HAEMOCYTE & TUMOR INTERPLAY IN A NEURAL STEM-CELL-DERIVED MODEL OF TUMORIGENESIS IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Drosophila melanogaster is an ideal system to study stem cell biology and innate immune responses. Through its genetic tractability and the evolutionary conservation of molecular effectors, the fly constitutes a workhorse for functional and lineage tracing studies. In this work, we built upon recent advances in innate immunity concerning tumorigenesis by incorporating a model of allograft series from neural-derived (primary) tumors. Powerful genetic tools allowed us to, temporally, obscure the lineage progression of the neural stem cell population (*neuroblasts*), specifically, in the larval brain. During larval neurogenesis, the majority of the adult neurons and glia are generated from asymmetric divisions of these neural stem-like cells. Neuroblasts (NBs) are endowed with *spatiotemporal* cues that unfold a highly stereotyped and dynamic transcriptional trajectory during the asymmetric segregation of their daughter cells, called *ganglion mother cells* (GMCs). This remarkable division mode ensures two distinct fates. First, NBs retain their stem-like characteristics, performing repeated rounds of asymmetric division. Second, the resultant GMCs are gradually specified and divide once more to generate post-mitotic neurons/glia. Notch-signaling and a plethora of conserved effectors are essential for the developmental orchestration of proper NB-lineage progression. Through RNA-interference (RNAi) we abrogated the self-renewal capacity of NBs by knocking-down the RNA-binding protein Imp (insulin-like growth factor II mRNA-binding protein) and the proto-oncogene Myc. In combination, we overactivated N-signaling within the NB lineages during larval neurogenesis. This resulted to a hyperplastic larval central nervous system that facilitated the foothold for our experimental method. By injecting these primary tumors in adult hosts, we set out to explore the tumor progression *in vivo*. Previous work in our laboratory from Eva Zacharioudaki and Chrysanthi Voutiraki has shown close interaction between tumor secondary masses and the cellular arm of the fly's immunity, called *haemocytes*. Part and parcel of the adult immunosurveillance system, haemocytes (plasmatocytes) exhibit phagocytic activity and active migratory behavior, mimicking the macrophage population of vertebrates. Focused on the neural stem cell-derived tumors and haemocyte interplay, we generated a survival screen of adult hosts deficient in haemocyte-specific genes (RNAi) and identified two scavenger-receptors as essential for preserving host lifespan during tumor progression. This work establishes an early view of the dynamics of tumor and haemocyte interaction and provides the framework of an *in vivo* model to decipher the fundamental mechanisms of tumorigenesis within an invertebrate system.

1. Introduction

1.1. Innate Immunity in the Invertebrate Model Organism *Drosophila melanogaster*: The Fly's Blood System & Its Two Pillars

Invertebrate immunity shares conserved evolutionary and functional similarities with vertebrate innate immune responses in the context of pathogen recognition, regulation of inflammation and wound healing[1–3]. Elegant studies in both vertebrate and invertebrate species, have largely shown a remarkable phylogenetical conservation of cellular and molecular mechanisms involved in host homeostasis. Evidently, in *Drosophila* the nature of these responses is dependent on the type of the initial stimuli. In the paradigms of fungal and bacterial infection -or in the extreme case of larval parasitoid manifestation- the immune response differentiates in terms of the molecular mechanisms that resolve each type of insult in flies.

The blood system of the fly is an open circulatory system called *haemolymph*. Despite its primitive origin, it functionally resembles the blood system of vertebrates. In the occurrence of injury or pathogenic entry, the haemolymph constitutes the first line of defense by providing a systems-level platform for recognition of circulating danger- and pathogen-associated signals, respectively. The cellular mediators of the fly's *immunosurveillance* response are collectively called *haemocytes* (blood cells) and can freely circulate in the haemolymph or localize in tissues and organs to function as resident cells, in the same fashion like their mammalian equivalents (macrophages). The fly's blood cells comprise three morphologically distinct categories that also differ in their expression profiles[4] and developmental specification[5]; lamellocytes, crystal cells and plasmatocytes. *Lamellocytes,* are the largest, size-wise, of the blood cells with an extended network of laminar processes. Upon extreme conditions of physiological infliction (injection of wasp parasitic eggs), they are activated and function to encapsulate debris or (extrinsic) foreign bodies that cannot be cleared by the remaining blood cell types. *Crystal cells*, are platelet-like cells that are enriched in crystallized vesicles of prophenoloxidase essential for melanization reactions during wound closure. Immune-activation leads to their rupture and subsequent containment of infectious agents (opsonization). Plasmatocytes are the only blood cell within the haemolymph and tissues of adult flies that exhibit phagocytic activity. In contrast to lamellocytes that encapsulate too-large-to-be-phagocytosed foreign bodies, plasmatocytes actively recruit cytoskeletal re-organization complexes and signaling cascades necessary to facilitate membrane protrusions to envelop bacteria, fungi or even apoptotic cells[6].. The 2nd pillar of the fly's immune-responsive system comprises the *fat body* tissue which combines the mode of action of the vertebrate liver and adipose tissue[7]. The fat body is responsible for the production and secretion of *antimicrobial peptides* (AMPs) in the haemolymph upon JAK/STAT induction mediated by plasmatocytes[8,9]. These small and potent cationic molecules can reduce the bacterial or fungal burden and, therefore, relieve the host of the infection.

1.2. The Role of *Haemocytes* in the Adult Fly (Host Homeostasis)

1.2.1. Two Flavors of Developmentally-orchestrated Haematopoiesis in *Drosophila*

Drosophila's blood cells are differentiated cellular immune effectors that originate from embryonic *prohaemocytes* and larval lymph gland *progenitors*. The *embryonic* origin of blood cells is

developmentally hard-wired and constitutes the backbone of larval hematopoiesis by contributing to the larval routine homeostasis. Embryonic plasmatocytes, along with the smaller embryonic populations of *lamellocytes* and *crystal cells*, are retained upon larval hatching (embryo-to-larva). This is referred to as the *1st wave* of haematopoiesis and ensures expansion of blood cells that will later populate the adult immunosurveillance system. The *2nd* and final *wave* of haemocyte production, originates from the synchronized differentiation of *larval lymph gland* progenitors. Notwithstanding their differential developmental and anatomical specification, the lymph gland progenitor population exponentially grows, although not indefinitely, and differentiates into haemocytes during late stages (3rd instar-pupa stages). Prior to metamorphosis, the lymph gland (lobes) will disintegrate and the totality of differentiated progeny (lymph gland haemocytes) generated will be retained together with the tissue macrophages (embryonic-larval), as a hybrid (intermixed) population in the adult fly. The existence of, both, embryonic plasmatocytes (known as tissue-resident macrophages) and haemocyte progenitors from the lymph gland niche (larva), support the notion of an evolutionary conserved binary *myeloid blood cell system* documented in vertebrate tissues[5]. This is also evident from the homology in the blood cell specification program (GATA-transcription factors) between invertebrates and vertebrates.

The adult fly has been under scrutiny in recent years concerning its utility as a model system to study blood cell proliferation upon environmental induction. Despite discrepancies originating from one particular study that claimed the initiation of a 3rd wave of haematopoiesis in the adult stage[10], a new study redefined the developmental trajectory of embryonic and larval (lymph gland) haemocytes that are retained throughout adulthood. Instead of an increase in the blood cell numbers upon adult maturation or bacterial infection, researchers described a stereotyped migratory redistribution of haemocytes which is finalized within the first week of adult life. Initially, adhering to larval fat body cells, the larval haemocytes that persist into adulthood, are afterwards released in the adult haemolymph upon cytolysis of the larval-originating fat tissue. Consistently, within the first week (*maturation*), the circulating blood cells, ultimately, reside in tissues and organs of the adult fly. Significant enrichment is present in the *respiratory* epithelia (trachea) and the coagent *fat body* (liver-adipose tissue) regions of the fly[8]. However, this restriction in localization within the host is not indicative of a dormancy-like state. Evidently, bacterial infection, independent of the site of permeation, induces the migratory recruitment of plasmatocytes, not accompanied by proliferation.

1.2.2. The Signaling Orchestration of Immune-activated Haemocytes

There has been a tremendous effort to decode the molecular machinery underpinning the cellular arm of innate immunity and humoral response in flies. Adult haemocytes (95% plasmatocytes) have been recently redefined as immune mediators of a multi-tissue promotion of host homeostasis through a plethora of converging signaling pathways such as Toll, JNK and JAK/STAT signaling[3,8,11].

Toll and Imd Pathways

Within the context of inflammatory response, several lines of evidence have demonstrated a key role of adult plasmatocytes following septic injury[12,13]. Circulating and tissue-resident haemocytes in the adult fly, owing to their potent phagocytic activity and their active recruitment, constitute the immediate effectors of the fly's immunosurveillance system. However, pathogenic entry of bacteria and fungi elicit different transcriptional outcomes depending on the type of recognition from haemocytes and the fat body, a potent immune-responsive tissue. Fat body cells, as well as

haemocytes, express receptors of the Toll and Imd pathways that converge in the upregulation of a plethora of secreted cytokines and peptides with antimicrobial activity (AMPs). Concomitantly, their downstream effectors contribute to the activation of 80% of all genes induced upon septic injury[14]. Toll receptors lack the ability to recognize molecular patterns associated with pathogens, in contrast to their vertebrate orthologues Toll-like receptors (TLRs), and instead depend on ligand-binding of the secreted cytokine *Spätzle* to initiate signaling. Specifically, within haemocytes, Toll and Imd signaling upregulate defense-responsive and stress-induced genes. The activation of these signaling cascades is necessary for maintaining primed haemocytes as initiators of a systemic response, a process apparently dependent on ROS-accumulation (discussed below).

Redox-sensitive Apparatus: Reactive Oxygen Species

Damage-associated signals (damage-associated molecular patterns, DAMPS) originate from the breached epithelial barrier that functions as the first line of defense against microbe infiltration[15]. Debris of apoptotic cells and *reactive oxygen species* (ROS), resultant from the breakage of the epithelial layer, act as inflammatory signals for haemocyte recruitment, in the same fashion that leukocytes and macrophages actively infiltrate damaged tissues of a mammalian host. Recently, one particular study shed light on the series of events that unfold after (septic) injury in adult flies[16]. Focused on the process of hemocyte-mediated resolution of trauma, researchers unlocked new insights into the role of ROS for haemocyte priming and cross-communication between immune-responsive tissues.

The apparent ROS release within the haemolymph, originating from the epithelia, is necessary and essential for the recruitment of circulating and, tissue-resident haemocytes adjacent to the wound. The redox-sensitive apparatus of haemocytes consists of many different enzymes that differ in their mode of action and localization. Two transmembrane NADPH oxidases, *Duox* and *Nox*, synergistically elevate the extracellular levels of ROS upon plasmatocyte recruitment. In contrast, the anti-oxidant secreted enzyme *immune-related catalase* (*IRC*) and cytosolic effectors, *catalase A* (CatA) and s*uperoxide dismutase 1* (SOD1), maintain an unresponsive-state of haemocytes upon trauma as shown by gain of function experiments. The resultant ROS overproduction following injury is attributed to the activation of circulating haemocytes, since many melanin precursors can be potentially utilized for ROS production. Within haemocytes, the biosynthetic cascade of melanin is based on the JNK-dependent proteolytic cleavage of the precursor molecule *pro-phenoloxidase* (PPO) to the bioactive form of the enzyme *phenoloxidase* (PO). Melanin is an essential factor for wound closure and forms as the final heteropolymeric product of a two-step catalytic process mediated by PO. The oxygenation of monophenols to O-diphenols, and sequential oxidation to O-quinones, produces the substrates for melanin's polymerization, therein many by-products of PO's activity can reinforce the ROS pool. Evidently, the diffused wave of ROS following trauma and its observed overproduction after haemocyte recruitment are necessary to promote host homeostatic responses through a *systemic wound response* (SWR).

The hierarchical scheme of redox-modulated SWR includes many intrinsically-driven signaling cascades in spatially distinct tissues of the host. ROS function initially as secondary messengers to recruit blood cells and impose cytoprotective transcription programs in nearby cells. The dissection of the molecular mechanisms that mediate a systemic response in adult flies during

the earliest events following trauma, have highlighted the role of haemocytes as potent and essential enhancers of ROS production.

Initially, H2O2 accumulates intracellularly of wound-recruited haemocytes, through the aquaporin-like protein *Prip* (*AQ1 mammalian orthologue*) located in the plasma membrane (passive diffusion)[16]. Duox oxidase contributes to the elevated H2O2 levels observed within haemocytes, leading to the transcriptional activation of the cytokine Upd3 (Toll-target). The link between ROS overproduction and Toll-activation is unknown, however, it seems to depend on the axis of the Src42A/Shark kinases and the scavenger receptor Draper. Collectively, the integration of DAMPs (ROS) within recruited haemocytes to the trauma region is necessary for the production of the inflammatory cytokines (Upd3). The Upd3 secretion binds to the receptor *Domeless* expressed in fat body cells and leads to the generation of AMPs through JAK/STAT-activation. These data support the hypothesis that haemocytes transduce an integrated sum of signals to generate a systemic response of different organs and tissues of the adult fly.

1.3. Genetic Tools for Spatiotemporal Manipulation of Gene Expression

The genetic tractability of *Drosophila melanogaster* allows us to perform unparalleled functional studies. Through the combinatory use of transgenic expression systems, site-specific recombination approaches and fluorescent protein genes, any cell or tissue, can be specifically labelled *in vivo* in a reproducible pattern. More importantly, the same technologies can be combined with gain- or loss-of-function tools (like RNAi, CRISPR) to enhance or suppress, respectively, the expression of a candidate gene [17,18].

The UAS/GAL4 binary expression system has been used extensively in *Drosophila* developmental studies in the past three decades. It consists of two-components; the yeast transcriptional activator GAL4 protein and a specific GAL4-binding sequence (UAS, *Upstream Activating Sequence*), driving a transgene's transcription start signal. The power of this technology lies in the fact that UAS/GAL4-mediated overactivation of effector-genes, is only limited by the availability of known promoter-fusion lines to mediate the cell-specific co-expression of the GAL4 protein. Additionally, a UAS/GAL4 repressible switch has been generated in the form of the *GAL80* suppressor protein. GAL80 can inhibit GAL4-driven expression when expressed simultaneously by binding to the GAL4 carboxy-terminal (the Gal4 activation domain). Temporal control, can be superimposed by expressing a temperature sensitive mutant form ($GAL80^{ts}$) that is active at the permissive temperature of 18°C but inactivated in the non-permissive temperature ≥ 29°C). This three-component expression system (UAS, GAL4, $GAL80^{ts}$) forms the backbone of technologies used for temporal gene-function analysis and cell-lineage tracing. Another technology, relies on a yeast recombinase enzyme (Flp, flipase) which recognizes a pair of 34 base-pair-long sequences called *FRT's* (Flipase Recognition Targets). These sequences can be artificially inserted into a transgenic construct and, depending on their orientation, flanked DNA-sequences can be excised upon flipase-recognition (known as "Flp-out"). There are specific considerations concerning the optimization of the Flp-out technique. Spatial manipulation of gene expression can differ depending on the defined promoter controlling flipase expression, the position and orientation of the FRT docking sites and the "flanked" (transgenic) sequence; insertion of a FRT-flanked stop codon

(polyadenylation signal) upstream of a transgene's coding sequence re-establishes transcriptional activation upon "flip-out" (for example, in the construct actin::FRT-stop-FRT::transgene). Alternatively, an FRT-flanked transgene can be excised by switching the coordinates of the FRT sites in the transgenic cassette (as shown in the construct, actin::FRT-transgene-FRT-stop).

