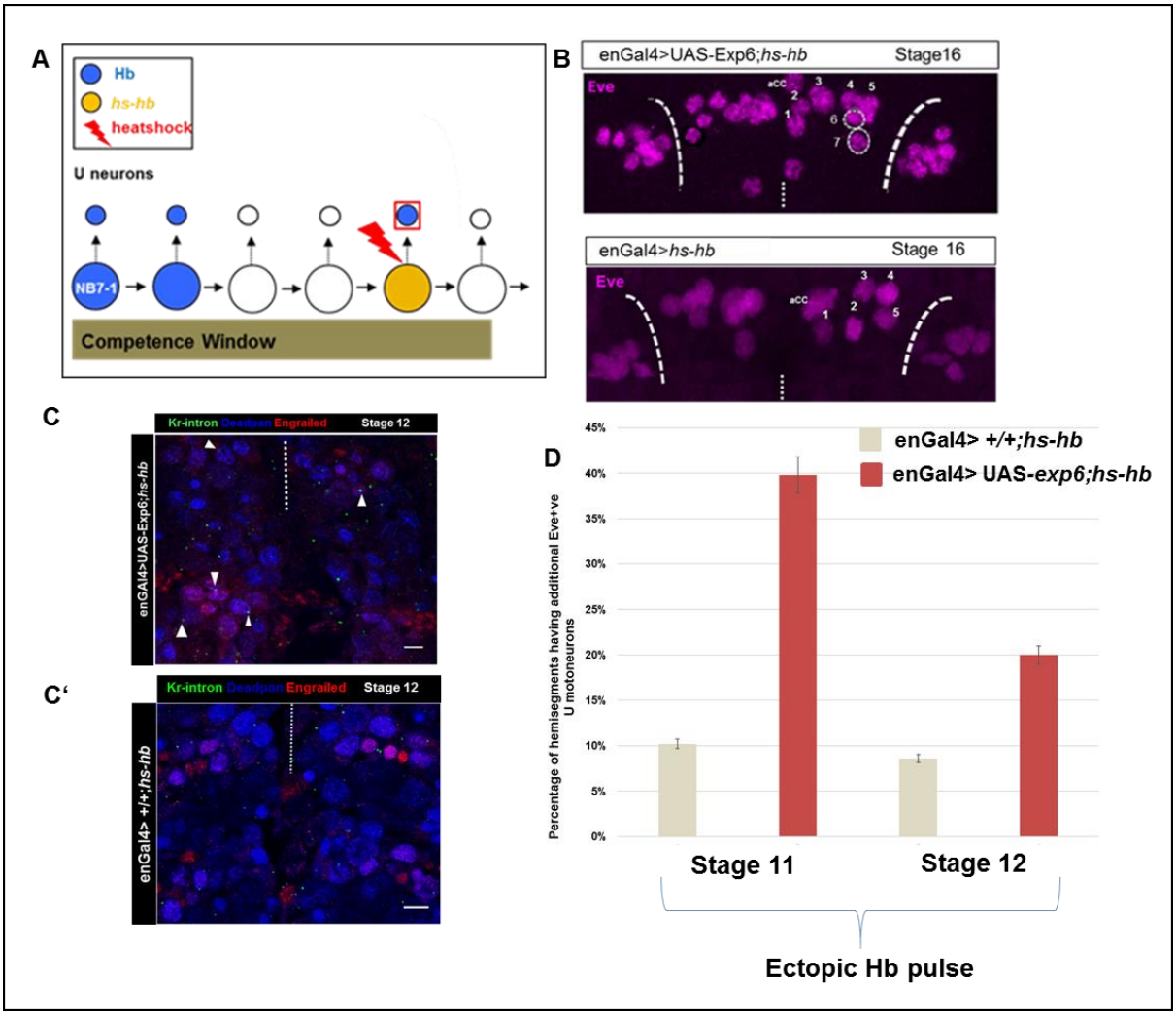
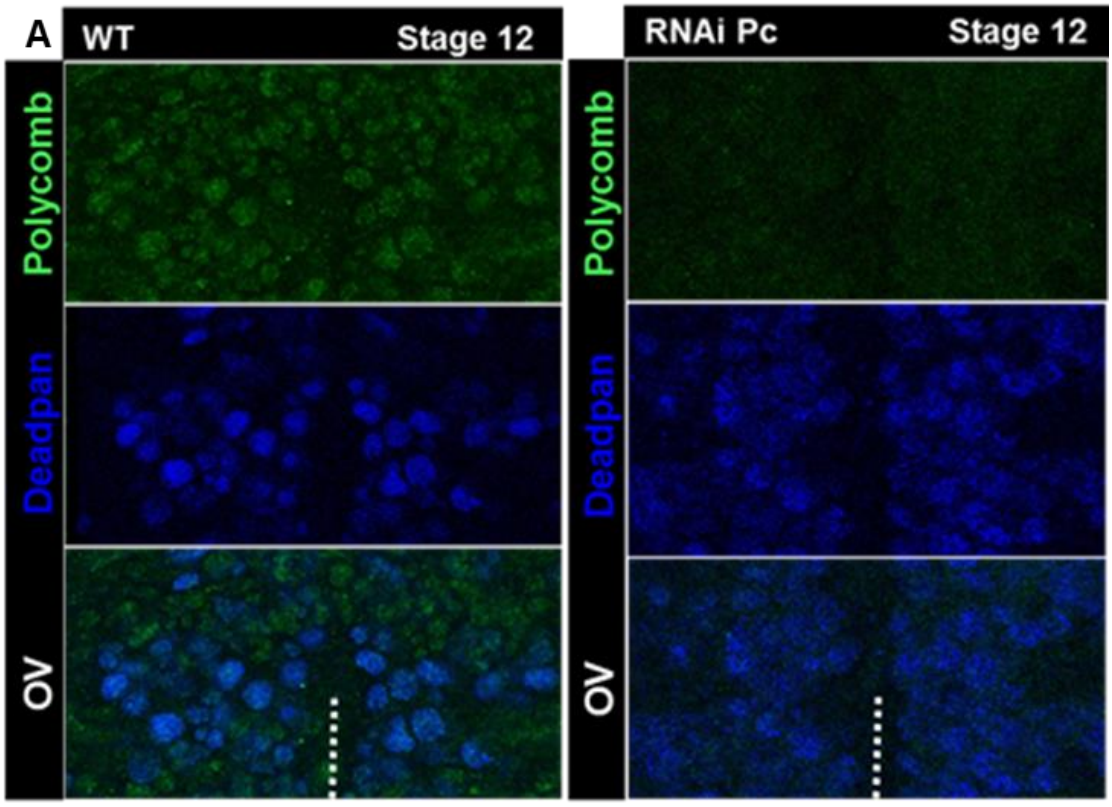


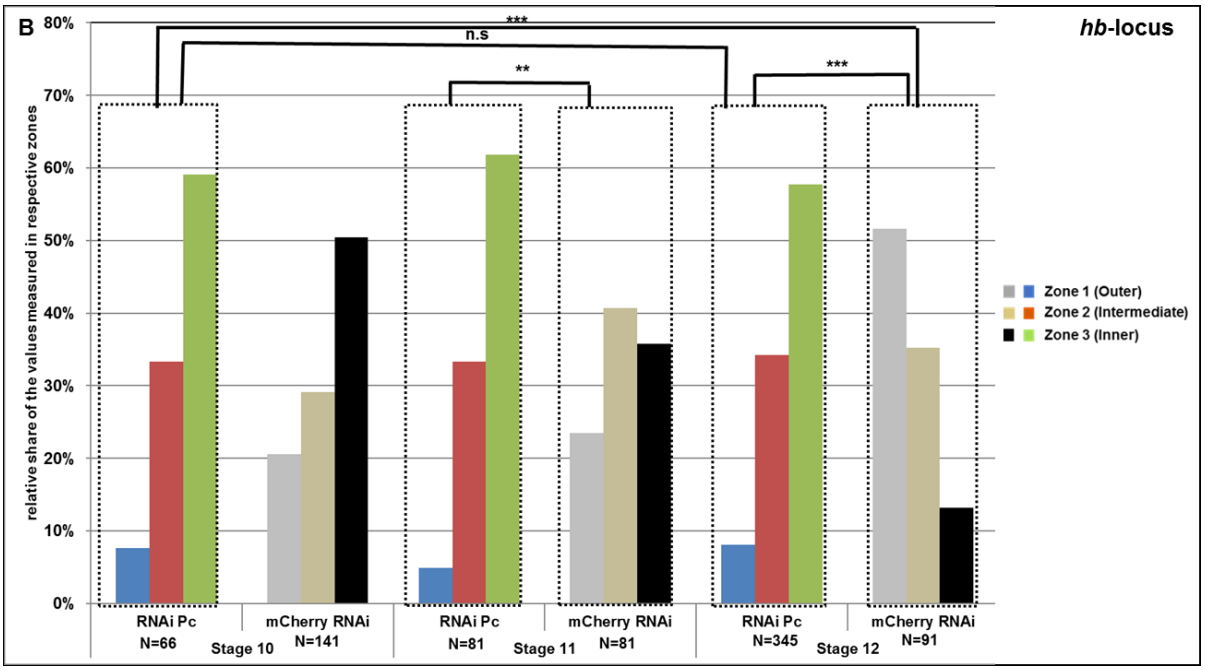
Fig 22 A. Schematic illustrating ectopic Hb expression in NB7-1 late in its early competence window (around stage 11 or stage 12). **B** One segment showing Eve staining in magenta. Additional U neurons in *en*Gal4xUAS-*exp6*;*hs-hb* marked with dotted circles. In most cases, no change in U neurons number was detected in control. **C and C'.** RNA FISH against Kr transcript green signal we could see endogenous Kr expression in *en*Gal4xUAS-*exp6*;*hs-hb* marked by arrowheads blue is a Deadpan marker for NBs and red is Engrailed antibody indicating engrailed positive NBs. Whereas no Kr intron signals could be seen in control shown in C' **D.** Percentage of hemisegments with additional U neurons at stage 16 after Hb pulse at stage 11 and 12 in WT (light color bar) or Exp6 overexpression (red bar). A total of 88 hemisegments from 4 embryos were examined in both cases at stage 11 and 100 hemisegments from 5 embryos at stage 12. Error bars represent SE. Scale bar 50 µm

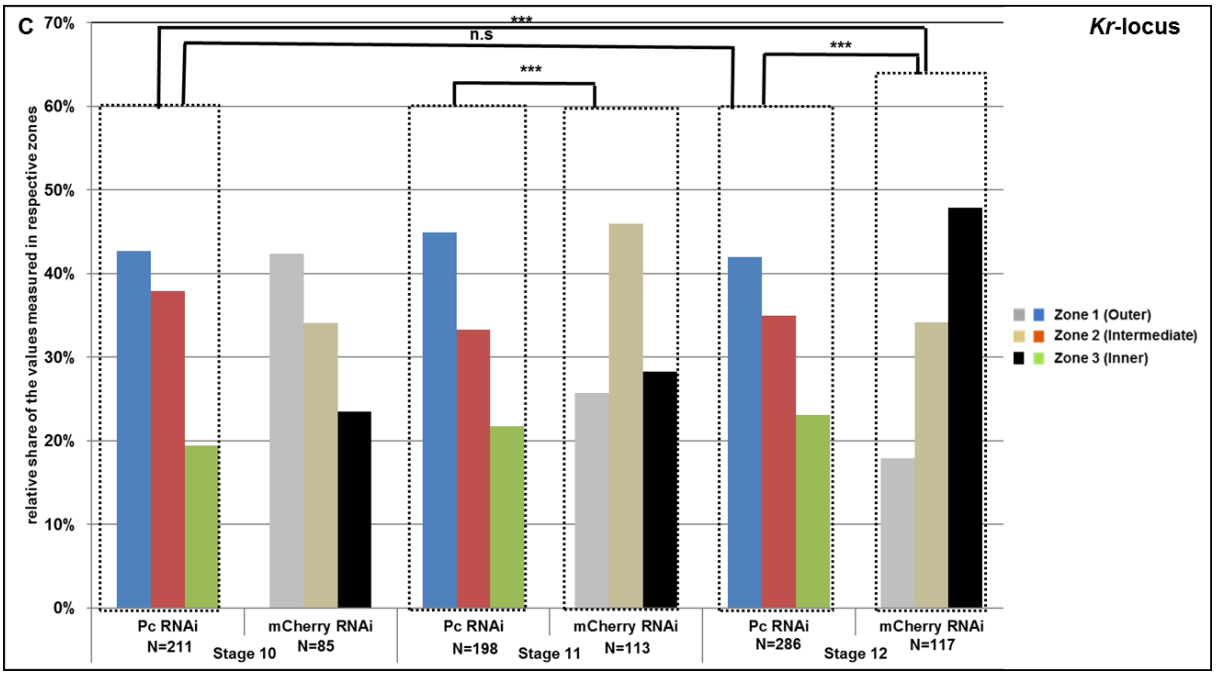
3.15 Re-localization of *hb*, *Kr* and *eve* is slowed down in Polycomb RNAi

Our results show that nuclear actin is a key factor in gene locus re-localization. It has been shown recently that in chromatin-remodeling complexes the ATPase activity of monomeric nuclear actin may be used as 'conformational switch' to facilitate interaction with chromatin and 'turn-on' the activities of the complexes (Shen et al., 2000). These chromatin re-modelers provide the mechanism for modifying chromatin and allowing transcription signals to reach their destinations on the DNA strand (Reviewed Kapoor and Shen, 2014). In search of the possible link between nuclear actin activity and epigenetic regulation in gene locus re-localization and silencing, we investigated the role of one such factor, Polycomb (Pc). Therefore we knocked down Pc using RNAi using MTD-Gal4 (MTD-Gal4xUAS-RNAi-Pc) and analyzed re-localization of *hb*, *Kr* and *eve* loci. At first, we tested if knockdown has worked using antibody staining against Polycomb protein. We could see clearly that Pc amount was reduced after RNAi as compared to WT (Fig 23A). Next, in case of *hb* locus, we could observe that in RNAi-Pc at stage 11 embryos 62% of measured alleles are in zone 3 as compared to mCherry RNAi (control) where locus is mostly in the intermediate state which depicts a statistical slowdown of re-localization. Also in stage 12 embryos of RNAi-Pc, almost 60% measured *hb* alleles were in zone 3 i.e. interior as compared to control where locus is peripheral and silenced, suggesting that RNAi-Pc resulted in halted *hb* locus re-localization. No difference could be seen in case of stage 10 measured alleles (Fig 23B). We could also see a similar effect in case of *Kr* locus: in RNAi-Pc at stage 11 45% of measured alleles are still peripheral as compared to control wherein most of the cases they are already re-located towards more interior. Similarly, at stage 12 in RNAi-Pc close to 42% of measured

alleles are in zone 1 and very few of measured alleles are in zone 3 or interior as compared to control where 15% of measured alleles are in zone 1 while 50% of measured alleles are in zone 3 (Fig 23C). No significant difference was observed in case of early stage 10 *Kr*-locus re-localization in both the genotypes (Fig 23C). In case of *eve*-locus, we also found similar result wherein RNAi-Pc at stage 11 embryos almost 40% of measured alleles are in zone 3 as compared to mCherry RNAi (control) where locus is mostly in the intermediate state which again depicts a statistical slowdown of re-localization. Also in stage 12 embryos of RNAi-Pc, almost 42% measured *hb* alleles were in zone 3 i.e. interior as compared to control where locus is peripheral, suggesting that RNAi-Pc resulted in halted *eve* locus re-localization. No difference could be seen in case of stage 10 measured alleles (Fig 23D). These results suggest that Polycomb might be involved in the epigenetic silencing of all three gene loci and is involved in the process of gene locus re-localization connected to this.




B
hb-locus
relative share of the values measured in respective zones
80%
70%
60%
50%
40%
30%
20%
10%
0%
***
n.s
**
***
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)
RNAi Pc
N=66
mCherry RNAi
N=141
Stage 10
RNAi Pc
N=81
mCherry RNAi
N=81
Stage 11
RNAi Pc
N=345
mCherry RNAi
N=91
Stage 12


C
Kr-locus
relative share of the values measured in respective zones
70%
60%
50%
40%
30%
20%
10%
0%
***
n.s
***
***
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)
Pc RNAi
N=211
mCherry RNAi
N=85
Stage 10
Pc RNAi
N=198
mCherry RNAi
N=113
Stage 11
Pc RNAi
N=286
mCherry RNAi
N=117
Stage 12