One of the great utilities of the Flp-out technique lies in its compatibility with gene expression systems such as the UAS/GAL4. By placing Flipase under the control of a heatshock promoter (hsflp), temporal control can be exerted in two -conceptually identical - ways. In the first paradigm, a transgenic cassette containing an upstream FRT-flanked poly-adenylation (pA) signal, is under the control of a UAS promoter. Even though GAL4 expression is independently controlled (through a constitutively or tissue-specific active promoter), the transcription stop signal will be eliminated only after heatshock-mediated activation of flipase (in the construct, hsflp; UAS::FRT-stop-FRT::transgene). Alternatively, the GAL4 coding sequence can be integrated within the transgenic cassette, downstream of the FRT-flanked stop codon (in the construct, hsflp; actin::FRT-stop-FRT::GAL4,UAS::transgene). Thus, flipase activation controls the time period of GAL4 transcription and, concomitantly, transgene overexpression. In both instances, heatshock-mediated flipase activation controls the temporal overexpression of a transgene. It is important to note, however, that the recombination event happens with incomplete efficacy, therefore the resulting animal (after heatshock) is a mosaic with random clones carrying the flipped-out transgene, whether Gal4 or UAS.

1.4. Neuroblasts & Where to Find Them: A Stem-like Population of the CNS

The invertebrate nervous system reflects an evolutionary conserved composite structure that lays out the fundamental underlying principles of neural circuitry and functional assembly, as seen in the higher order brain organization of vertebrate species. Despite their vast differences in size connectivity, neuronal and glial cell type diversity, and in many properties of electrophysiology, invertebrate CNS has been under vigorous study for biological processes regarding stem cell biology, asymmetric cell divisions (ACDs) and developmental patterning of neural precursors.

The CNS of *D. melanogaster* is composed of neurons, glial cells and a progenitor (stem cell-like) population, referred to as *neuroblasts*. By definition, stem cells are characterized as pluripotent cells, endowing their progeny with distinct cell fates whilst maintaining their unlimited self-renewing capacity. Through a series of asymmetric cell divisions (discussed below), neuroblast lineages will eventually shape the larval and adult brain in a highly reproducible and stereotypic fashion [19].

1.4.1. Origins: Neuroblast Specification

Neuroblasts (NBs) originate from a pool of developmentally equipotent cells in the ventral neuroepithelium of the developing *Drosophila* embryo. Early in embryogenesis, cells of the *ectoderm* are specified according to positional information provided by patterning gene cascades during the establishment of the anteroposterior and dorsoventral body axes[20]. Following a developmentally fine-tuned wave of proneural gene expression, neighboring neuroectodermal cells cluster into interspaced regional patterns and compete against each other for acquiring two distinct

fates; epidermal or neuronal. From each proneural cluster, only one (dominant) cell will acquire the neuronal fate (future NB) in a process called lateral inhibition and mediated by the Notch signaling pathway. Once specified, this single NB will segregate from the surface interiorly in a morphogenetic movement called *delamination*. The remaining cells in the cluster will acquire epidermal fate and form the ventral epidermis[21].

1.4.2. Overview: Notch Signaling in a Nutshell

Notch signaling (N-signaling) regulates a myriad of developmental choices in mammals and invertebrates, alike, and as any other major developmental module there is ample evidence of its role in disease and cancer. All ligands and receptors of Notch pathway are transmembrane proteins (apart from some ligand paralogues of the nematode organism *Caenorhabditis* elegans). Specifically, *Drosophila* harbors only one Notch receptor and two distinct ligands Serrate/Delta, whereas in the mammalian system, Notch1-4 paralogues and various ligands of the Delta-like (DLL) and Jagged (JAG) protein families contribute to a more complex signaling outcome. Importantly, different members of the Notch family of ligands and receptors, exhibit distinct properties concerning the strength and duration of the signaling cascade, their expression patterns as well as the rate of their recycling from the endocytic trafficking system.

The ligand-mediated Notch activation is based on the generation of an attractive force during the cell-to-cell interaction, originating from ligand endocytosis. This mechanical interaction is necessary and sufficient to reveal a highly folded extracellular region of the Notch receptor, called S2 cleavage site and occluded by the Negative regulatory region (NRR)[22]. The exposed S2 site can then be recognized and cleaved by the transmembrane metalloprotease ADAM, rendering the remaining intramembrane cytoplasmic fragment of the Notch receptor a recognition site for the γ-secretase complex. Within the signal-receiving cell, both sequential proteolytic cleavages are required for the production and intracellular release of the bioactive form *Nicd* (Notch Intracellular Domain), which constitutes the main effector of the Notch pathway [23,24]. *Nicd* then enters the cell nucleus and interacts with a protein machinery composed of the DNA-binding proteins *CBF1–Su(H)-LAG1* (CSL) and the co-activator *Mastermind* (Mam). The *Nicd*-CSL-Mam apparatus promotes gene expression by binding CSL specific cis-regulatory elements (enhancers). Through co-recruitment of chromatin remodeling complexes, different local epigenetic modifications can be facilitated [24–28]. Hence, in the nuclear context, the repertoire of Notch-responsive genes can differ greatly according to the cell-type- and stage-type-chromatin landscape.

1.4.3 Neuroblast Lineages Arise from Asymmetric Cell Divisions

Anatomy of Drosophila's CNS

The entirety of the larval and adult CNS of *Drosophila* is the product of a highly stereotyped sequence of repeated divisions of neural progenitors. *Drosophila*'s CNS is composed of two brain regions; the *Central Brain* (CB), with its two lobes, and the *Ventral Nerve Cord* (VNC), which is further subdivided into the abdominal and thoracic ganglia. To make a simplistic vertebrate analogy, the CB is the computational and sensory integration brain region, whilst the VNC resembles the spinal cord, in that, it is responsible for propagation of sensory stimuli and motion.

Embryonic & Larval Waves of Neurogenesis

NB lineages arise in two sequential waves of neurogenesis. The 1st wave of *embryonic neurogenesis*, initiates right after NB embryonic delamination and involves the production of the majority of the larval CNS, much of which will be retained in the adult fly (after metamorphosis). After a progressive restriction of asymmetric divisions, owning to gradual loss of cellular volume, embryonic NBs enter quiescence (G0) and stop dividing. The 2nd wave of larval (post-embryonic) neurogenesis, initiates with the cell-cycle restart of quiescent NBs after larval hatching and involves the generation of all the remaining neural and glial types, amounting up to 90% of the adult CNS[19,29,30]. Before metamorphosis, larval NBs exit mitotic proliferation, mediated by steroid hormone (ecdysone) signaling.

Importantly, in each step of the way, NBs generate progeny in a highly hierarchical manner (lineage *specification*) and terminate their proliferation (*differentiation or apoptosis*), by being responsive to extrinsic (hormonal) and positional developmental cues. This strict sequence of events is referred to as *spatiotemporal pattering* of NB lineages. Albeit, uncertainties remain concerning the lineage-specific developmental trajectories, recent efforts have exponentially advanced our knowledge for larval NB lineage identities[31].

Division Mode of (Stem-like) NBs

In *Drosophila*, NBs differ in their division cycles according to the spatial origin of their specification. Collectively, these neural precursors contribute to a variety of differentiated neural types through a*symmetric cell divisions* (ACD). This remarkable division mode results to the segregation of daughter cells that are diversified depending on the order of their birth. Asymmetrically dividing NBs, require *ab initio* establishment of an *apical-basal polarity* axis. This polarization event, orients the mitotic spindle (deciding the plane of division) and secures the sequestration of *basal cell fate determinants*. During mitosis, these segregating determinants will be inherited, unequally, between the two daughter cells. Following asymmetric division, the larger in size cell will form the daughter NB, retaining the ability to self-renew, and the smaller cell, called *ganglion mother cell* (GMC), will divide once more to generate a pair of post-mitotic neurons (or glia)[19,32]. This scheme of divisions is the backbone of every embryonic and larval (*Type I*) NB lineage. Hereafter, we will focus on the 2nd wave (post-embryonic) of neurogenesis in *Drosophila* CNS.

Two Types of Larval NBs

In larval neurogenesis, stem-like progenitors in the central brain are subdivided into two types of NBs. Despite sharing the same pattern of ACDs, type II NBs are characterized by an additional transit-amplifying step of proliferation. The newborn daughter cells are referred to as i*ntermediate neural progenitors* (INPs) and after maturation (mINPs), they will self-renew three to five more times to produce a single GMC per division. The overlapping self-proliferative capacity of NBs and mINPs, contributes to an increased order of magnitude of GMCs production -compared to single (type I) progenitors- expanding the post-mitotic pool of neurons and glia from each type II lineage.

1.4.4. Temporal Patterning: The "Salt & Pepper" of Lineage Diversity

When one compares the division programs of embryonic versus larval NBs, the inability of the former to regrow after each proliferation and the existence of type II larval-specific lineages in the latter, partly, explain the vast differences in post-mitotic progeny generated in each developmental stage. However, variety in neuronal identities goes beyond numerical differences in lineage descendants. Instead, all NB lineages are endowed with temporal patterning cues during development to generate the enormous cellular *diversity* needed in a nervous system.

Temporal patterning is a dynamic developmental process in NB lineages, that, uniquely, specifies the identity of post-mitotic progeny, using a cascade of birth-order-specific transcription factors[33]. Depending on the developmental stage (embryo or larva) or NB anatomical location (mushroom body, optic lobe etc.), the sequential expression of these *temporal transcription factors* (tTFs) may differ. In the example of larval CB and VNC, there are two main tTF windows of expression that fine-tune the transcriptional program of (type I) NB lineages, with their presence being paralleled by neuronal identity series (discussed below).

During the embryo-to-larval transition, quiescent embryonic NBs of the VNC, re-enter the cell cycle, whilst retaining the last embryonic tTF expression, namely *castor* (Cas)[34]; Cas is the first transcription factor of the larval temporal series, followed by *Seven-up* (Svp). Taken together, the sequential *Cas-to-Svp* progression within NBs, specifies a temporal window that depends on the expression of the protein *Chinmo*, which subsequently specifies the generation of *early-born* post-mitotic neurons[35]. The step-wise transition and unidirectionality of the series is dictated by transient *cross-regulatory* interactions between temporal transcription factors, like Cas and Svp, which result in permanent chromatin changes in their offspring, directly influencing their particular neuronal/glial subtypes. This synergistic module of feed-forward and mutually repressive interactions between tTFs, consists of strictly *intrinsically-driven* transcriptional switches within NBs. Recent studies have also reinforced the fact that, in some cases, NBs remain responsive to extrinsic cues for temporal lineage succession as well as for their terminal differentiation (cell-cycle exit)[35,36].

Balancing Self-renewal & Differentiation in Aging NBs

In the early larva, Cas's expression leads to the permanent expression of the transcription factor *Grainyhead* (Grh) which is retained in all larval NBs, whilst ensuring the inhibition of the embryonic-specific *Dichaete* (D) expression. Later, Svp's expression triggers a switch in the expression of two mutually antagonistic RNA-binding proteins, *Imp* (IGF-II mRNA-binding protein) and *Syncrip* (Syp). The sequence of the D-to-Grh (Cas-mediated) and Imp-to-Syp (Cas-to-Svp-mediated) transitions is essential to establish the proliferative properties of larval NBs and direct fate commitment to their neural lineages. Importantly, due to their dynamic expression and the reproducible patterns of NB lineages, tTFs and their effectors (Imp/Syp) can function as stage-specific markers of the developmental trajectory underlying lineage progression[33,37]. In the absence of Cas-to-Svp transition, either through Cas misexpression or Svp down-regulation, the early temporal program continues aberrantly due to the inactivation of Syncrip. This translates to late-born identities (dependent on Syncrip) being abolished, while early-to-mid larval NBs (expressing Imp) retain their stem-like characteristics and *continue proliferating* even during adulthood[38].

Consistently, two overlapping developmental modes of NB behavior exist during larval neurogenesis. The first in the hierarchy is the *self-renewal-permissive* state that encompasses all larval stages (early-to-late). During this temporal window, NBs present a high proliferative capability, although with a significant degree of asynchronization between various types of larval stem-like cells, which gradually declines towards late-larval stages. This self-renewal state depends on the expression of proteins promoting growth and stem cell identity. Chinmo and Myc mRNAs are both positive targets of the temporal effector Imp during early larval neurogenesis. Both gene products originate from proto-oncogenes that facilitate the regrowth of each NB after ACDs, a salient feature for enabling cell division, as seen in embryonic asymmetric divisions[37,39]. Secondly, the *differentiation-permissive state* initiates with Syncrip expression and defines the late larval stages (prior to metamorphosis). It is important to note that the two-states are not mutually exclusive. During the ACDs of early-larval NBs, the progeny can differentiate into *Chinmo-positive* neurons. However, the Imp-to-Syp transition gradually exhausts the self-renewing potential of larval NBs and allows their terminal differentiation in later (pupal) stages upon induction from a steroid hormone (*ecdysone*) and other signals. This event characterizes the termination of NB lineages, leading to a progressive depletion of the progenitor stem-like pool[40]. There has been some evidence of neurogenesis in adult flies, albeit, limited and NB-independent[41]. Hence, there is a fine line between restrictive and unchecked perpetuation of neural stem cells during development. Several lines of evidence have incriminated the NB juvenile-state state as a window of opportunity for NB hyperproliferation during the larval stages (*tumorigenesis*)[38,42].

The self-renewal state of NBs in early larval neurogenesis, is a critical point for proper CNS development of the adult fly. One could portray the window between the re-activation of the (dormant) embryonic NB population and its terminal differentiation (pupal stages) as its putative developmental boundaries. It is worth noting that mitotic proliferation is intrinsically regulated in each stage-specific NB and the pace of their proliferative capacity is dependent on systemic nutrient status[30,32]. Illuminating the temporal effectors that orchestrate normal progression of NB-lineages is of utmost importance for understanding neural-derived tumor formation (neuroblastomas), as we will discuss below.