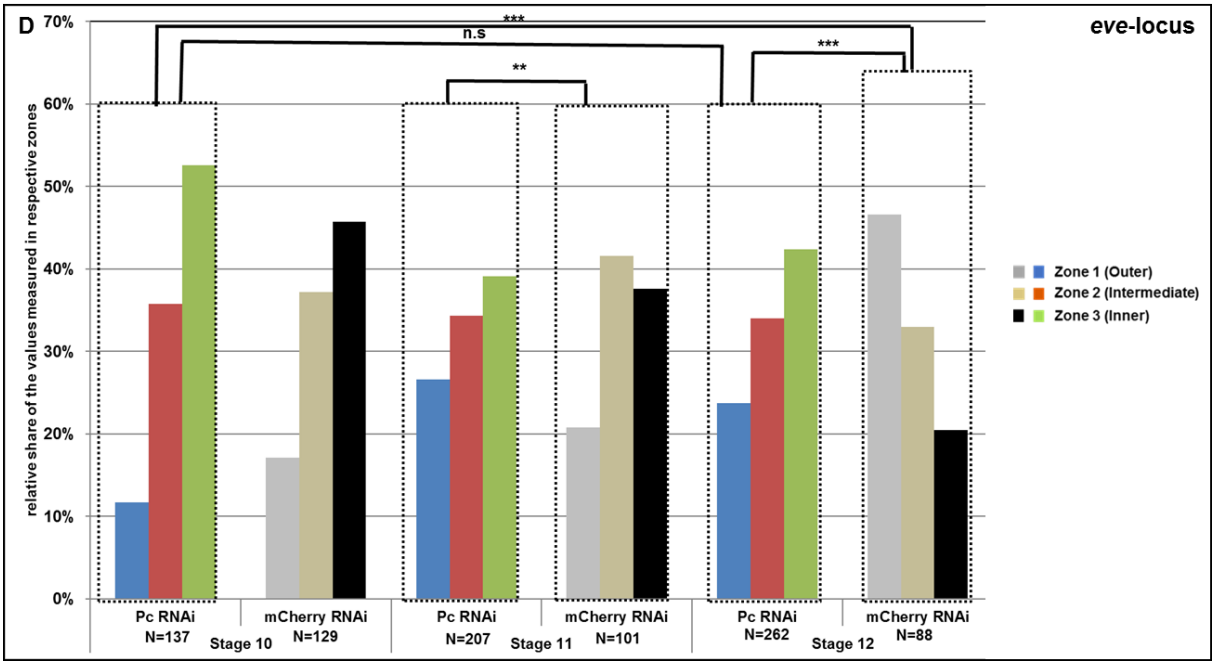
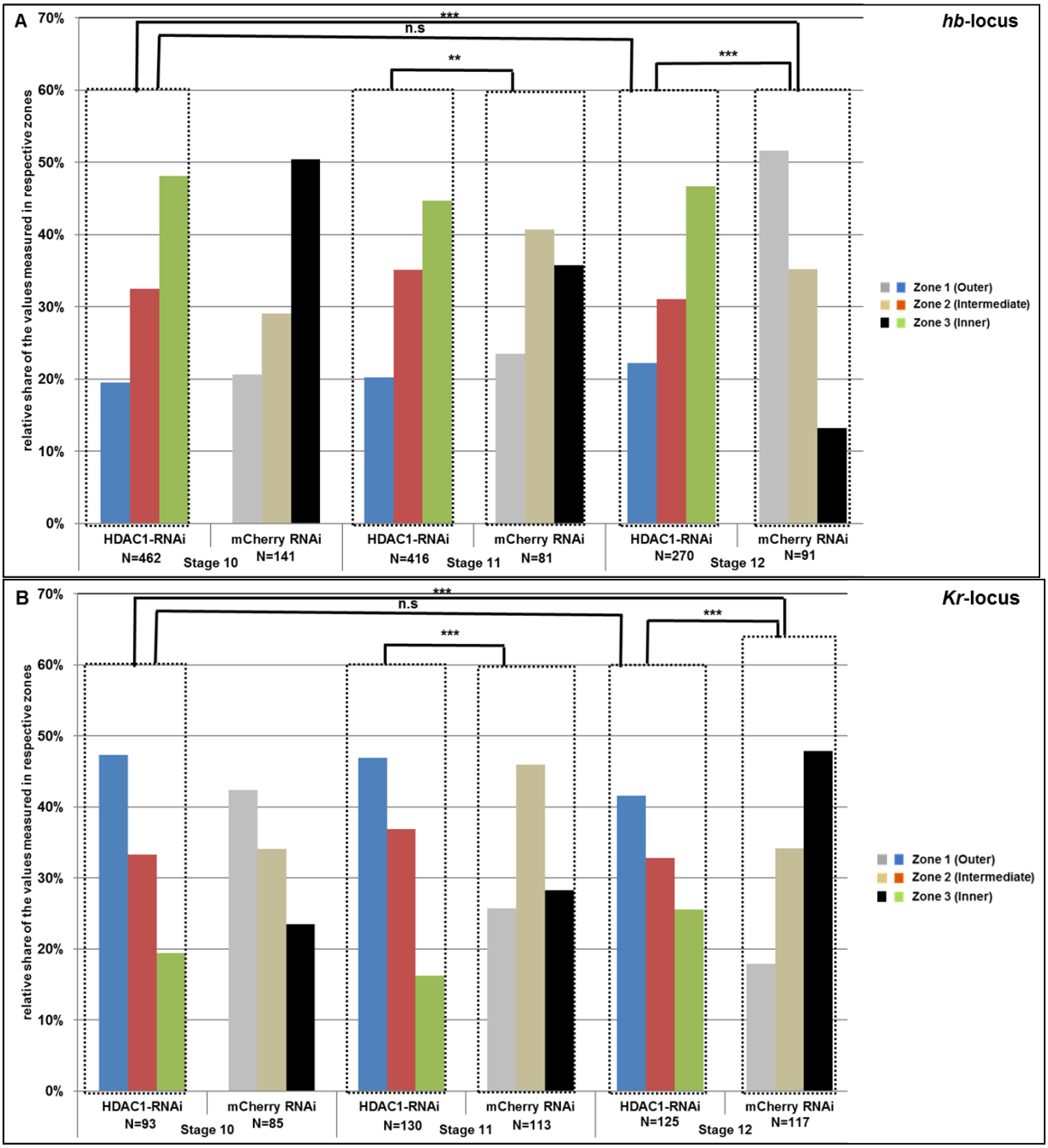


Fig 23 A. Knockdown of Polycomb tested using Pc antibody. In left panel Pc staining in the green can be seen clearly within NBs stained by Deadpan in blue, whereas RNAi-Pc driven by MTD-Gal4 showed a strong reduction in Pc staining seen in the right panel. **B, C and D** Graphical analysis showing a comparison of the distribution of *hb*-locus, *Kr*-locus and *eve*-locus after MTDGal4-driven RNAi-Pc to control mCherry RNAi driven by MTD-Gal4 in different zones 1, 2 and 3 in stage 10, stage 11 and stage 12. It can be clearly seen that re-localization for all three loci is indeed significantly slowed down in RNAi-Pc as in stage 11 and stage 12 *hb* and *eve* are found in nuclear interior i.e. zone 3 as compared to control where its peripheral and silenced whereas *Kr* is found at periphery as compared to control where it is already into nuclear interior. c. The chi2 test was carried out to check statistical significance ($p<0,001$). The chi2 test was carried out to check significance ($p<0,001$). These results one can say that Polycomb might epigenetically silence all three loci and when knocked-down the *hb* and *eve* loci stay interior and *Kr* towards periphery thus hinting towards ongoing epigenetic silencing.

3.16 Re-localization of *hb*, *Kr* is slowed down in HDAC1 (Rpd3) RNAi

Histone deacetylases (HDACs) proteins remove acetyl groups from lysine residues and are mediates post-translational modification of histones into a de-acetylated and more repressive state (Dokmanovic et al., 2007). Recently it was shown in the vertebrate system that nuclear actin is able to bind active HDAC1 and HDAC2 complex and that polymerizing nuclear actin increases HDAC activity resulting in decreased histone acetylation (Serebryannyy et al., 2016) resulting in silencing of the gene. I have found out that increase polymerization of nuclear actin results in premature silencing of *hb* and *Kr* loci thus hinting towards the possible

interaction between nuclear actin and HDAC1. Therefore we analyzed the effect of HDAC RNAi on re-localization of *hb* and *Kr* gene loci. At first knockdown of HDAC1 (MTD-Gal4xUAS-RNAi-HDAC1) was tested using mRNA in situ-hybridization against HDAC1 RNA and we could see a reduction in mRNA levels in HDAC1-RNAi as compared to WT suggesting degradation of RNA after knocking down HDAC1 (Fig 24C). Next when tested for *hb* locus re-localization a significant change was seen in repositioning because it was halted or slowed down in HDAC1 RNAi at stage 11: 45% of measured alleles are found in zone 3 and 20% are in zone 1 while in control NBs from the crossing MTD-Gal4xRNAi-mCherry show only 20% of cases in zone 3 and nearly 40% of cases in zone 1. Also, at stage 12 it was seen that 47% of measured alleles in HDAC-RNAi are in zone 3, 20% are in zone 1 whereas in case of control only 12% of cases are in zone 3 and nearly 52% of cases it is zone 1. No significant difference was seen in the position of *hb* locus at stage 10 in both the genotypes (Fig 24A). Also, in case of *Kr* locus similar effect was seen where it tends to stay towards periphery or zone 1: at stage 11 almost 50% cases of measured alleles were seen in zone 1 and only 20% are in zone 3 as compared to control where 35% of measured alleles are already in zone 3 and also 43% are in zone 2 or intermediate stage. At stage 12 locus re-localization is slowed down where 42% of alleles are in zone 1 and 25% are in zone 3 in HDAC-RNAi as compared to control 12% of measured alleles are in zone 1 and most of the measure alleles 52% are in zone 3 or in nuclear interior. No significant difference was recorded for stage 10 in both the genotypes (Fig 24B). Together we can conclude from above results that knockdown of HDAC1 indeed slows down gene locus re-localization and that this protein is involved in this process.



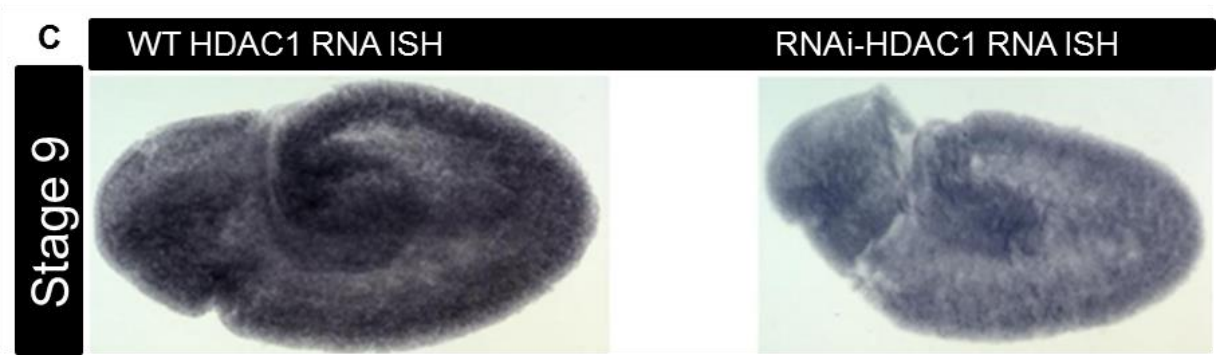
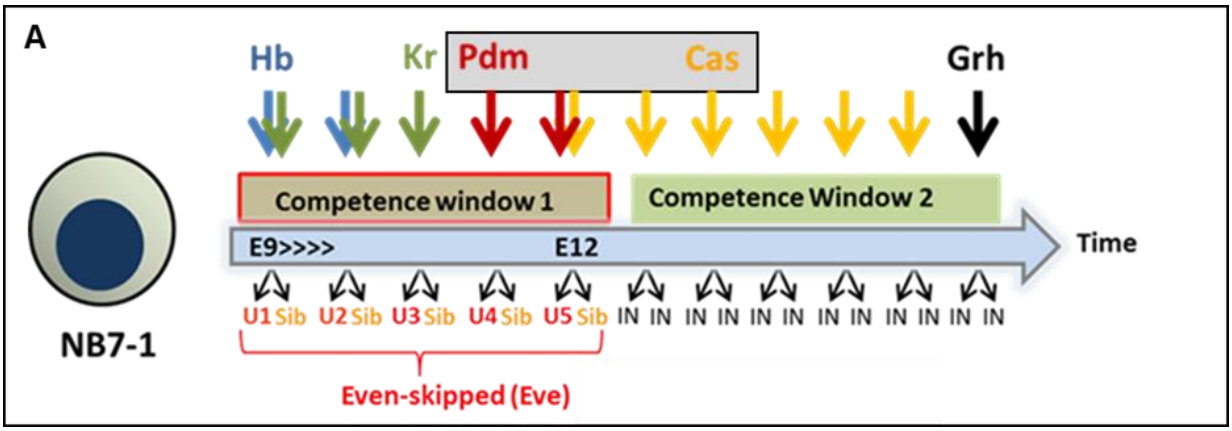


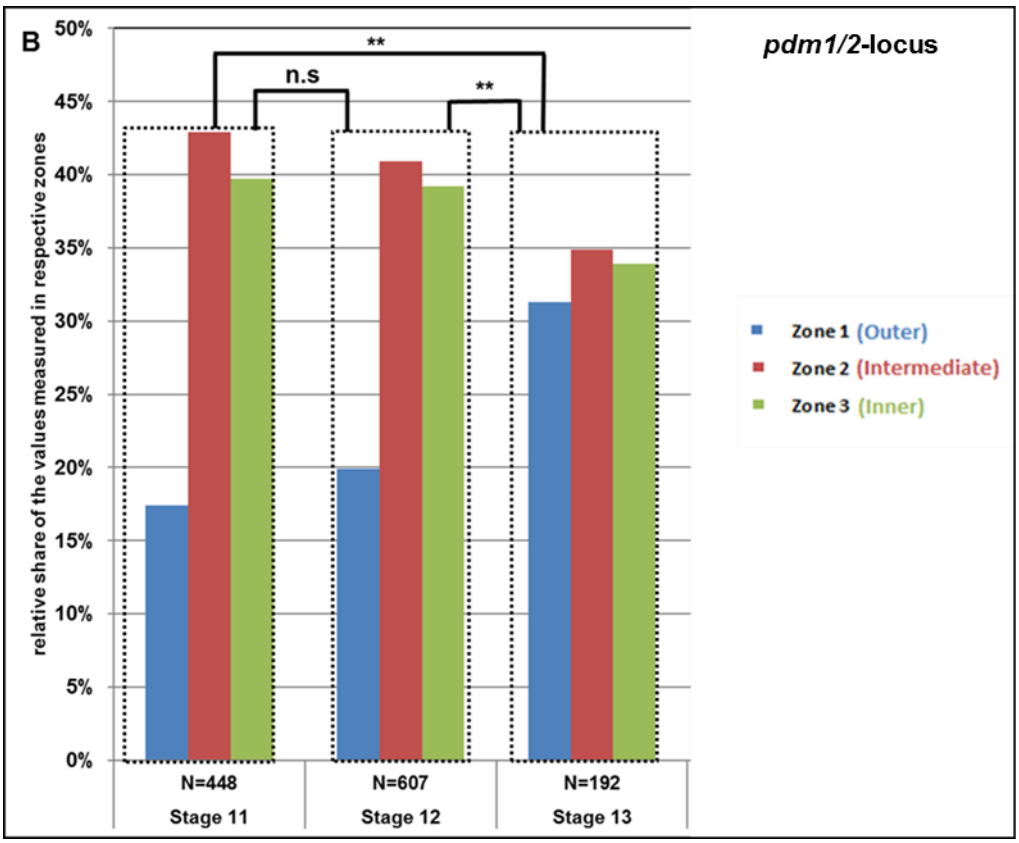
Fig 24. A and B Graphical analysis showing a comparison of the distribution of *hb*-locus, *Kr*-locus after MTDGal4-driven RNAi-HDAC1 to control mCherry RNAi driven by MTD-Gal4 in different zones 1,

2 and 3 in stage 10, stage 11 and stage 12. It can be clearly seen that re-localization for *hb* and *Kr* loci is indeed significantly slowed down in RNAi-HDAC1 as in stage 11 and stage 12 *hb* is found in nuclear interior i.e. zone 3 as compared to control where its peripheral and silenced whereas *Kr* is found at periphery as compared to control where it is already into nuclear interior. The chi2 test was carried out to check significance ($p<0,001$). **C.** RNA-ISH against HDAC1 RNA. It can be seen that HDAC1 RNA is been degraded (Light signal) as compared to WT (Dark signal). These results one can say that HDAC1 might be also important factor necessary for epigenetic silencing of the gene locus.

3.17 *pdm1/2* loci re-localizes with a small shift towards nuclear interior while *cas* do not re-localize during development

As with Hunchback (Hb), Krüppel (Kr), Pdm1/Pdm2 (Pdm), Castor (Cas), are four temporal transcription factors are consecutively expressed within the early competence window in Drosophila neuroblasts (summarized in Fig. 1 and 25A; Kambadur et al. 1998; Isshiki et al., 2001). I questioned whether the loci the later expressed transcription factors *Pdm1/2* and *Cas* also show a re-localization behavior and maintained in their neuronal progeny. It has been reported that Pdm and Cas are required for the development of late-born motor neuron identity within the NB7-1 lineage: Pdm specifies U4 motor neuron fate, whereas Pdm and Cas together specify U5 motor neuron fate (Grosskortenhaus et al., 2006). Here we analyzed the re-localization of third and fourth transcription factors i.e. *pdm1/2* and *cas* within NBs of stage 11 to stage 14 in WT. For *pdm-1/2* locus, the graph shows that in stages 11 and 12 the distribution is predominantly towards inner nuclear regions (zone 2 and 3) while in stage 13 it is almost evenly distributed. There is a small shift of the *pdm-1/2* gene locus from the interior to the periphery at stages 12 and 13, as well as between 11 and 13 (Fig 25B). In contrast, the *cas* locus was found constantly within the interior of the nucleus in all stages analyzed with no significant difference in re-localization behavior (Fig 25C). In summary, we could predict that *pdm1/2* might be initially inactive at early stages and is at the periphery. It moves slightly towards interior region later in development and is possibly active during this phase. In contrast, it is not possible with the results shown to make a concrete conclusion on the state of activity and the occurrence of a re-localization of *cas* locus during the neurogenesis.