1.4.5. Illuminating The Role of Imp in *Drosophila*

Imp (insulin-like growth factor II-mRNA-binding protein) is an RNA-binding protein that recognizes cis-binding localization elements (LEs) in the 3’ untranslated region (3’UTR) of target mRNAs. The tethering of single stranded nucleic acids is attributed to its four KH-type RNA-binding motifs that are evolutionary conserved in vertebrate orthologues such as the ZBP-1 (Zip-code protein), in the chicken and rat. Imp was first recognized as an essential factor for normal axial polarization in the *Drosophila* egg and embryonic viability[43]. Parallel to its functions as a binding partner of *oskar* mRNA (germline determinant in the posterior egg cytoplasm), several lines of evidence have described its role for synaptic terminal growth in presynaptic boutons and its necessity for establishing plasma membrane protrusions during cell migratory behavior[44,45].

Imp is expressed in the cytoplasm in many different embryonic and adult tissues. During the cellular blastoderm phase of *Drosophila*’s embryogenesis, its apically-localized ubiquitous expression pattern, eventually, is restricted in the ventral neuroepithelium, from which NB precursors will arise. Imp expression is retained throughout late larval VNC neurons that will

innervate the peripheral tissues of the adult fly[46]. In the larval CNS, other than endowing competence in early NBs for proliferation, Imp holds a prevalent role for remodeling of axonal terminal projections. Thus, in neuronal synapses the coordination of local mRNA biosynthesis with the cytoskeletal machinery is imperative for retraction, pruning and subsequent regrowth of axonal projections. In neurons Imp has been shown to function as a major coordinator of the retrograde/anterograde transport of remodeling synaptic factors[47]..

1.4.6. The (Temporal) Oncogenic Effector Myc

A salient feature of tumor onset and progression relies on the ability of cancer cells to exploit readily available cellular components and co-opt, the intrinsic molecular circuitry to their advantage. There is a long-standing bibliographic consensus depicting *Myc* (dMyc or diminutive in *Drosophila*) as a potent oncogenic factor, attributed partly to its evolutionary conserved role in anabolic pathways regarding ribosome and protein biosynthesis[37–40]. Decades of accruing evidence have established the deregulation of its mammalian counterparts (c- and N-Myc) as potentiators of tumor development and malignant transformation[42]. One of the earliest studies in the fly, characterized dMyc as a non-obligatory component in the cell cycle control machinery and highlighted its impact on the rate of cellular growth (cellular mass), rather than the rate of cell cycle progression (cell number) in mitotically active cells[42]. Eventually, dMyc was incriminated for its pleiotropic function in organogenesis and embryonic neurodevelopment, highlighting its essentiality for cellular outgrowth and cell division[43].

dMyc is a single copy gene encoding a bHLH transcription factor that transcriptionally regulates a set of available growth-promoting genes that can propagate proliferation, when aligned with metabolic intracellular demands and the derepression of cell cycle inhibitors [39,41,44]. Hence, through dMyc, nutrient-sensing is coupled with intrinsic transcriptional changes that favor cellular growth depending on nutrient availability[41]. In the context of regulating stem cell proliferation, the dMyc's target network includes polarity and mitotic spindle genes, as well as, positive interactions with components of chromatin remodeling complexes[39,48]. The repeated rounds of asymmetric division of larval NBs depend on cellular regrowth. Additionally, the establishment of a polarity axis that segregates basal determinants for progeny differentiation is essential for normal lineage progression. Hence, dMyc coordinates the expression and interacts with proteins that maintain and shape the proliferation capabilities of larval NBs. However, dMyc loss of function experiments in larval NBs have showed mild differences in stem-cell size accompanied with unaltered lineage progression[49,50]. These lines of evidence, highlight the unexplored transcriptional regulatory networks that propagate the stem cell self-renewal state and the dispensability of dMyc for larval neurogenesis. Importantly, upon perturbation of the specification program of NB progeny, dMyc expression in post-mitotic neurons is essential for enabling dedifferentiation from which ectopic tNB cells arise (tumor-like NBs)[51].

1.4.7. Imp Promotes dMyc's Translation

Intriguingly, the regrowth of larval NBs which is, partly, dependent on dMyc's function involves the expression of the temporal effector Imp[52]. Individual NBs are highly asynchronous throughout their lineage progression. However, the conserved heterogeneity of the neural stem cell pool needs to be refined during larval neurogenesis for normal CNS development despite differences in division rate and the time of their termination. Accordingly, Imp protein levels, in all type I NBs,

correlate with the juvenile state, which gradually decline towards later larval stages, leading to NB reduction in size and differentiation of asymmetrically segregated progeny. Imp imposes post-transcriptional control over the dMyc's function through binding to its mRNA (3' untranslated region). This interaction is essential for promoting dMyc's translation and permit growth of NBs.

1.4.8. Notch Signaling in Larval NB Self-renewal

In the endeavor to unravel the transcriptional and gene networks during NB lineage progression, recent studies have demonstrated an indispensable role of normal N-signaling during larval neurogenesis[53–55].

Deadpan (Dpn) and E(spl)-mγ are transcriptional repressors that belong to the bHLH-O subfamily of mammalian *Hes* paralogues. Dpn expression is retained in the quiescent NBs during the embryo-to-larva transition and throughout NB lineage propagation. Null mutations in Dpn result into a *hypoplastic* phenotype of the developing larval CNS[21,56], which is defined by defects in the normal neural circuitry of the animal and lethality in pupal stages. Apparently, Dpn overexpression has a weaker impact on NB hyperproliferation compared to systemic Notch overactivation, as shown by the overexpression of a cropped transmembrane region of the Notch receptor which mimics the effect of the ligand-binding activation (NΔecd). Specifically, perturbations in controlled Notch activation result in devastating and varying effects in the larval NB pool. Systemic down-regulation of N-signaling during post-embryonic neurogenesis exterminates completely type-II and many of the type-I NB lineages. Conversely, overactivation of N-signaling disturbs each type's asymmetric divisions, multiplying the progenitor pool at the expense of post-mitotic progeny generation in type-II NBs, but with a weaker and more variable outcome in type I progenitors. All these lines of evidence, showcase the importance of regulating and restricting N-signaling in larval NBs for proper CNS development.

1.5. Aim of Our Study

1.5.1 A Neural-derived Model of Tumorigenesis in *Drosophila melanogaster*:

Perturbations in stem cell asymmetric divisions of neural stem cells can manifest in tumor formation[57]. Previous work from many labs have generated key-findings for normal progression of NB lineages during larval neurogenesis[53,54,58,59]. Notch signaling, is imperative for maintaining the self-renewal capacity of neural progenitors, through the expression of a plethora of downstream effectors like Deadpan and dMyc. Any perturbation of Notch signaling in NBs either through NΔecd ubiquitous overactivation, misexpression of Hes Notch targets or abnormal segregation of fate determinants to descendants, can dramatically affect normal CNS development. One of the major research works in recent years in our laboratory has focused on utilizing these aberrations in N-signaling within NB lineages to create hyperproliferative larval CNSs (or clones), in an effort generate transplantable tumours. Promising RNAseq (unpublished) data have demonstrated the extensive interaction between the tumor and the microenvironment within adult flies, upon serially transplanted tumors originating from hyperplastic larval CNSs. Specifically, dMyc and Imp have been shown to be upregulated in the 4th allograft (T3) compared to the first event of tumor injection

(T0). In an effort to unravel the role of these growth-promoting factors in tumorigenesis, we decided to knock-down their expression in Notch-induced hyperproliferating NBs. In parallel, I participated in a screen to genetically alter the haemocytes of the host, ultimately aiming to address their role in tumour growth.

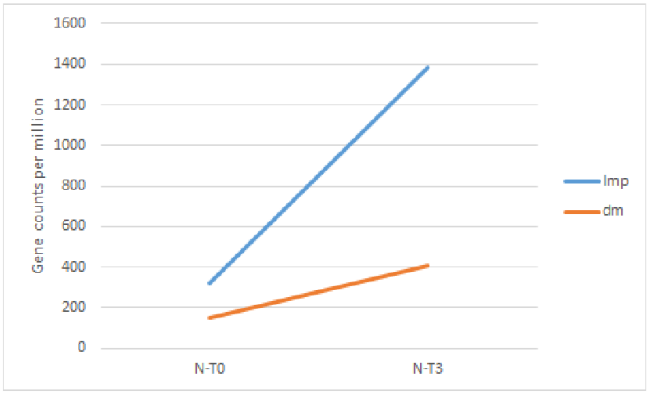
1.5.2 Tumorigenesis in Adult Flies (A Model of Allograft Transplantable Tumors)

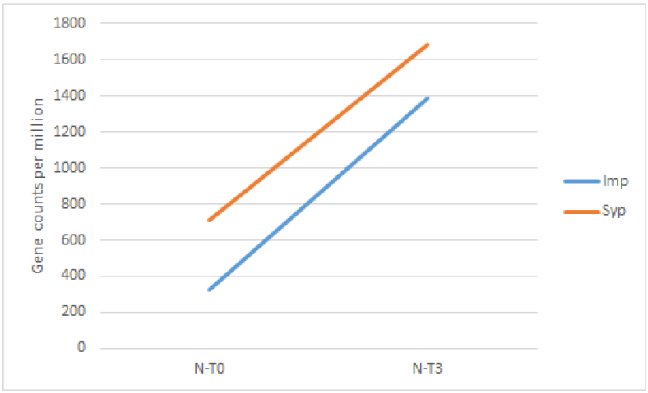
Historically, *Drosophila melanogaster* has been utilized as a powerful model system to unravel basic principles underlying innate immunity regarding infection and inflammation. In the last two decades research focus has started shifting in the pursuit of molecular mechanisms that promote tumorigenesis. Despite the anatomy of the fly's blood system (lacking a vascular system) and the absence of adaptive immunity (no documentation of somatic hypermutation and DNA re-arrangements in blood cells) there has been progress in understanding the aspects of tumor manifestation through studies incorporating genetic tools to mimic the sequence of events that potentiate tumors as seen in mammals. Around 50% of all proteins involved in human diseases, such as cancer, share homology with Drosophila proteins[60]. In support to this notion, the genetic tractability of the fly, the multitude of conserved signaling cascades and the existing knowledge of the fly's innate immunity, provide an ideal system to dissect the onset and progression of cancer cells.

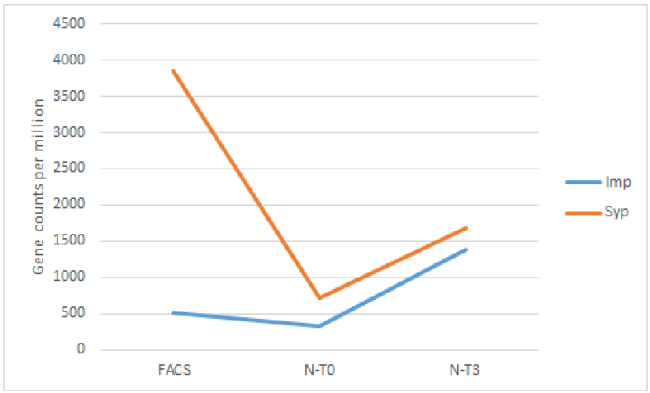
Tumorigenesis is a multifaceted and multistep process which by definition presupposes four criteria[61]: intrinsically-driven overproliferation, unresponsiveness to apoptotic and growth control (extrinsic) cues and, depending on the tissue of origin, transition to mesenchymal metastatic behavior. By default, each developmental decision exposes cells to aspects of these, otherwise, spatially controlled and temporally segregated programs. However, the hallmarks of malignant transformation are interdependent. Multiple alterations are needed to exert the phenotypic outcome of malignancy. This has been shown extensively in both fly and vertebrate studies. One of the earliest studies of Caussinus and Gonzalez[62] in larval NBs proposed that aneuploidy is formulated over time through accumulating mutations within a subset of tumor cells that expand and immortalize within the host microenvironment. Aneuploidy in primary tumors in the fly fails to be addressed even to this day[32], despite occurring evidence of tumorigenesis in wild type larval cells and aging flies[63]. Additionally, malignant transformation of cells involves many aberrations in the regulation of cell cycle progression, polarity establishment and control of mitotic proliferation. Accordingly, perturbations in the asymmetric segregation of neural progeny (cell-autonomous division) or in the orientation of the mitotic spindle (niche-controlled division) of neural stem cells disturb proper linage progression. This results to highly proliferative aggregates of cells due to abnormal development within their foci[32,57,64,65]. The hyperproliferative phenotype of larval NBs can be mimicked upon *Numb* and *Brat* (basal cell fate determinants) loss-of-function mutations. Following NB asymmetric divisions, both proteins are unequally segregated in GMCs and their progeny, in order to specify their differentiation program[66]. In order for differentiation to be permitted in progeny, they function, partly, by establishing a negative regulatory loop of Notch activation. Brat directly represses Deadpan translation, whilst Numb facilitates the endocytosis and decommission of the Notch receptor. These events ensure the repression of the stem cell identity and facilitate the specification program that is inherited within the descendants of NBs[32,66,67].

Human cancers are characterized as multi-layered unfolding in the span of decades within the host's tissue microenvironment. Surprisingly, the fly with an average lifetime of 6 to 8 weeks, is emerging as a workhorse for the identification of relationships between tumors and the host's microenvironment as well as the intracellular prerequisites enabling tumor initiation and outgrowth. Initially, early endeavors in *Drosophila* studied the relationship between organ-autonomous developmental patterning (larval eye disc differentiation) and the host's microenvironment (global or hormonal control) by introducing transplanted tissues in larvae and later in adult flies[68]. Recently, pioneer studies in the fly's eye imaginal discs, identified cooperative forces between oncogenes (Ras^{V12}) and tumor suppressor genes (*scrib*) to drive tumor malignancy have been identified in epithelial cells [69–71]. To date, the introduction of tumorigenic larval tissues within adult hosts and the generation of allograft transplantable tumors has become established as an "a la carte" model of tumorigenesis[62,72].