A
Hb
Kr
Pdm
Cas
Grh
Competence window 1
Competence Window 2
E9>>>>
E12
Time
U1 Sib U2 Sib U3 Sib U4 Sib U5 Sib IN IN IN IN IN IN IN IN IN IN
NB7-1
Even-skipped (Eve)


B
pdm1/2-locus
relative share of the values measured in respective zones
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
n.s
**
**
Zone 1 (Outer)
Zone 2 (Intermediate)
Zone 3 (Inner)
N=448
Stage 11
N=607
Stage 12
N=192
Stage 13

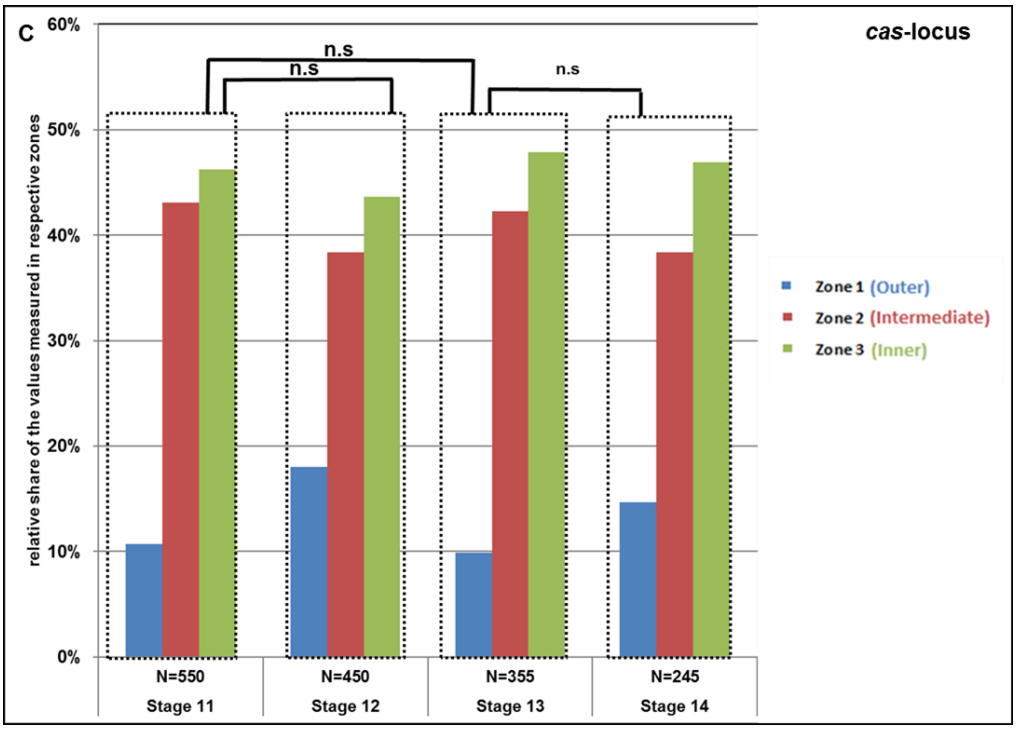


Fig 25 A. Schematic illustrating NB7-1 competence phase. Four transcription factors, Hunchback (Hb), Krüppel (Kr), Pdm1/Pdm2 (Pdm) which mark first window where five U motoneurons are generated which are positive for Even-skipped (shown in red), Castor (Cas) which starts its expression after the first window. **B.** Graphical analysis showing re-localization of *pdm-1* locus highlighted in schematic in A. It can be seen that in stages 11 and 12 the distribution is predominantly inward, whereas in stage 13 it is almost evenly distributed. There is a small shift of the *pdm-1/2* gene locus from the interior to the periphery at stages 12 and 13, as well as between 11 and 13. **C.** In case of *cas* locus re-localization, it is found constantly interior i.e. zone 3 of the nucleus in all stages analyzed with no significant difference in re-localization pattern. The chi2 test was carried out to check significance ($p<0,01$). The chi2 test was carried out to check significance ($p<0,001$).

4 Discussion

4.1 Regulation of neuroblast competence

How important role does competence window play during development? It is necessary to maintain both plasticity and limitations for generation of different cell types. Another potential function is that consecutive competence windows may allow the same cell fate determinant to generate different cell types. Stem cells or neural progenitors generate different cell types which can be in reproducible order in the retina, cerebral cortex and in some cases in the spinal cord (Turner et al., 1998; Cepko et al., 1996; Zhou et al., 2002). Such cellular diversity occurring from single progenitor is achieved in response to signals from different internal as well as external factors like transcription factors, environmental stimuli etc. (Bohner et al. 1997; Hanashima et al. 2004; Pearson and Doe 2004). In the vertebrate nervous system, there are examples of the role of progenitor competence in the generation of such cellular diversity. The cerebral cortex is derived from progenitor cells whose progeny migrate to distinct layers depending on their time of birth (McConnell 1992). Several experiments have proven that these early cortical progenitors are multipotent; however, the ability to make early cell types is gradually lost as progenitor's age, in a process known as "loss of progenitor competence" (Desai and McConnell 2000). In our system *Drosophila*, a similar loss of competence has been observed in well-characterised lineage of neural stem cell or known as neuroblasts 7-1 (NB7-1) (Pearson and Doe 2004, Kohwi et al., 2013). NB7-1 generates five Eve positive U motoneurons. These five U motoneurons are well characterized by sequential expression of transcription factors Hb, Kr, Pdm, and Cas. It has been studied that the first transcription factor Hb is necessary and sufficient to decide the early born cell fate. Hb loss of function mutants lack early-born U1/U2 neurons, and prolonged Hb expression within this NB will generate many additional Eve-positive U1-like neurons until end of embryogenesis (Isshiki et al., 2001; Novotny et al., 2002). Importantly, re-expression of Hb after the NBs fifth division has no effect (Pearson and Doe, 2003). This shows that NB7-1 loses its competence to generate Eve-positive motoneurons in response to Hb activity latest after five divisions and this is referred as 'neuroblast early competence window.' This is analogous to the activity of Ikaros in the vertebrate retina and cortex development (Elliott et al., 2008; Alsiö et al., 2013). For example in cortex development sustained Ikaros expression extend the window of early deep layer neurogenesis at the expense of late upper-layer neuron production in and re-expression of Ikaros in older progenitors cannot induce specification of the early-

born neuron types anymore (Alsiö et al., 2013). Next, there is another example in a vertebrate system which shows that competence of radial glial cells (RGCs) to produce different progeny changes over time. These cells produce Cajal-Retzius neurons and deep layer neurons, a phenomenon known as direct neurogenesis (Guillemot, 2005). This process is followed by generation of superficial layer neurons mainly via intermediate progenitor cells (IPCs) in a process called indirect neurogenesis (Sessa et al., 2008). During late development, RGCs gradually terminate neuronal production in favor of gliogenesis. Similar temporal pattern has also been shown in culture for NPCs purified from the embryonic mouse cortex (Qian et al., 1998, 2000; Shen et al., 2006), or differentiated from mouse/human embryonic stem cells (ESCs; Eiraku et al., 2008; Gaspard et al., 2008). This timely transition of NPC competence was studied in vivo using a heterochronic transplantation approach where early born NPCs of donor ferret cortex were transplanted into the ventricular zone of older recipients and this resulted in the generation of late-born superficial layer neurons, on the contrary, old NPCs transplanted into a younger host failed to generate early-born deep-layer neurons. These results proved that both intrinsic and extrinsic signals together regulate the transition of NPC competence, which is restricted over time (Ki‐Jun Yoon et al., 2018). Although, such loss of competence is well studied how this can be reversed still remains unclear. Here, we showed that such loss of competence can be regained by combined ectopic expression of transcription factors Hb and Kr. As shown continuous Hb expression in early time window do extend competence and additionally it was seen that Kr is also expressed continuously along with Hb (Isshiki et al., 2001 and our data). This led us to hypothesize that additional factor Kr is required along with Hb to extend the competence since single ectopic expression of Hb and Kr late in development didn't result in additional U motoneurons production. Similarly, NB3-1 is known to generate the well-characterized RP1, RP4, RP3 and RP5 motoneurons (Bossing et al., 1996; Landgraf et al., 1997; Schmid et al., 1999). Hb and Kr also specify early temporal identity in NB 3-1 lineage, extending their role as multi-lineage temporal identity factors to a different spatial domain of the CNS (Tran and Doe, 2008). RP1/4 are Hb^{+} Kr^{+}, RP3 is Hb^{-} Kr^{+}, and RP5 is Hb^{-} Kr^{-} and these motoneurons additionally are also positive for $Hb9^{+}$ and $Islet^{+}$ markers (Tran and Doe, 2008). We could also show a similar phenotype in case of NB 3-1 lineage where additional neurons could be produced late in the window after combine expression of Hb and Kr, also single ectopic expression didn't result in any change. However these neurons are positive for only three markers i.e. Hb, Islets, and Hb9+, therefore, this makes it really difficult to determine exact fate. Also, we could observe some additional neurons which were only positive for Hb and Hb9+ and no Islet+ staining was seen. To get

more information regarding such fates one has to follow genomic analysis in future. Altogether from these results, we can propose that Hb and Kr indeed interact and bind together and Kr is target gene that is involved in extending neuroblast competence along with Hb. But how Kr is able to carry out this function is still an open question? For example it has been shown in vertebrates that controlled expression of one of such transcription factor KLF4 (Kruppel like Factor 4) which is a Yamanaka factor and is a part of a machinery to reintroduce pluripotency where it interacts with other transcription factors like Oct4, Sox2, and c-Myc to carry out this process (Zhang et al., 2010). However, it's unclear yet what exactly are the concrete activities of these factors necessary to carry out such process of reintroducing pluripotency. Therefore in our case additional characterization of Hb and Kr function in the CNS will be required to be done in future by implementing genomic studies using Chromatin immunoprecipitation (ChIP) to identify Hb and Kr binding sites within the genome, or DamID (Southall et al., 2013) experiments to identify all the genes regulated by Hb within the CNS. Also one can detect target genes in presence of both Hb and Kr together, in absence of Hb and presence of Kr and vice versa. Such relative analyses might help to interpret the complex gene interactions involved in neuroblast competence regulation. Polycomb group complex silenced the proneural bHLH genes to end neurogenesis, highlighting the role of epigenetic mechanisms in the competence switch (Hirabayashi et al., 2009). Similar detail study of how a change in competence can be regulated by epigenetic complex like PRC will give insight into the mechanism of neuroblast competence.

4.2 Gene loci re-localization within NBs correlating with competence

Research from past decades has proved that, in the eukaryotic nucleus, the DNA double helix wraps around histone proteins in repeating units of nucleosomes to form chromatin, which in turn is folded into multi-level higher-order structures. Advances in microscopy techniques, in particular, those based on fluorescence in situ hybridization (FISH) and live-cell imaging of in vivo tagged genomic loci, have made it increasingly clear that eukaryotic genomes are non-randomly organized inside the nucleus. There is evidence showing non-randomness of the 3D organization of nucleus which consists of following aspects, 1) Different chromosomal territories (CT) are occupied by each chromosome. 2) The eukaryotic genome is divided into different euchromatin and heterochromatin regions. The heterochromatin is a region characterized by its transcriptionally repressed state and highly condensed structure, whereas

euchromatin is transcriptionally active and less condensed. 3) Individual chromosomes, genes, and genomic elements undergo repositioning within the nucleus, which has been shown to correlate with genomic properties as well as with genomic functions such as transcriptional activity and replication timing (reviewed in Duan and Blau 2012). Interior of the nucleus is considered euchromatic while lamina or periphery a compartment is known to be involved in transcriptional repression. In order to experience a gene silencing, there are evidences in multiple organisms showing that repositioning of a gene to the nuclear lamina can cause transcriptional repression (Pickersgill et al., 2006). It has been suggested for a long time that this non-random distribution of heterochromatin and euchromatin has a function and that attachment of chromatin to the nuclear envelope is important to obtain the three-dimensional organization of the chromatin fibers (G Blobel, 1985). There are examples in several animal models where there is a change in the repositioning of gene locus has been depicted one such example of repositioning was shown in snails where it was determined that there is specific temporal repositioning or relocation of gene loci within interphase nuclei after in vitro schistosome exposure. It was shown that significant gene loci repositioning within nuclei tightly correlated with gene expression, example in a vertebrate system depicts repositioning of key differentiation gene loci Pax6 and Irx3, where surrounding chromatin undergoes both de-compaction and displacement towards the nuclear center coincident with transcriptional onset (Patel et al., 2013). It has been well documented that chromatin in the nucleus undergo rapid constrained Brownian motion and can move over several microns on a longer time scale, also it has been proposed that it can undergo diffusive random walk motion within the nucleus (Marshall et al., 1997). Thus, genes may move back and forth between the nuclear periphery and interior in each individual nucleus over time. However, such diffusive motion is limited in a way that even if chromatin can diffusively move in a free manner the given region for chromosome movement is confined to small sub-region within the nucleus. This limited diffusional movement does indeed reconcile the dynamics of chromatin with a specificity of its position and inferred from functional studies within the genome (Marshall et al., 1997). Here, we analyzed re-localization pattern in NB population using zone model system (Hediger et al., 2004) for *Kr* gene and another downstream gene *eve* (Even skipped) during change in competence i.e. from stage 9/10 which is marked by onset of competence phase until stage 12 of embryogenesis which marks the end of the neuroblast competence window to specify early-born cell fates. Interestingly, we found that *Kr* gene locus re-localizes from peripheral region and is active (shown by FISH against *Kr* intron) at stage 9/10 of embryogenesis and moves towards the interior of the nucleus at end of competence at stage 12 and this position is

maintained further as analyzed until stage 14. This re-localization pattern is unusual since most of the cases it has been shown that gene is active in the inner nuclear region and moves towards periphery as it silences (Chubb et al., 2002; Gasser, 2002). On the other hand, *eve* locus also found to be re-localized and in normal way where it is in nuclear interior and active and moves towards periphery to be silenced in multiple NB population during a change in competence. Next, we could observe that re-localization of *Kr* locus and *eve* locus can be inhibited by continuous Hb activity (enGal4xUAS-Hb2,3) during early competence window. This correlates strongly with previously published observations showing continuous Hb expression maintains Kr expression and also extends neuroblasts competence by producing additional Eve +ve U motoneurons. In contrast to the well-studied zinc finger transcription factor Hb, only little is known about the repressing and downregulating mechanism regarding the temporal identity gene Kr. It was shown that Pdm is able to repress Kr, however, at the same time, it was seen that within *pdm* loss-of-function mutants, Kr gets downregulated as well, an observation leading to the presumption of another factor being involved in the downregulating mechanism of Kr (Grossekortenhaus et al., 2006). Also, in absence of Hb both the loci showed accelerated movements, *Kr* locus was found in the interior right from stage 9/10 and was stable in this position throughout, similarly *eve* locus was peripheral in early stage 9/10 and was observed at same position until stage 12. This again correlated with the observation that in hb^{15} mutants the window is shortened as early cell fates are missing. These observations suggest the assumption that once Hb is turned off, Kr might re-localize towards a certain nuclear compartment in the inner center of the nucleus and *eve* towards periphery in order to become silenced permanently. Furthermore, a combined, late re-expression of Hb and Kr (casGal4xUAS-Hb/Kr) was shown to ectopically generate Eve+ postmitotic neurons. This re-expression resulted in change in re-localization of both *Kr* and *eve* genomic region towards the periphery and nuclear interior at stage 13, an observation further supporting the model of a transcriptional silencing for *Kr* and *eve* at end of competence whereas when competence is regained after ectopic expression of Hb and Kr together both of them are transcriptionally active. Altogether these results strongly correlate to our observation that Hb and Kr together re-activates competence window late and supports our hypothesis of a specific and goal-driven re-localization of *Kr* and *eve* loci during a change in competence as NBs ages. Next, a similar change in nuclear localization of *hb* locus from the interior towards periphery (nuclear lamina) was shown by (Kohwi et al., 2013), where they observed a strong correlation of silencing of *hb* locus with the termination of the early competence window. The *hb* locus positions overlap with the nuclear lamina after the end of

the early competence window in stage 12. However, it is important to know that overexpression of Hb resulted in no change in *hb* locus re-localization itself, it was silenced and found to be in the periphery at end of competence (Kohwi et al., 2013). This suggests that Hb can specify early-born neuronal identity but cannot extend the competence window for postmitotic *hb* expression. Together we can conclude that within the early competence window there are multiple gene loci re-localizations in different directions and *Kr* and *eve* loci re-localizations are dependent on Hb activity during a change in competence. It has been shown that mammalian retinal and cortical progenitor cells also change competence over time, producing sequentially different cell types (Cepko et al., 1996; McConnell, 1989). In the future, it would be interesting to determine whether the genes expressed in early-born cortical or retinal cell types are also re-localizing in mammalian neural progenitors at the end of competence. Additionally, we also analyzed re-localization of *pdm1/2* and *cas* loci, which are third and fourth transcription factors in a cascade. *pdm1/2* locus showed slight shift later in development, prior to this, it is at the periphery and might be inactive at an early stage i.e. stage 11. It moves slightly towards interior region later in development and is possibly active during this phase, however, we did not analyze the expression of Pdm transcript. In contrast, it is not possible with the results shown to make a concrete conclusion on the state of activity and the occurrence of a re-localization of *cas* locus during the neurogenesis because it was constantly seen into the interior of the nucleus in all stages analyzed.