Comparison of Imp-dMyc Expression Profiles in our 1st (T0) and 4th (T3) Allografted Stem-derived Tumors (Genetic background of NΔ-signaling in Larval NBs)







2. Materials and Methods

2.1. Fly Strains & Crosses

Drosophila stocks are described in Flybase and were obtained from the Bloomington *Drosophila* Stock Center (BDSC) unless otherwise indicated. For the study of adult haemocytes, the list of RNAi stocks (Table 1.) includes constructs for knocking-down proapoptotic genes (*hid* and *bax*), phagocytic receptors (members of the NIMC family *nmc1* and *nmc4*, *eater* and *draper*), genes regulating inflammatory responses (*eiger, tep4),* the *Mmp2* metalloprotease, redox genes and genes responsible for ROS generation (*nox, duox and* aquaporin-like protein *prip*), components of cytoskeleton assembly complexes (*rudhira* and *scar/WAVE*) and the downstream effector *src42A* of an inflammatory signaling cascade. The plasmatocyte-specific expression was mediated by crossing HmlGal4;UAS-(2X)GFP female flies (hemolectin-driver of Gal4 expression) with male flies from each individual RNAi line. Fly crosses for the RNAi screen were grown on standard cornmeal-yeast-agar medium at 18°C under 60% humidity for ~18 days and were transferred at 29°C right before metamorphosis (late pupal stages). Controls included LacZ, and the *yw*;wRi stock under the control of a UAS promoter. For the generation of overproliferating third instar larval CNSs for the first injection series (T0), we crossed female flies carrying *tubGal80ts,UAS-stingerRFP; grhGal4* with male flies carrying UAS- NΔecd. Crosses were maintained at 18°C for 7 days, then transferred to 30°C for 48 hs before dissection. For the generation of FLP-out clones in third instar larval CNSs in a genetic background of Notch hyperactivation (NΔecd) we first crossed UAS-NΔecd (and the control UAS-LacZ) with three RNAi lines (UAS-ImpRi, -wRi and -MycRi). We generated the *UAS-NΔecd;UAS-ImpRi*, the *UAS-NΔecd;UAS-wRi* and *UAS-NΔecd;UAS-MycRi* stocks with their respective controls *UAS-LacZ;UAS-ImpRi, UAS-LacZ;UAS-wRi* and *UAS-LacZ;UAS-MycRi.*

2.2 Flip-out clones

Male flies from the hs-FLP; act-FRT::stop::FRT-Gal4, UAS-GFP stock were crossed with female flies carrying the appropriate UAS combinations for generating knocked-down transgenes, namely wRi ImpRi and MycRi (all in the 3rd chromosome). Each RNAi transgenic lines also contained the transgenes of NΔecd or LacZ (2nd chromosome) under the control of the UAS promoter. Progeny underwent heat shock for 45 min or 1 h at 37oC at 24-72 h after egg lay (AEL) depending on the experimental setting. For confocal imaging, phenotypes were analyzed 3 days after this heat shock-mediated (flip-out activation) onset of transgene expression. For the injection series, CNSs from each genotype (*UAS-NΔecd;UAS-ImpRi, UAS-NΔecd;UAS-wRi* and *UAS-NΔecd;UAS-MycRi*) were dissected 4 days after 1h of heat shock-mediated (flip-out activation) to induce larger clonal lineages.

TABLE 1 STOCKS & RNA-INTERFERENCE LINES FOR SURVIVAL ASSAYS

Fly line	Bloomington ID	Full genotype_Bloomington	Chromoso
UAS-NimC1-RNAi	25787	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01793}attP2	3rd
UAS-eater-RNAi	25863	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01884}attP2	3rd
UAS-crq-RNAi	40831	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01997}attP40	2nd
UAS-NimC4-RNAi	61866	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ23355}attP40	2nd
UAS-mmp2-RNAi	65935	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC06206}attP2	3rd
UAS-Scar-Ri	51803	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03361}attP40	2nd
UAS-Duox-Ri	33975	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00934}attP2	3rd
UAS-Prip-Ri	44464	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GLC01619}attP2	3rd
UAS-src42A-Ri	55868	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04138}attP2/TM3, Sb[1]	3rd
UAS-Nox-Ri	32902	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00691}attP2	3rd
UAS-Tep4 RNAi	67218	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC06319}attP40	2nd
UAS-Eiger-Ri	58993	w[*]; P{w[+mC]=UAS-eiger.IR}3/TM3, Sb[1] Ser[1]	3rd
UAS-Pvr DN	58431	w[1118]; P{w[+mC]=UASp-Pvr.DN}D7	3rd
UAS-Pvr DN/Cyo	58430	w[1118]; P{w[+mC]=UASp-Pvr.DN}D1/CyO	2nd
UAS-draper-Ri	36732	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01623}attP2	3rd
M28	by A.Giangrande	Hemolectin-gal4 ; UAS-2xEGFP (2)	
yw;wRi	by Eva		
UAS-rudhira-Ri	36846		
w; UAS-LacZ	by Eva		
Tep4 RNAi	67218	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC06319}attP40	Valium 20
src42A-Ri	55868	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04138}attP2/TM3, Sb[1]	3rd
Scar-RNAi	36121	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01536}attP40	

2.3. (Allograft) Transplantation Series

Transplantation assays were performed as previously described[72]. Donor larval brains were made using FLP-out GFP clones or *tubGal80ts,UAS-stingerRFP; grhGal4*. CNSs were dissected, sliced into single brain lobes, loaded into a fine glass needle and implanted into the abdomen of female host flies using a nanoinjector (Nanoject II Auto-Nanoliter Injector, Drummond Scientific Company, 3-000-205A). Host flies carrying allografts were kept at 30°C (GFP/RFP allografts) and examined daily for viability and the presence of GFP/RFP in their abdomen and other tissues. Malignant GFP/RFP-positive tumour pieces (T0) were dissected out of the abdomen of host flies and either re-transplanted into new host flies (T1) or fixed with 4% formaldehyde (for 25 min at room temperature) and used for immunohistochemistry experiments according to standard protocols[73].

2.4. Survivals

Flies were collected every day after eclosion (corresponding to the first day of life, D1), and both sexes were kept in the same vial (29°C) for at least 3 days -prior to female selection (fertilized female flies). Vials were refreshed every two days and observed daily for deceased flies (Uninjected flies n>200 per genotype). Post-injection hosts (fertilized females at day 5 or 6, D5-6) were kept at 29°C and observed daily for fluorescence emergence (GFP/RFP); first day of tumor initiation corresponded to the first day of fluorescent puncta observed in tissues of the hosts. Vials were refreshed every two days and observed daily for deceased flies (fluorescent and non-fluorescent flies).

2.5. Immunohistochemistry (IHC)

Fixation and IHC of larval and adult tissues were performed according to standard protocols. In particular, the fixative solution used for all IHC experiments consisted of 10XPBS, dH20 (nanopure) and 10% formaldehyde solution (FA) and was diluted down to a concentration of 1XPBS/4%FA. Larvae CNSs were fixed for 20' in room temperature (R.T) shaking on a rotator. Adult tissues (*belly* staining) were fixed for 35' in R.T shaking on a rotator. At least three rinses with 1xPBS were performed following fixation. For blocking, the solution (1XPBT) consisted of 0,5%BSA and 0,1%Triton-100X in1XPBS buffer. Larvae CNSs were blocked for at least 1hour in R.T. Adult tissues were incubated overnight. Primary & secondary antibody incubations were also performed overnight for adult tissues. Conversely, only the primary incubation was overnight for larvae CNSs. The incubation with the secondary antibody was performed for only 1 hour. A least three 30minute-long washes of sterile 1XPT (0,2%Triton-100X in1XPBS) were performed in R.T after each respective antibody-incubation. Mounting medium for both larval and adult dissected tissues used was 80% glycerol in PBS + 5% n-propyl gallate to stabilize the fluorochromes (NPG) for confocal imaging.

2.5.1. Methodology of Dissections

Dissection of Larval CNSs

The dissection of the larval CNS [Central Brain (CB) and Ventral Nerve Cord (VNC)] was conducted using two fine forceps, one for holding down the larvae and the other for performing micro-movements under a conventional stereoscope. Larvae were bathed in 1xPBS (non-sterile) during dissection. The procedure goes as follows:

In each larva a transverse incision is made posteriorly (around the two thirds of the total larval length). This will form the entry point for one of the forceps, in order to invert inside-out the larval cuticle (like a sock). The exposed CB, VNC and the peripheral ganglia are left intact on the larval carcass -prior to fixation and throughout the IHC protocol-, dissecting out tissues of the fat body and the tracheal system (since these tissues non-specifically bind and effectively dilute the antibody-solution). Before mounting, the CB and VNC are separated from the larval carcass, removing the attached imaginal discs (antennae/eye, leg etc.) and the peripheral ganglia (the former will hinder confocal imaging of larval CNS and the latter will create background noise). Then the larval brain is mounted and placed on a frosted slide with the brain dorsal side facing down. This orientation ensures that the region closest to the coverslip -corresponding to the CB lobes- will have greater pixel resolution and this is important for subsequent quantification analysis after confocal image capturing. The coverslip is then coated perimetrically with nail polish to seal off air and humidity and allow slide storage at freezing temperatures (-20℃).

Dissection of Adult Abdomens

Accordingly, to the standard fixative and IHC protocols of larval CNS, adult flies are first anaesthetized with ice cold temperature and then sequentially bathed in 70% ethanol (drowning and semi-fixation of flies) and 1XPBS. Using fine forceps, the belly is dismembered from the head/thorax regions with extreme care -avoiding injury of the crop organ (storage organ of hydrolytic enzymes)- and a small incision is made superficially in the middle part of the belly. This step ensures fixation and permeabilization to occur even in the deepest layers of the tissue. For stainings, bellies are placed on a 48-well (round bottom) plate for incubation with primary and secondary antibodies. For mounting, the bellies are finely dissected into smaller pieces of tissue inside the mounting NPG medium.

2.6. Cryosectioning

Flies were anaesthetized with ice cold temperature and sequentially bathed in 70% EtOH and 1XPBS. Proboscis then was dissected out in order to allow the fixative to infiltrate the interior without damaging the anatomical architecture of the fly. For fixation, the flies were submerged in 4%FA/1XPBS for a total duration of 150 mins. The fixed flies were then washed three times with 1XPBS for at least 30 mins per wash. For cryo-protection of the fixed flies, O/N incubation with 30% Sucrose was performed. To immobilize the flies for cryosectioning, a bed of OCT was initially prepared (flash-freeze with dry ice), and then an overlay of liquid OCT allowed flies to be submerged and oriented accordingly for cryosectioning (flies were positioned ventrodorsally and laterally). Frozen flies were then stored at -80℃ at least one day prior to cutting (to secure the integrity of OCT's cryopreservation and enhance OCT-tissue solidification). Horizontal and

sagittal sections of fly tissue were obtained ranging between 30~50µm and the cryotome slices were collected on super frost slides. After drying the sections in R.T for 1 hour, IHC was performed as detailed above for DAPI.

2.8. Clonal Analysis

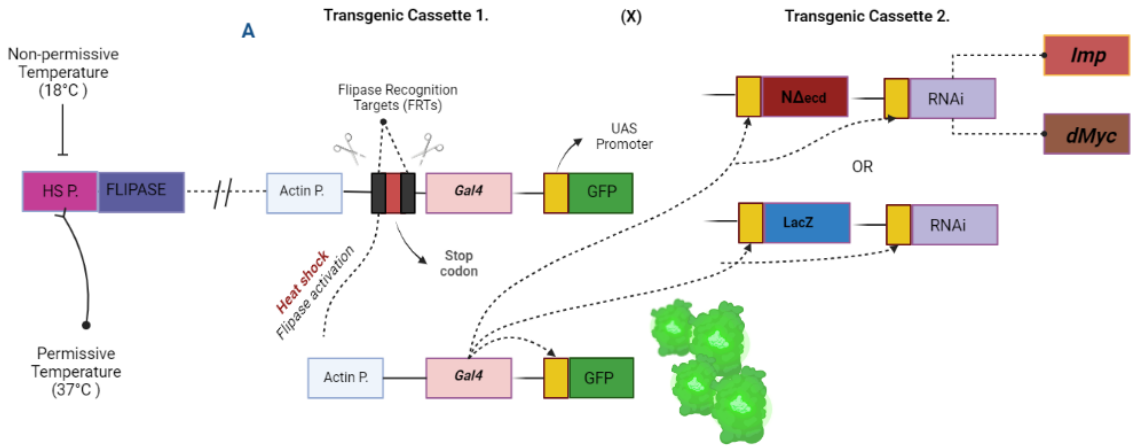
Mitotic clones were induced using the FLP/FRT technique. Flies were raised at 25℃ and hsFLP was induced by heat-shocking first to second instar larvae (Day 3) of the following genotypes for 45 mins or 1 hr at 37℃: *UAS-NΔecd;UAS-ImpRi*, *UAS-NΔecd;UAS-wRi* and *UAS-NΔecd;UAS-MycRi* and their respective controls *LacZ;UAS-ImpRi*, *UAS-LacZ;UAS-wRi* and *UAS-LacZ;UAS-MycRi*. Quantifications of NB lineages proceeded by hand in 3D or 2D (single Z-stacks) by incorporation of Stardist (Fiji plugin) in confocal images of immuno-stained larval CNS[74].

3. Results

3.1 Illustration of our Functional Model System

The self-renewal capacity of NBs collectively promotes cellular growth and inhibits the differentiation-permissive fate that is inherited in NBs' progeny. The maintenance of the transcriptional program that facilitates NB proliferation depends on N-signaling during larval ACDs of neural progenitors. Overactivity of the Notch pathway, results to aberrant proliferation and concomitant loss of post-mitotic neurons, leading to a hyperplasic CNS phenotype. These animals often show lethality prior to adulthood or, in mild cases, exhibit NB overproliferation even into adulthood. Imp plays a crucial role during early larval stages in asymmetrically dividing NBs. Although the exact molecular circuit remains elusive, its interplay with *Chinmo* and *dMyc* -both gene products of proto-oncogenes- suggests the existence of a stem-like regulatory network of transcription factors. Imp is an RNA-binding protein and, thus, could potentially function to regulate the fate of a number of RNAs. Indeed, in a recent study, Imp was identified as essential for dMyc's mRNA stability to promote its translation and expression in early-larval dividing NBs[52]. Arguably, these interactions could potentiate the regrowth and enhanced self-renewal capacity of young NBs.