4.3 *Kr* locus re-localizes unusually from periphery to nuclear interior

Kr locus re-localizes in multiple NBs starting in the periphery and is active transcriptionally with a final localization in the nuclear interior. This finding was surprising since both *hb* and *eve* were seen to be localized in the inner NB nucleus by early embryogenesis at stage 9/10, and re-localizing towards periphery at end of Stage 12. There are examples where similar re-localization has been observed one such example is gene *ferritin* in snails that is initially at periphery and re-localizes into nuclear interior concurrently with up-regulated gene expression (Knight et al., 2011). Another interesting example is, transcriptionally activated *var* genes in the malaria parasite Plasmodium falciparum (Apicomplexa), are located at the nuclear periphery (Duraisingh et al., 2005). Next, it was shown in vertebrates that chromatin around key differentiation gene loci Pax6 and Irx3 undergoes both de-compaction and displacement towards the nuclear center coincident with transcriptional onset (Patel et al.,

2013). In plants, it has been shown that genes are been active upon their recruitment to the nuclear periphery. In Arabidopsis thaliana, the chlorophyll a/b-binding protein (CAB) gene locus, which contains a 7-kb cluster of three members of the CAB gene family (CAB1–CAB3) on chromosome 1, re-positions to the nuclear periphery in response to red or far-red light (Feng et al., 2014). In the dark, these genes are repressed and physically retained in the nuclear interior of mesophyll cells by a number of master repressors of photoreceptor signaling [DET1, COP1 and phytochrome-interacting factors (PIFs)] (Feng et al., 2014). In case of *Kr* locus re-localization one possible explanation might be because of its telomeric position, it was shown by Therizols et al. 2006, that telomeric regions in Saccharomyces cerevisiae are tethered to the nuclear periphery which is required for an efficient DNA single double break repair of subtelomeric regions. Similar observations were as well made for *Drosophila* telomeres, which were often seen to localize in the nuclear periphery (Loidl, 1990, Hiraoka et al., 1990). There has been one more observation in yeast that active genes tend to interact with nucleoporins, Nuclear Pore Complex (NPC) at lamina are composed of different nucleoporin proteins. These same genes also interact with transcriptional regulator Rap1 and with nuclear transport receptors (Kap95, Cse1, XpoI/CRM1) (Casolari, et al., 2004). These nucleoporins then move dynamically between NPC and nucleoplasms directing towards the possibility of nup and chromatin interaction that takes place partially or completely within the interior of the nucleus (Reviewed in Kalverda et al., 2008). This could also be the case with *Kr* locus, it may move towards the certain nuclear body in the inner nuclear region and get silenced, however, we do not have concrete proof yet and further analysis like staining with an antibody specific for nuclear bodies might provide us the hint. Another possibility would be a directed re-localization of *Kr* towards a sub-nuclear region located in the inner nucleus towards the transcriptionally silenced region. One probable candidate as essential for such a sub-nuclear compartment might be a Polycomb body since strong Polycomb group (PcG) binding sites were found within the *Kr* locus thus hinting towards possible epigenetic mechanism involved in silencing (Schwartz et al., 2006). Further, we analyzed re-localization of neighboring genes to *Kr*, CG9380 which re-localizes only into periphery from stage 9/10 to stage 12 and second gene which is intermediate to Kr and CG9380, CG30429 which localizes or is found constantly into the nuclear interior (Bachelor thesis Julia Kautz and Nadine Körtel). This provided us with an insight or a hint that there might be the occurrence of chromosomal looping in course of development. To conclude, it appears as if the *Kr* locus re-localization in NBs occurs directed with the objective to be

transcriptionally silence and position itself into the inner nuclear region and this is indeed dependent on the activity of Hb.

4.4 Svp (*Drosophila* COUP-TF) acts as an important timing factor

Previous studies demonstrated that Svp a homolog of mammalian COUP-TF is expressed in a brief pulse in the majority of early embryonic neuroblasts, where it suppress the activity of Hb, thereby allowing for the switch to the next stage of temporal competence. Loss of Svp function increased the number of early-born cell types and it was also shown that Hb expression was maintained in many lineages (Kanai et al., 2005; Mettler et al., 2006). Similarly, it has been shown in a vertebrate system that COUP-TF1 and COUP-TF2 nuclear receptors, function as a 'timer' that switches progenitors from neurogenesis to gliogenesis. Both of these factors are transiently expressed in neural progenitors near the end of the neurogenic phase, and their loss prolongs neurogenesis at the expense of gliogenesis (Naka et al., 2008). COUP-TF1 has also been important factor necessary in the switch from early-born to late-born cortical neurons (Faedo et al., 2008). Thus, Svp in *Drosophila* and the COUP-TF in the vertebrates seem to have conserved roles as switching factors in neural progenitors. Therefore, with this known information we proceeded to check the effect of loss of Svp on *hb* gene locus re-localization, since early overexpression of Hb results in extension of competence window but resulted in no change in *hb* locus re-localization, it was silenced and found to be in periphery at end of competence (Kohwi et al., 2013). Interestingly, we found that in Svp mutant *hb* re-localization was halted or stopped until late embryogenesis, pointing towards the notion that Svp indeed acts as a ‘timer’ or ‘initiator’ of silencing process and this might be taking place epigenetically. In vertebrates, there is an evidence showing COUP TFI/II knockdown resulted in the maintenance of epigenetic silencing at the *Gfap* gene, which resulted in the loss of astrogliogenesis (Naka et al., 2008). However, in our system presence of Svp, *hb* activity is silenced leading to end of the temporal window, whereas the absence of Svp maintains *hb* active leading to an extension of competence. In future, it will be necessary to further study if any epigenetic mechanism exists and this can be done using genomics e.g. by identifying factors that interact with Svp using ChIP seq analyses. All together we can conclude that Svp might be acting as an important temporal identity factor which acts as a switch or trigger to initiate the silencing process.

4.5 Mechanism behind re-localization of genes, nuclear actin potential factor

Until now we could show a strong correlation between the end of competence window accompanied by re-localization of gene loci. However, less is known about the possible mechanism behind such chromosomal reorganization within the nucleus. Our data obtained after manipulating nuclear actin points in the direction that it indeed has a role to play in the repositioning of genes. The first evidence of nuclear actin was found nearly 34 years ago by Scheer et al 1984, however, they could neither reject the contamination with cytoplasmic actin, nor could they visualize filamentous actin in the nucleus, which was commonly observed in the cytoplasm. Nuclear actin has only been recently found to be present in *Drosophila* cells, where it associates with all three RNA polymerases, regulates the activity of transcription factors and is crucial for chromatin remodeling complexes (Dopie et al., 2012; Philimonenko et al., 2004; Zhao et al., 1998). Transport of such macromolecules like actin between the nucleus and the cytoplasm requires nuclear pore complexes such as transport receptors. One such essential nuclear export receptor is Exportin 6 (Exp6) or Ellipsoid Body (Ebo) in *Drosophila*, which mediates nuclear export of Profilin-Actin complex (Stüven et al., 2003). *Drosophila* and human Exp6 shares approximately 20 % of identical amino acids, however, the function in actin export seems to be conserved. Knocking down Exp6 results in accumulation of actin in the nucleus (Dopie et al., 2012) in contrast overexpression of *exp6* driven by MTD-Gal4 enhanced the nuclear actin export that resulted in significant decrease of nuclear actin molecules (Fig 17E and E') (Thran et al., 2013). We analyzed repositioning of *hb, Kr* and *eve* loci in Exp6 overexpression and Exp6 loss of function, and found that movement for all three loci was significantly halted or slowed down, suggesting that change concentration of nuclear actin levels in nucleus indeed does have an impact on re-localization. To further support this idea we also analyzed other actin-binding protein that is involved in nucleo-cytoplasmic shuttling of actin molecules. For example, in *chickadee* loss of function mutant where export of actin is lost all three gene loci showed acceleration. These observations indeed could prove that nuclear actin plays a role in chromosomal re-organization. However, we could not show a change in nuclear actin levels within these factors since staining nuclear actin in our previous experiments didn't show any conclusive results. Further, we showed that re-positioning of the locus is a pre-requisite for silencing and determines the competence window size with the experiment where additional U motoneurons were generated in response to a transient pulse of ectopic Hb protein late in NB7-1 competence window at stage late 11 and stage 12 in Exp6 overexpression (reduced nuclear