Initially, in our study, we set out to explore the possible role of Imp and Myc, in a genetic background of Notch hyperactivation in larval NB lineages. To that end, we combined the Flip-out system with a constitutively active form of the Notch receptor *(NΔecd*) together with a dsRNA construct targeting either Imp or Myc. As a negative control, we used an RNAi construct against the *white* gene, thereby maintaining the same number of UAS transgenes in each experimental genotype. This additional step allowed us to attribute any differences observed, solely, to the down-regulation of each target gene (and not to attenuation of the Gal4 potency, due to its binding to multiple UAS targets). Accordingly, UAS-LacZ expression instead of UAS-NΔecd was used as a control condition to identify the role of Imp and Myc down-regulation in the normal NB lineage progression (during normal N-signaling). hsFLP expression was induced during late 2nd instar larval stage which occurs, roughly, at day 3 *after egg deposition*, AED. At day 6, after extensive larval feeding, we isolated late-3rd instar larvae and dissected their CNSs for confocal imaging. All cells labelled green, must express GAL4 since GFP is under the control of the UAS promoter (Figure 1B). It is worth noting that all progeny of the initial GFP^{+} cell will retain transgene expression even upon return to 25^{o}C (temperature prior to hs), when flipase is no longer expressed, since excision of the FRT-STOP_FRT cassette is irreversible. Thus, this experimental setting of FLIP-out allows tracing of cells in GFP^{+} clones that are lineage-related.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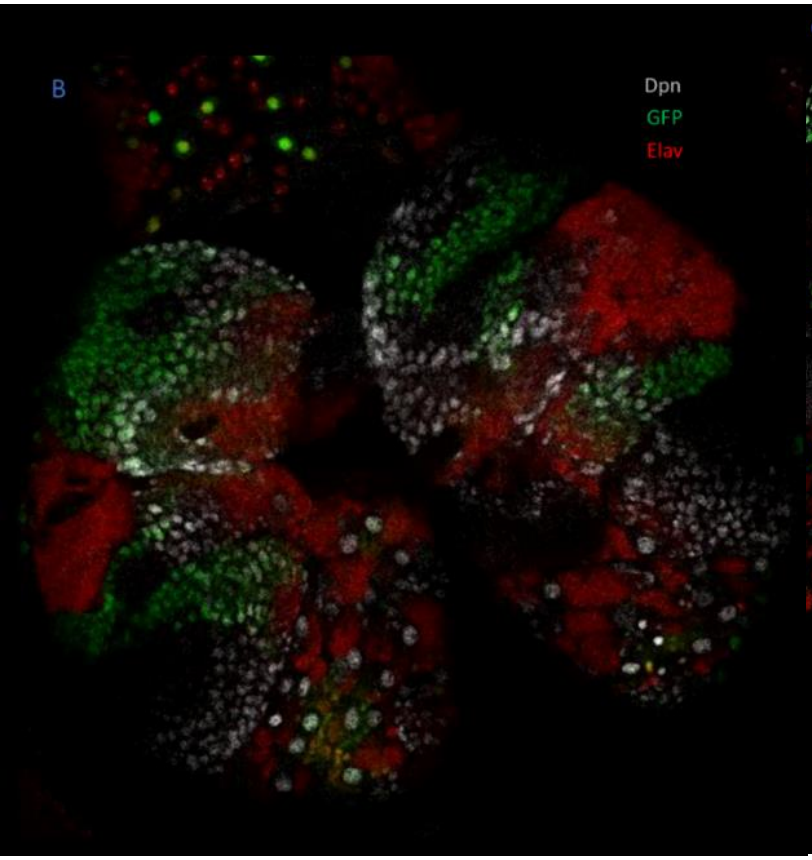
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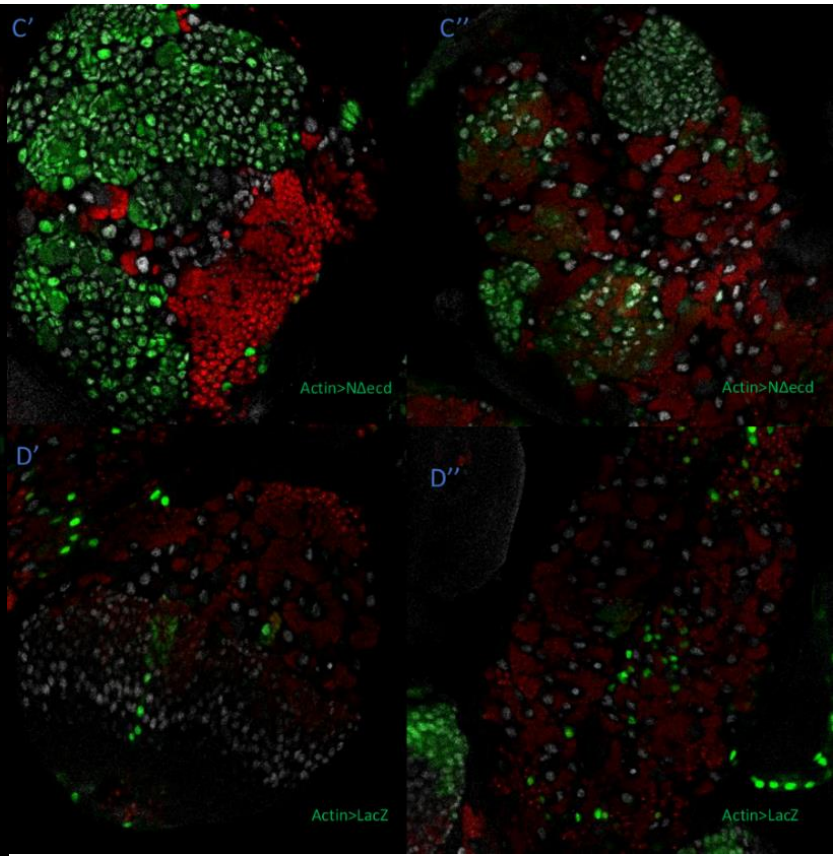

C'
C''
Actin>NΔecd
Actin>NΔecd
D'
D''
Actin>LacZ
Actin>LacZ

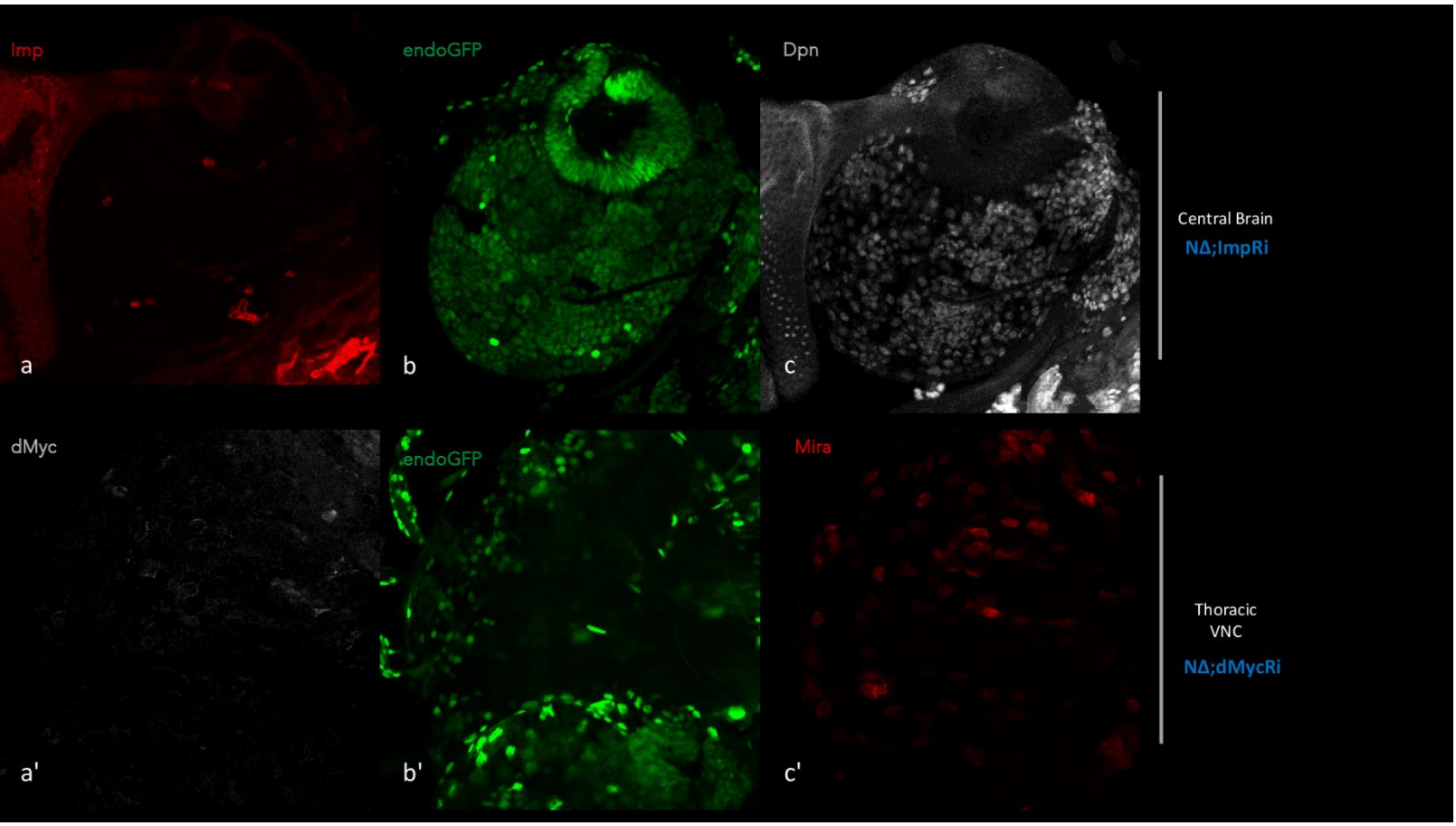
Fig. 1. Notch-driven (NΔ) Hyperplasia of NB Clonal Lineages During Larval Neurogenesis (Flip-out-mediated).

(A) Schematic representation of our experimental model. Randomly-generated clones of lineage-related cells were marked with GFP^+ upon cell division of a progenitor cell in early larval neurogenesis. We incorporated the FLP-out system to exert temporal control over the expression of our transgenic cassettes. The flipase coding sequence was integrated downstream to a heat shock promoter, which becomes active only during heat stress (37 °C). After heat shock, the excision of a stop signal, allowed expression of the GAL4 transactivator. Day 3, AED (after egg deposition) reflects the day of heat shock induction. CNSs were dissected at day 6 (late 3rd instar larvae). (B) The CNS of *UAS-LacZ;wRi* late 3rd-instar larva (control genotype). The central brain and its two brain lobes stand out from the thoracic VNC (thVNC) from the densely stained (red) optic lobes with the neural marker *Elav* (embryonic lethal abnormal vision). NBs (grey) stained with the stem cell marker Deadpan (Dpn) surrounded by their post-mitotic neurons (red). GFP is randomly expressed by the FLPout system. (C', C'') Type I NBs (grey) in the Central Brain (CB) and thVNC, respectively, of *UAS-NΔecd;wRi* animals. Notice the size difference of Dpn^+/GFP^+ (grey & green) clones of NB lineages, compared to the respective clone size in control *UAS-LacZ;wRi* CNS (D', D''). The NΔ-induced hyperproliferative phenotype is characterized by the presence of supernumerary NB-like cells. This tumor-like accumulation of stem cells, comes at the expense of normal generation of progeny marked with the post-mitotic neural marker Elav (red).

3.2 Knock-down of Imp or Myc in Larval Neuroblasts Relieves NΔ-Hyperproliferation

We used the FLP-out system to create clones (GFP^+) of cells that expressed UAS-NΔecd together with either UAS-ImpRNAi or UAS-MycRNAi in a stochastic fashion. The verification of the knocked-down genes was established by co-immuno-staining for the product of each respective gene (Imp & Myc) with a stem cell marker (Dpn^+ cells) that delineated the stem-like pool of the larval CNS, as well as, with the basal cell fate determinant Miranda (Mira) that is localized cortically only in NBs. We observed ubiquitous and unaffected expression of Myc and Imp in our NΔecd-driven hyperplastic control animals (*UAS-NΔecd;wRi*) in NBs localized, both, in the thVNC and CB; Myc was only detected in NBs, whereas Imp was also detected in progeny cells (Figure 2A, 2A' and 2I). This phenotypic consequence is consistent with Imp and Myc promoting growth and sustaining the proliferative capabilities of tNBs (tumor-like NBs). Conversely, we failed to detect almost any expression of Imp in *UAS-NΔecd;ImpRi* larval CNSs (Figure 2B, 2B' and 2II, 2II'), whilst dMyc detection was unhindered in the majority of the GFP^+ clones.

dMyc's persistence in Imp-deficient NBs is contrary to Imp's role in stabilizing dMyc's mRNA and promoting its translation in *Drosophila*'s NBs. There are several lines of evidence that characterize dMyc as one, of the more than a hundred, direct downstream target genes of N-signaling[54]. Consequently, we hypothesize that the Imp-RNAi-dependent translational defect on dMyc is compensated through continuous Notch activation in our NΔ-genetic background. A very prominent size reduction in Type I lineages, was seen in *UAS-NΔecd;dMycRi* larval CNSs (Figure 2C, 2C' and 2I, 2II'). These animals showed dramatic reduction in Myc expression levels, however, the expression patterns of Imp protein, seemingly, were unaffected.