actin levels). This indeed supports the idea of the causal relationship between gene locus re-localization and NB competence. There are several examples depicting the important role of nuclear actin in gene locus re-localization in a vertebrate system. Also, it has been shown that polymerization and non-polymerization of nuclear actin indeed have significance in this re-localization process. For instance, in inducible transgene Chinese Hamster ovary cells it was observed that tethering the transcription acidic activation domain (AAD) of the viral protein VP16 to a peripheral site led to the repositioning of the chromosome site from the nuclear periphery towards the nuclear interior and this repositioning was halted after expression of non-polymerizing mutant (G13R) and accelerated when nuclear actin was strongly polymerized (S14C) (Chuang et al., 2006). Also, Dundr et al (2007) revealed the occurrence of directed movement over a distance of 2-3µm by examining the interaction between the Cajal bodies (CBs) and U2 genes and they too could observe that expression of G13R actin mutant inhibited the repositioning of the locus. For further examination of the role of actin/myosin involvement in the gene locus movement, Hu et al. (2008) chose ERα-mediated inter-chromosomal interactions. They treated E2-stimulated breast epithelial cells the drugs latrunculin, which blocks actin polymerization and jasplakinolide, which in turn inhibits actin depolymerization. In both cases, the ERα-mediated inter-chromosomal interactions and even the activation of ERα target genes are prevented. In order to further support this phenomenon in our system we also generated flies, which express a dominant/negative (D/N) G14R (Glycine to Arginine) and a gain of function (GoF) mutated form of actin S15C (Serine to Cysteine), both lines tagged with GP-NLS sequence, similar to vertebrate system. We found that all three *hb, Kr* and *eve* loci in G14R mutant undergo slow re-positioning and in case of S15C mutant the movement was faster. These phenotypes were indeed similar to vertebrate system showing this function of actin is conserved from mammals to insects. All these studies mentioned above, point out that actin-dynamics in the nucleus have a direct or indirect effect on gene positioning and long-range movements of chromatin. However, how does nuclear actin perform this function? As mentioned in examples above nuclear actin and myosin provide molecular motors driving directed movements of gene loci towards a target region. Nevertheless, direct evidence for such a mechanism is currently lacking, however, there could be another possible scenario to explain this. For example, it might be that actin dependent chromatin remodeling leads to a relaxation of chromatin structure allowing looping of an activated locus. Diffusing chromatin loops might then collide with other nuclear compartments until specific molecular interactions stabilize a defined association (Reviewed by Carmo-Fonseca, 2007). Next, our data shows that re-localization of *Kr* and *eve* loci are

dependent on Hb activity. As when Hb is overexpressed re-localization of both of these loci is halted and slowed down and in absence of Hb premature acceleration is seen. It could be explained as above that in continuous Hb expression chromatin is loosened allowing locus to be active and reduced movements and in case of its absence, it might lead to tightening of chromatin and faster movements resulting premature silencing. But, it still remains enigmatic that how Transcription factor and actin interact? One can say that there are certain epigenetic factors that might play role in this situation. Altogether it shows that transcription factors also control, either directly or indirectly, chromatin dynamics leading to particular higher-order activity-dependent nuclear arrangements of chromatin. It will be fascinating in future to investigate in detail the exact mechanisms underlying such chromatin movements associated with gene activation. In our model system i.e. embryos it was rather difficult to stain nuclear actin hence for detailed understanding of nuclear actin, especially in the context of actin polymerization, it is critically important to develop new probes and systems to monitor nuclear actin dynamics (Belin et al., 2013; Plessner et al., 2015) and to distinguish between nuclear and cytoplasmic actin. It is equally important to use genome-wide or large-scale approaches to identify interactome of nuclear actin (Rohn et al., 2011; Samwer et al., 2013; Dopie et al., 2015). Structural insights into nuclear actin (Cao et al., 2016) will also accelerate our understanding. Another important fact in all the studies by Chuang et al., 2006, Dundr., et al 2007 and Khanna et al., 2014 shows the gene locus movements in interphase nuclei, however, in our case NBs are in the constant division so it is indeed difficult to predict precise cell cycle stage of NBs. Our preliminary data did point in this direction wherein it was seen that in String mutants where the G2/M transition is blocked the re-localization was halted (Nadja Dinges master's thesis) and it has been shown by Tumbar and Belmont that chromosome sites move into the nuclear interior in early G1 and early S phase. The arrest of String mutant NBs in a G2 state does, therefore, inhibit the mitotic division of the cell, preventing NBs from entering a G1 or S phase, in which chromosome movement was found to take place. Another example is re-localization of *hb* locus is halted in Pebble mutant where cytokinesis is blocked resulting in multinucleated cells (Verena Engelhardt bachelor's thesis). However, further detail studies are necessary to understand the exact role of cell cycle progression in our model system and how does it affect directly the movement.

4.6 Potential epigenetic regulation in silencing of loci

Until now we could see during development that change in competence is correlated with change in positions of *hb, Kr* and *eve* loci, where they are active at the start of competence phase and moves towards specific regions within the nucleus of NBs to be possibly silent when the window is closed. We could predict that nuclear actin might be possible factor necessary for such repositioning. Additionally, polymerization of actin does play important role in this process, as strongly polymerized actin increases HDAC-1 activity resulting in decreased histone acetylation (Serebryannyy et al., 2016) which might result in silencing of the locus. Class I histone deacetylases (HDACs) are known to remove acetyl groups from histone tails. This releases positive charges on the histone tail and resulting in tight DNA wrapping, thus preventing transcription factor binding and gene activation. This epigenetic change thus halts transcription machinery ultimately affecting cell proliferation and survival (Dokmanovic et al., 2007). RNAi knockdown of Rpd3 (*Drosophila* HDAC1) resulted in slow down or halt of *hb, Kr* loci, thus suggesting that silencing might be epigenetically regulated. However, it is still to be proved in *Drosophila* if such interaction between actin and HDAC exists, but since these proteins are well conserved in many species such phenomenon is still plausible. We also tested the knockdown effect of another epigenetic factor Polycomb (Pc) on gene locus re-localization and found a similar result that repositioning of all three loci is slowed down. There could be following explanation for this phenotype, it was shown by Touma et al. that a loss-of-function mutation of the Polycomb repressor complex extends the competence of Kr to induce Eve+ U3 neurons, which, in turn, suggests that *eve* becomes silenced by PcG bodies. In addition to this, it has been shown that *Posterior sex combs* and *polyhomeotic* interact with *Krüppel* and enhance embryonic phenotypes of *hunchback* and *knirps*; and in *polyhomeotic* mutants, Eve expression is significantly high as compared to the normal situation n (Mckeon et al., 1994) thus suggesting that indeed silencing of *eve* is epigenetically govern. Looking at all these possibilities it can be predicted that our results do point to the direction of ongoing epigenetic silencing of all three gene loci. However, the more detailed analysis is necessary to understand this process, which can be done by carrying out NB specific ChIP analysis and look for epigenetic marks present on all three loci.

4.7 Working hypothesis

I speculate following two possible mechanisms that might occur in parallel

Mechanism 1:

Loss of competence for neuroblasts can be regained by combined ectopic expression of transcription factors Hb and Kr late in development. During this, we also observed a shift in gene loci that is precisely correlated with change in competence. *hb* locus re-localize from the nuclear interior at the start of window to the periphery at end of competence (Kohwi et al., 2013), *Kr* locus re-localizes within the relevant time window but in the opposite direction: initially it is detected in the periphery but changes into the nuclear interior. *eve* locus is re-localizing from nuclear interior towards nuclear periphery in most of NB population similar to *hb*. Interestingly the movement of the *Kr* and *eve* locus can be inhibited by continuous Hb/Kr co-expression. This suggests that both genes, *Kr* and *eve*, might get silenced and that this could be dependent on the observed developmentally regulated re-localization patterns. However, in contrast to *eve* and *Kr*, the re-localization of *hb* cannot be blocked by continuous Hb/Kr co-expression suggesting that there are differences in the regulation of these processes. However we found out that Seven-up (Svp) *Drosophila* COUP-TF functions as an important timing factor that possibly could act as a trigger to initiate the process of silencing.

Mechanism 2:

In quest of the mechanism responsible behind the re-localization, we found evidence that polymerization of nuclear ß-actin is involved in such events. So how potential silencing of observed gene loci is taking place? In vertebrates, polymerize nuclear actin interacts with HDAC1 and increases its activity (Serebryannyy et al., 2016). Our data shows that polymerization leads to accelerated gene locus re-localization and RNAi of HDAC1 leads to slow down of repositioning. We also showed that Polycomb-RNAi resulted in slow down of gene locus re-localization. Question raises that is there possible interaction between Pc and HDAC1? It has been shown that HDAC1 (Rpd3) interacts with Polycomb for silencing of homeotic genes (Chang YL, et al., 2001), so in our case it could be possible that such interaction is taking place. Furthermore, it has been shown that during early patterning in embryos: Hb recruits Pc in order to silence its target genes e.g. homeotic genes (Kehle et al., 1998). So it could be possible that such mechanism exists in our case. In conclusion, we can say that within early competence window there are nuclear actin dependent gene loci re-

localizations taking place. And there is potential epigenetic regulation which can be inhibited partly or reverted by combined Hb/Kr activity.

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