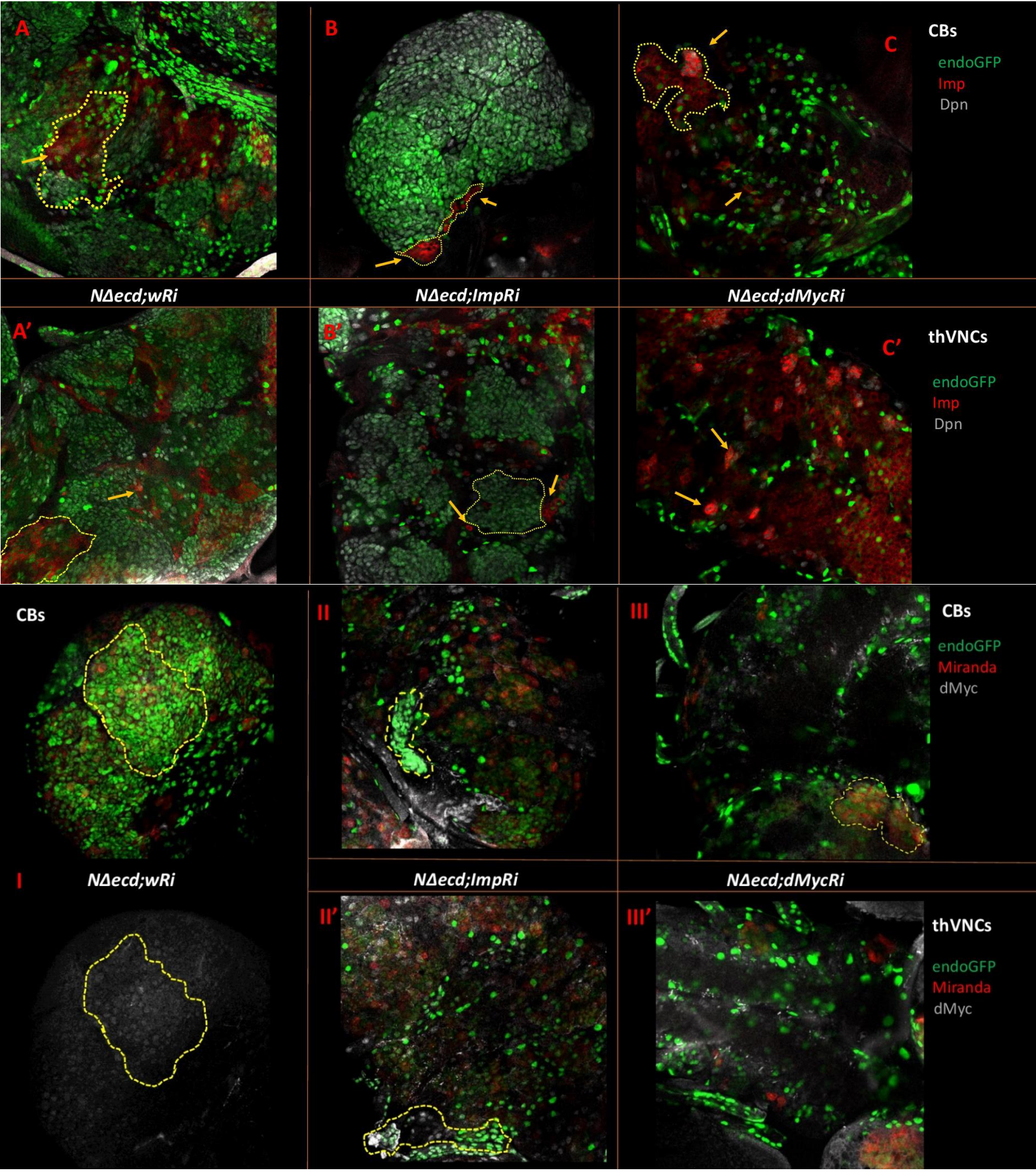

A
B
C
CBs
endoGFP
Imp
Dpn
NΔecd;wRi
NΔecd;ImpRi
NΔecd;dMycRi
A'
B'
C'
thVNCs
endoGFP
Imp
Dpn
CBs
II
III
CBs
endoGFP
Miranda
dMyc
I
NΔecd;wRi
NΔecd;ImpRi
NΔecd;dMycRi
II'
III'
thVNCs
endoGFP
Miranda
dMyc

Fig. 2. Diagnostic Stainings for Imp- and Myc-Knock-down in Flip-out Mitotic Clones of NΔecd-NB Lineages.

Confocal Images of CBs and thVNCs for the functional verification of our experimental (knocked-down) models. (a-c) Confocal images upon dissection of a single Central Brain of the NΔ;ImpRi genotype. Note the absence of Imp expression (red) in all GFP^+ regions (identical Z-stacks). (a'-c') Note the absence of Myc expression (grey) in the thVNC of the NΔ;dMycRi genotype (identical Z-stacks). (A-C, A'-C') Larval CNSs from each genotype were co-immuno-stained with the pan-NB nuclear marker *Dpn* (NBs) and the RNA-binding protein *Imp* which is localized cortically. Endogenous GFP from our transgenic expression upon Flip-out can also be observed. *NΔ;wRi* and *NΔ;dMycRi* showed extensive expression of Imp in GFP^+/Dpn^+ clones (A, A'). Conversely, in *NΔ;ImpRi* GFP^+ clones, the expression of Imp was dramatically diminished (B, B'). (I-III, II'-III') Larval CNSs co-immuno-stained with the cortical stem cell marker *Miranda* (NBs) and the dMyc transcription factor. *NΔ;wRi* and *NΔ;ImpRi* displayed unhindered expression of dMyc. On the contrary, in *NΔ;dMycRi* larval CNSs we failed to detect any expression of dMyc in all observed GFP^+ clones. The dotted lines reflect regional examples in larval CNSs from which our observations were deduced. Arrows point to individual examples of GFP^+ expressing cells and the state of protein expression patterns from each genotype. As dMyc is expressed at low levels, the quality of its immunodetection is mediocre.

In order to, measure the extent of reduction in the hyperproliferative behavior of NBs by the down-regulation of Imp or Myc, we quantified the number of Dpn^+ cells in each clonal area (GFP^+ cells) of NB-specific lineages. Additionally, we co-immuno-stained with the neuronal marker Elav to navigate between different lineages and NB-descendants, in an effort to restrict our measurements to lineage-specific alterations of NB ACDs (Figure 4). We only scored Type I NBs in (both) central lobes and the thVNCs of each larval CNS. This exclusion was based on existing evidence for the peculiarities of Type II NBs (8 per lobe) and optic lobe NBs, concerning their response to Notch activation. Finally, in our quantification we included animals that clonally downregulated Imp or dMyc without perturbing the normal N-signaling - *UAS-LacZ;ImpRi* and *UAS-LacZ;dMycRi*, respectively- as our control groups.

In agreement with the bibliographic documentation, the (control) groups exhibiting normal N-signaling (*UAS-LacZ*) showed a strict appearance of one (Type I) NB per lineage in all clones counted (Figure 3). This held true for both thVNC and CB NBs. In a small minority we could detect 2 $NB^{DPN\text{-}positive}$ cells per clonal area, differing greatly in their size (data not shown). This phenomenon could be either due to coalescence of two neighboring clones, or due to perdurance of Dpn expression in a recently born GMC (ganglion mother cell). As already reported, the larval CNSs of *UAS-NΔecd;wRi* animals, displayed substantial explosion in NB-like cells compared to the wild type (wt). This extreme NB overproliferation was reduced in *NΔ;ImpRi* with a higher percentage of intermediate level of NB expansion (2-11 NBs) at the expense of larger clones (12 to >20NBs). *NΔ;dMycRi* CNSs displayed an even more severe reduction in NB hyperplasia, with a significant increase in single-NB clones, and an almost complete elimination of large (>20NB) clones. It is worth noting, that in all instances of tumor-like expansion (tNBs), Type I NBs exponentially grew at the expense of their post-mitotic descendants and expanded in towards neighboring areas of other NB lineages. This resulted to an aggressive and hyperproliferative phenotype of NBs (Figure 4) in the CBs and thVNCs, alike.

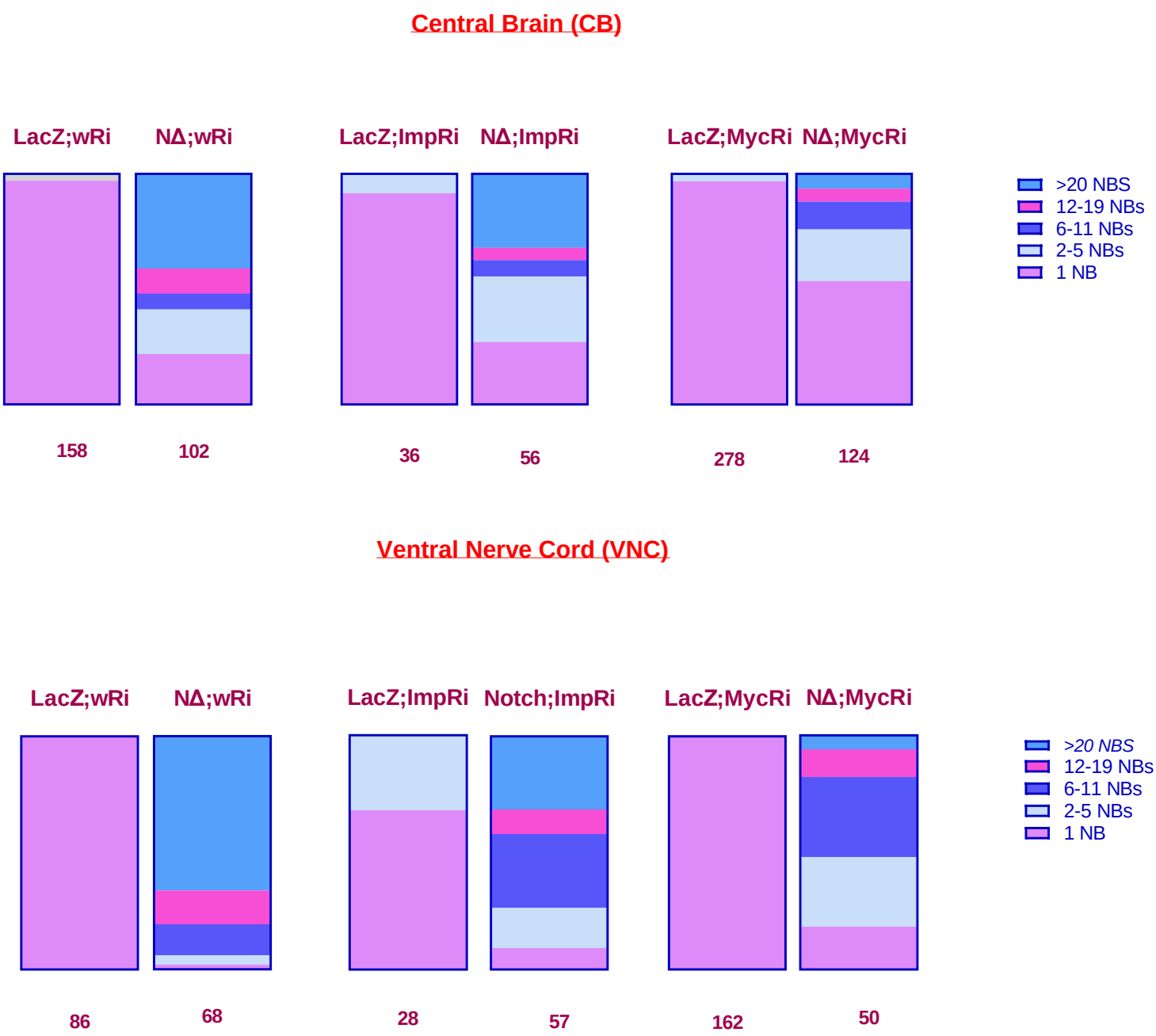
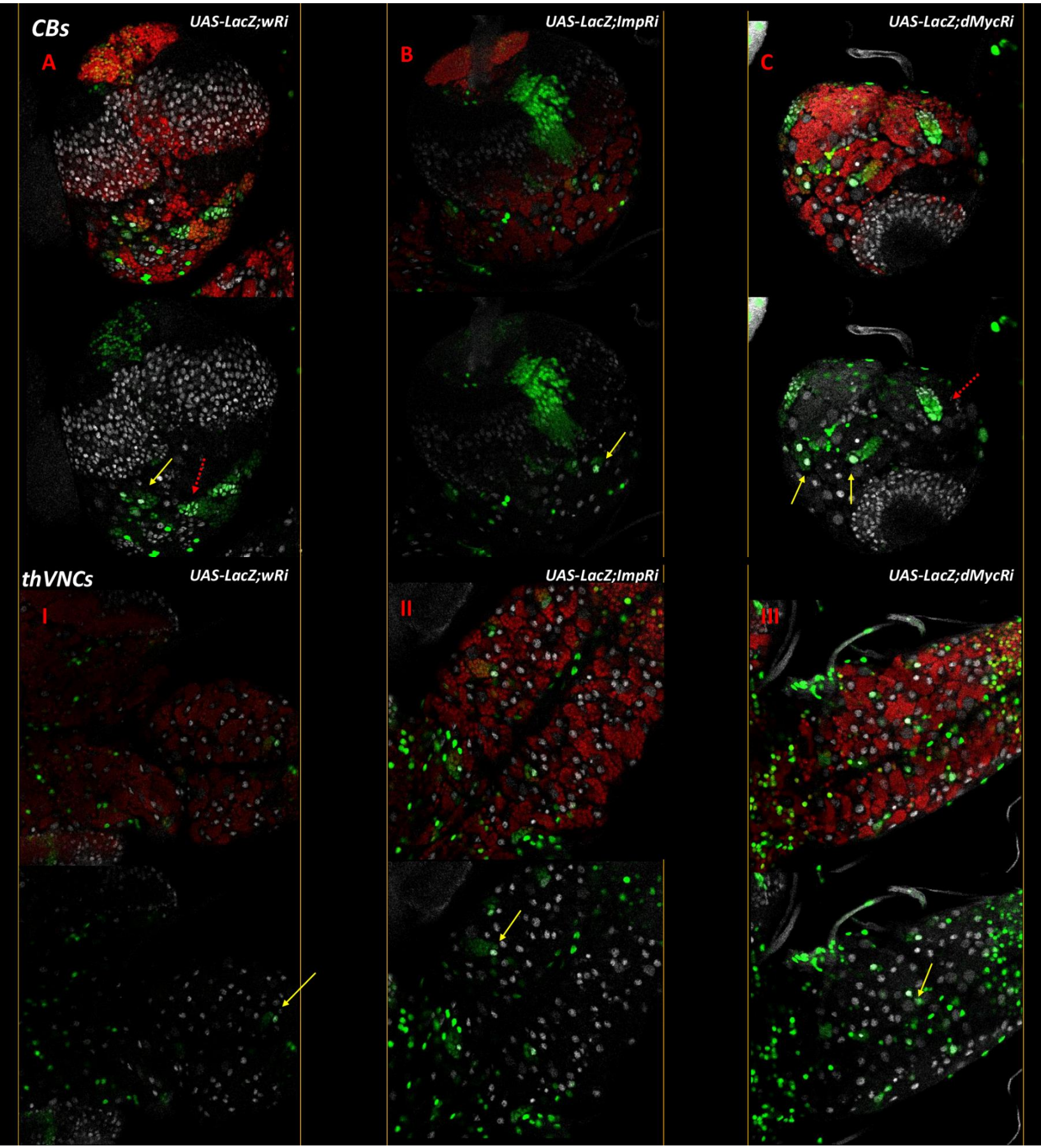


Fig. 3. Alleviation of the Extreme NΔ-Hyperplasia upon Imp- or Myc-Knock Down
Quantification of Type-I NBs (Dpn^{+}) in $GFP^{-positive}$ clonal regions from stochastic Flip-out excision events in progenitors and their emerging lineages in late-larval (3^{rd} instar) CNSs. We fragmented the NB numbers per clone into five sequential categories in order to characterize the extent of hyperplasia across our different genotypes. Numbers indicate the measured clones per genotypes.


CBs
A
UAS-LacZ;wRi
B
UAS-LacZ;ImpRi
C
UAS-LacZ;dMycRi
thVNCs
I
UAS-LacZ;wRi
II
UAS-LacZ;ImpRi
III
UAS-LacZ;dMycRi

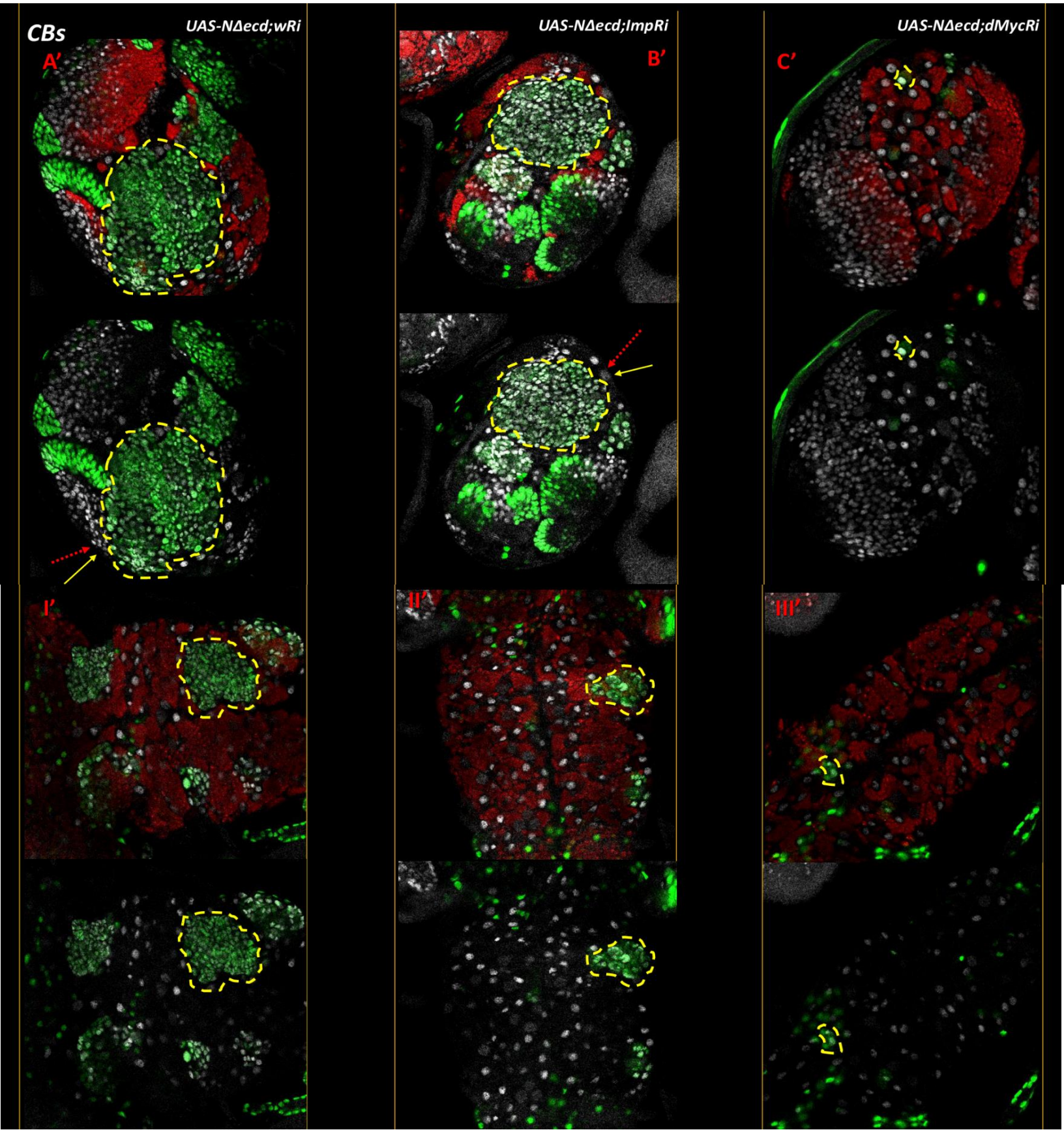
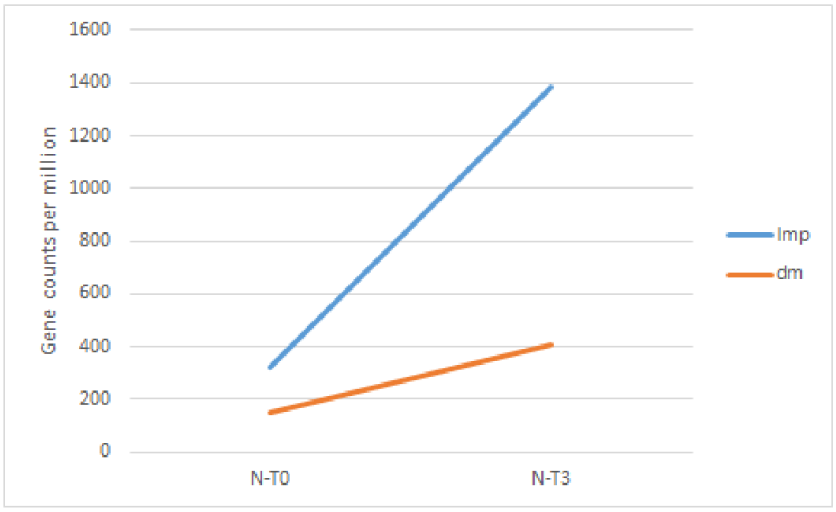

CBs
UAS-NΔecd;wRi
UAS-NΔecd;ImpRi
UAS-NΔecd;dMycRi
A'
B'
C'
I'
II'
III'

Fig. 4. Confocal Characterization of NB-lineages upon Imp- and Myc-Knock Down in the Context of Normal and NΔ-signaling.
(A-C) Confocal images of CBs from *UAS-LacZ* animals. In accordance with our quantifications in Figure 3, all genetic manipulations during normal N-signaling exhibit normal NB numbers (1NB/clone) independent of targeted knock-downs of Imp and dMyc. (I-III) Confocal images of thVNCs from *UAS-LacZ* animals. Consistently, thVNCs from each respective genotype show normal NB numbers. (A'-C') Confocal images of CBs from *UAS-NΔecd* animals. Overgrowth of tumor-like NB-lineages with multiple Dpn^+ progenitor cells in each clonal area is seen. This stem-like hyperplastic phenotype is gradually attenuated upon ImpRi. In dMycRi CNSs (C', III'), an almost complete rescue of normal NB numbers is observed. (I'-III') Confocal images of thVNCs from *UAS-NΔecd* animals. Yellow arrows indicate Type-I NBs in all genotypes. Red-dotted arrows show Type-II-specific lineages, composed of multiple Dpn^+ *mature intermediate progenitor cells* (mINPs) in the wt control. Dotted yellow lines demarcate the boundaries of GFP^+-lineages from seemingly a single NB-lineage. Regions that we could not conclusively assign to Type I or II NBs were excluded from the quantification presented in Figure 3 and are marked here with, both, yellow and red (dotted) arrows. Note the expansion of aberrant NBs at the expense of the production of post-mitotic neurons ($Elav^+$, red marker) from each lineage.

3.3 Injections of NΔ-Hyperplastic Larval Brains in Host Flies: A Model for Ascertaining Tumorigenesis

Previous work in our laboratory has suggested the overexperssion of Imp as indicative of tumor progression during sequential transplantation of NΔ-tumors (allograft series) (Figure 5A). We set out to investigate the Imp expression patterns in the abdomen of wild-type flies (w^{1118}) that have been sequentially injected with tumor cells from hyperplastic CNSs of NΔ;wRi larvae. According to our allograft method, single (NΔ) central lobes were injected in the first allgoraft (T0). Following tumor expansion we subsequently dissected the host bellies, dissociated tumor cells (*collagenase treatment*) and injected, roughly, 500 cells to healthy young adult flies (2^{nd} passage, T1). Consistently, bellies of T1 were dissected and so forth, till we reached the 4^{th} allograft (T3). All allografted bellies from T1-3 exhibited the characteristic cytoplasmic localization of Imp (RNA-binding protein) which was enriched in all GFP^+ tumor regions (Figure 5B). These findings are qualitative and, thus, we cannot ascertain any quantitative differences concerning tumor (tNBs) behavior during our series of transplantations. However, it may be plausible to suggest that Imp expression accompanies tumor progression, indicated by the overlap of Dpn^+/GFP^+ in all observed tumor masses within each allografted host.

A


1600
1400
1200
1000
800
600
400
200
0
Gene counts per million
N-T0
N-T3
Imp
dm

B

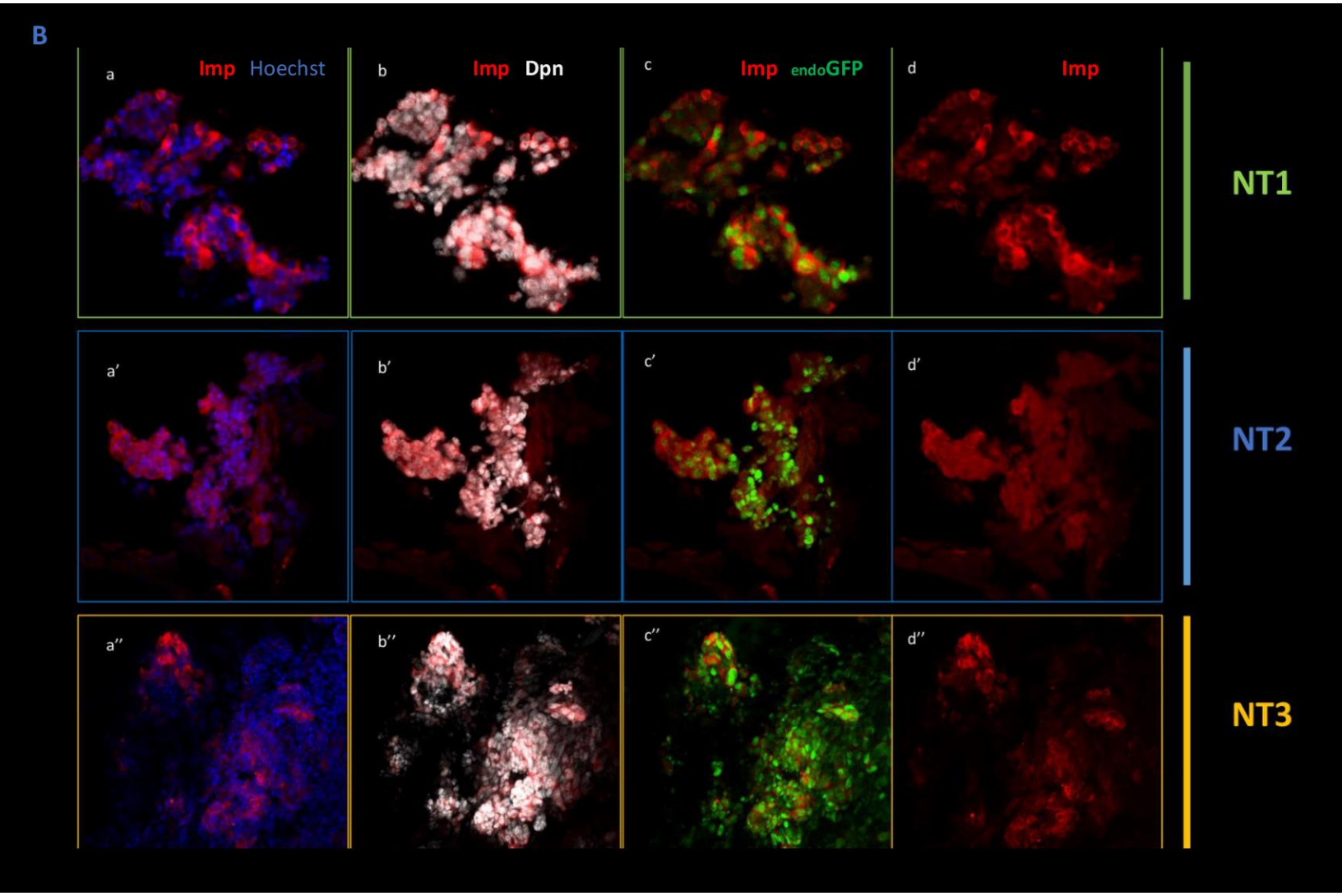

a
Imp Hoechst
b
Imp Dpn
c
Imp endoGFP
d
Imp
NT1
a'
b'
c'
d'
NT2
a''
b''
c''
d''
NT3

FIG. 5 IMP EXPRESSION PATTERNS IN TRANSPLANTABLE ALLOGRAFT SERIES OF *NΔ;wRi*-BEARING HOSTS

(A) RNAseq data from extracted ***NΔ;wRi*** tumors from the abdomen of the 1st (NT0) & 4th (NT3) allograft of injected adult hosts. Imp and Myc upregulation is observed during the serial transplantation of tumor cells. (B) Confocal images (40X) of abdominal extracts from the 2nd (NT1), 3rd (NT2) and 4th (NT3) allografts, immuno-stained for Imp and Dpn (NB) expression. Nuclei are stained with Hoechst. Endogenous GFP (tumor cells) is driven by the NB-specific driver grainyhead (grhGAL4). Tumor cells (GFP) overlap with Dpn expression, indicating overproliferation of ectopic tNBs. Imp expression accompanies the extended tumor masses within the body cavity, in all allografted hosts.

Following up on our previous finding, we injected the NΔecd tumours with impaired Imp or Myc function into adult flies in order to gauge their tumorigenicity. Our "control" group of aberrant NΔ-signaling *(UAS-NΔecd;wRi)*, exhibited the most aggressive phenotype of tumor initiation (Figure 6A). The median time to GFP^+ emergence of this genotype was 2,7 days compared to hosts injected with *UAS-NΔecd;ImpRi* larval CBs which averaged to 5,9 days to GFP^+ emergence. Surprisingly, from all the animals (n=44) injected with *UAS-NΔecd;dMycRi* lobes, not once did we observe formation of tumor masses (figure 6E), indicated by green fluorescence, in any of the tissues of adult hosts. This finding was very surprising, since in larval brains we did observe the existence, albeit limited, of extreme (>20NB) tumor-like lineages of aberrant NBs. However, this never manifested into tumorigenic formation in adult hosts. Following up our experimental workflow, we assayed the lifespan of the tumor-bearing hosts from our two remaining NΔ-genotypes (Figure 6B,C). Intriguingly, the lifespan differed greatly between the different genotypes. Concerning the hosts with the earliest GFP^+ emergence (*w*Ri), the median lifespan of tumor-bearing hosts was 12 *days post-injection* (DPI). Conversely, *Imp*Ri-injected hosts survived 26,5 DPI on average. These observations regarding the (~100%) increase in lifespan and retarded GFP^+ emergence after lobe injection, is indicative of a differential capacity of each respective tumor to expand and upset host homeostasis, leading to lethality. Between our two data sets (quantifications in Fig.3 vs survivals in Fig 6.), the only parameter that differed, was the day we dissected the larval CNSs (Day 6 and Day 7, respectively), prior-to-injection. This is an important parameter since lineages will keep propagating and expanding in order to populate the entirety of the larval CNSs, as shown by our confocal images (Fig. 2 and 4), presumably only restricted from arising cell-to-cell antagonistic relationships[65]. Yet it is important to recognize the correlation between tumor progression and total NB-behavior between our experiments

Most strikingly, regarding the shape of the statistical distribution between our tumor-bearing hosts of *w*Ri and *Imp*Ri genotypes, we identified significant differences (Figure 6A-C). There is a wide distribution, concerning both tumor onset and tumor lethality, in *Imp*Ri-bearing hosts. Arguably, this may suggest differences in the pacing of tumorigenic progression within *Imp*Ri-injected hosts compared to the homogeneity shown in our *w*Ri "control" group (aberrant NΔ-signaling). The subsequent downregulation of Imp translates as a perturbation in the temporal program of NBs during larval neurogenesis. As such, depending on the stochastic induction of our

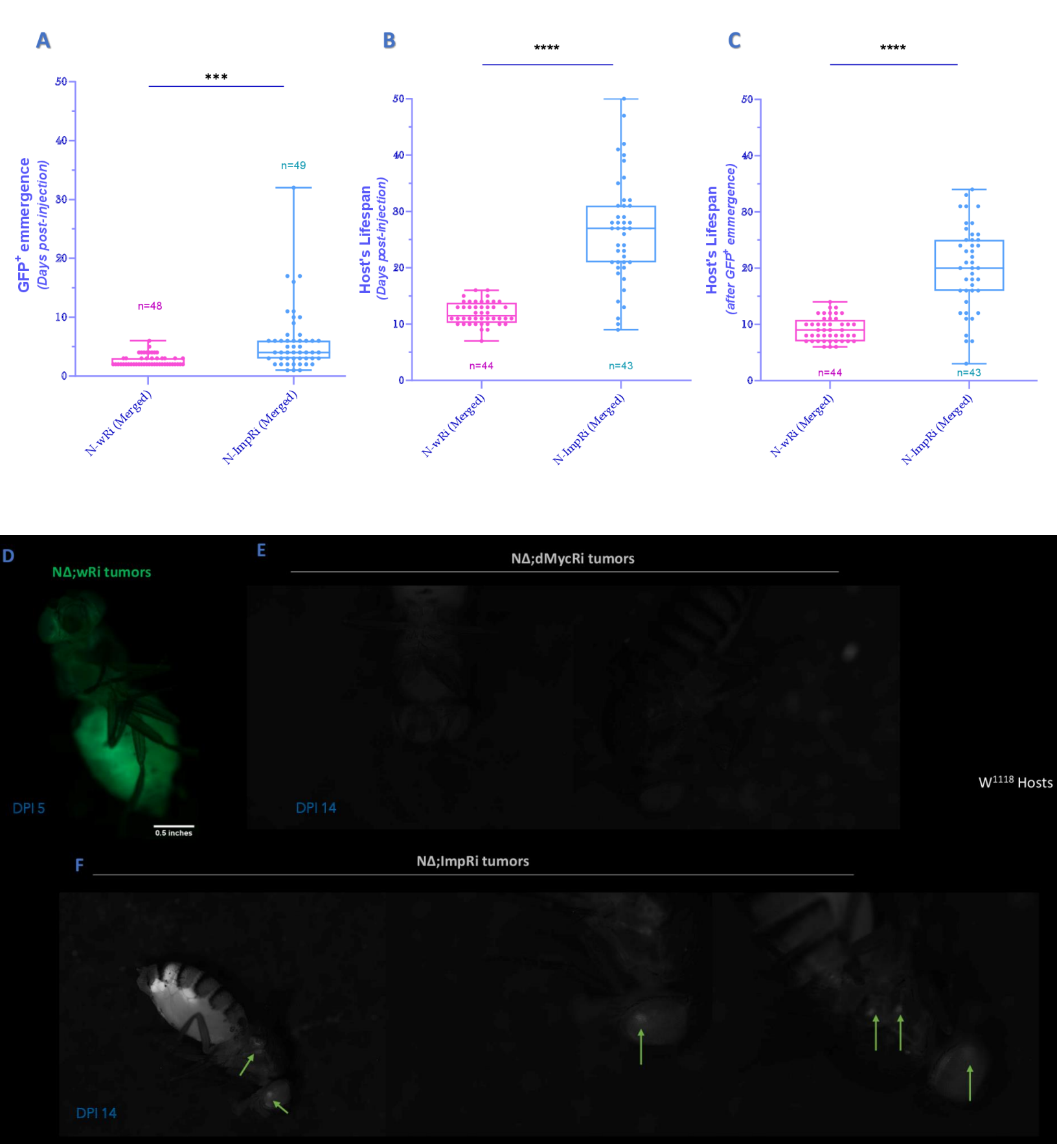
transgenic expression in developing NBs, which by definition are highly asynchronous in the completion of their temporal program, we hypothesize that our genetic manipulation of Imp's expression patterns might have enhanced the differential progression of (FLIPed-out) NB lineages.

FIG. 6 INJECTIONS OF NΔ-HYPERPLASTIC LARVAL BRAIN LOBES IN ADULT (T0) HEALTHY HOSTS TO ASSESS TUMORIGENIC POTENTIAL

Four-day old w^{1118} adult flies, pre-fed with yeast, were injected with single central lobes from tumorigenic late 3rd instar larval CNSs using a nanoinjector (see Materials). Four biological experiments have been performed for each genotype. (A) Box plot portraying tumor initiation. Injected flies with larvae central brains were observed daily for emergence of green fluorescence as indicative of tumor onset. Median of *NΔ;ImpRi*-bearing flies (n=49) amounted to 5,8 days post-injection (DPI). Median of *NΔ;wRi-bearing* (n=48) flies amounted to 2,7 DPI. (B) Box plot showing death after injection (DPI). Median of *NΔ;ImpRi*-bearing flies at 26,5 DPI. Median of *NΔ;wRi* tumor-bearing hosts amounted to 12 DPI. (C) Box plot showing the temporal dynamics of tumor progression by measuring the lethality due to tumor initiation (Day 1 of GFP^+ emergence). Median of *NΔ;ImpRi*-bearing flies reached 20 DPG. Median of *NΔ;wRi* tumor-bearing hosts amounted to 9 DPG. In each box plot, whiskers show 2,5 and 97,5 percentiles, boxes are the upper and lower quartiles and each dot represents a single fly. (D) Mesoscopic images of *NΔ;wRi*-bearing flies after excitation with a UV lamp (488nm) of the endogenous green fluorescent protein within the adult tissues (5 DPI). Note the extended tumor distribution within the eye and abdomen of the host. (E) *NΔ;dMycRi-bearing hosts* failed to show any tumor initiation up until their death following injection (greyscale,14 DPI) (Total GFP= 0%, 0/44). (F) Indicative tumor masses within the eye and laterally of thorax due to GFP expression (in greyscale, green arrows). Auto-fluorescence in the abdomen is attributed, presumably, to fluid accumulating due to the injury upon our injection series (14 DPI).

NΔ;ImpRi-bearing flies showed Total GFP% = 95% (48 flies showed tumor from the 51 injected flies). *NΔ;dMycRi*-bearing flies showed Total GFP% = 0% (0 flies showed tumor from the 44 injected flies). *NΔ;wRi*-bearing flies showed Total GFP% = 87% (48 flies showed tumor from the 55 injected flies).

Statistical analysis was performed with t-test: $p<0.05$ (*), $p<0.005$ (**), $p<0.001$(***) and $p<0.0001$ (****).

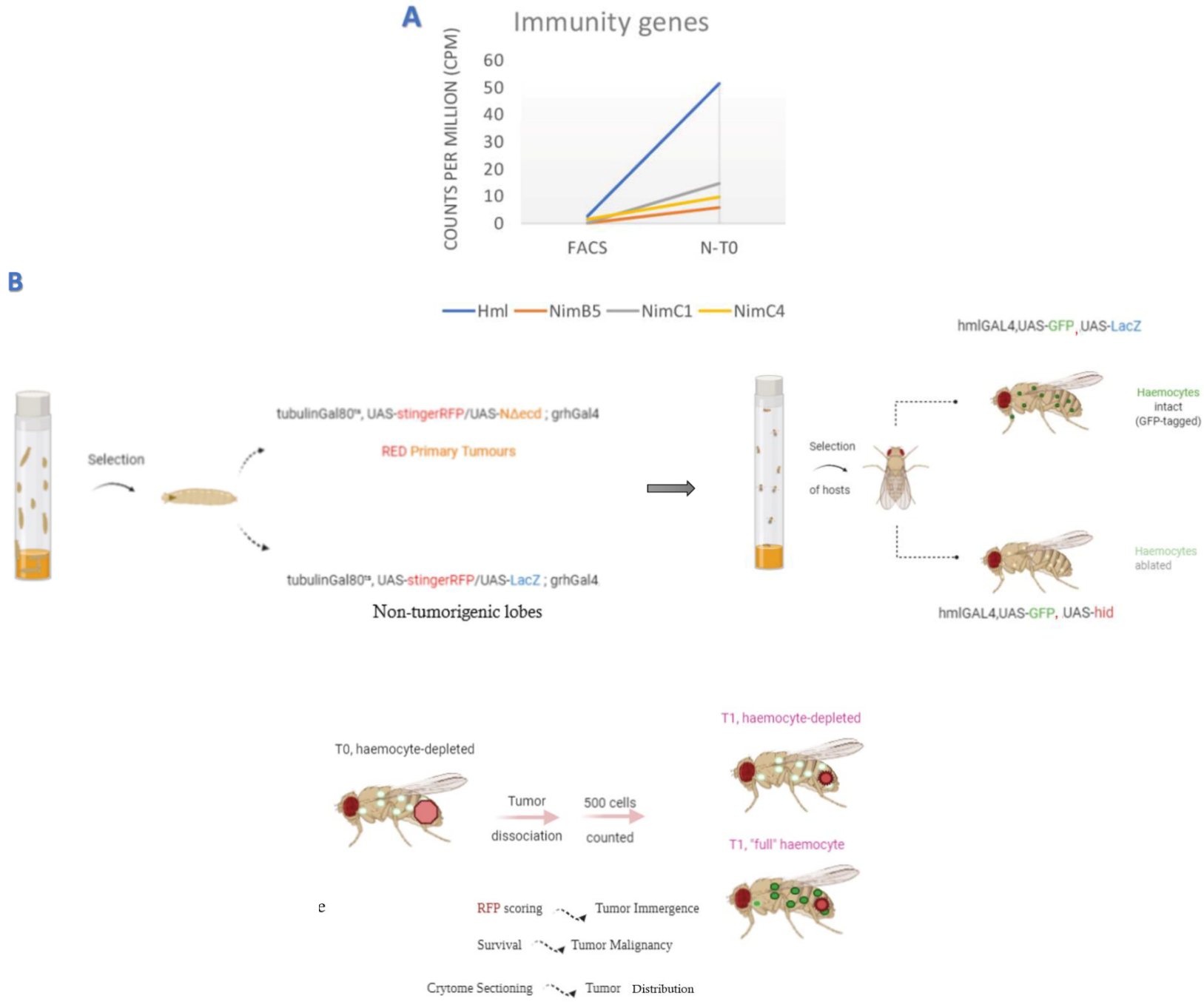

A
***
GFP+ emmergence
(Days post-injection)
n=48
n=49
N-wRi (Merged)
N-ImpRi (Merged)
B
****
Host's Lifespan
(Days post-injection)
n=44
n=43
C
****
Host's Lifespan
(after GFP+ emmergence)
n=44
n=43
D
NΔ;wRi tumors
DPI 5
0.5 inches
E
NΔ;dMycRi tumors
DPI 14
W1118 Hosts
F
NΔ;ImpRi tumors
DPI 14

3.4.1 Generation of Donor Flies (T0) for Allograft Transplantation

Focused on our previous findings, we have illuminated the temporal progression of tumor cells to be dependent on the genetic background. In agreement with the attenuation of NΔ-hyperplasia upon Imp and Myc knockdown in larval CNS, our *in vivo* model showed a drastic reduction in tumor aggressiveness. Recent work in our laboratory, has identified haemocyte involvement during tumor progression (Fig. 7A). To that end, we set out to explore the relationship at play following tumor injection in our allograft model. Firstly, we focused on the perspective of the tumor. We generated donor flies (1st allograft) by injecting their abdomen with primary tumors (brain lobes) that overactivated NΔ-signaling (UAS-NΔecd) in all NB lineages through the grainyhead promoter (grhGAL4). This provided a more potent tumorigenic outcome than our previous FLIP-out genetic approach which was dependent on stochastic clonal induction. To that end, we combined the (temperature-sensitive form of the GAL80 inhibitor within our grh;GAL4 construct to inhibit precocious activation during embryonic stages and avoid lethality. When larvae hatched, we incubated our vials in the permissive temperature (GAL80-inactivation at 29°C) for 2days and hyperplastic CNSs were generated for subsequent dissection and allografting. In an attempt to avoid "cross-contamination" of our T1 allograft with haemocytes populating our T0 allograft we used T0 hosts that were haemocyte- deficient via hml>hid expression (Figure 7E). After injection of single brain lobes into these haemocyte-ablated hosts, we dissected the abdomens of tumor-bearing hosts after several days, judged by a co-expressed stinger-RFP under the control of the UAS promoter. Following the dissociation of the recovered tumor masses, we allografted ~500 tumor cells in T1 RNAi-expressing flies.

Fig. 7. Generation of Donor Flies (T0) and Primary hyperplastic tumors in larvae CNSs for Sequential Allograft Transplantation (T1)

(A) RNAseq data following T0 generation of donor flies as shown by previous unpublished work in our laboratory. High-enrichment in *hemolectin* expression proposes recruitment of haemocytes within the tumor microenvironment upon FACS (Fluorescence-activated cell sorting) and RNA-sequencing of extracted tumor masses within adult T0 bellies. (B) The experimental design of growing donor flies (haemocyte-ablated) by overexpressing the pro-apoptotic gene Hid (hemolectin-driven GAL4 expression). Primary tumors were created by overexpressing the truncated NΔecd receptor specifically in NBs, driven by grainyhead (grh), during early-to-late larval stages. Temporal expression of our transgenes is permitted upon temperature switch (18°C-to-29°C) for GAL80^{ts} inactivation. After injection of singular central lobes in the abdomen of adult (donor) flies, several different directions can be taken for furthering our understanding of tumor progression.


A
Immunity genes
COUNTS PER MILLION (CPM)
60
50
40
30
20
10
0
FACS
N-T0
Hml
NimB5
NimC1
NimC4
B
hmlGAL4,UAS-GFP, UAS-LacZ
Selection
tubulinGal80ts, UAS-stingerRFP/UAS-NΔecd ; grhGal4
RED Primary Tumours
tubulinGal80ts, UAS-stingerRFP/UAS-LacZ ; grhGal4
Non-tumorigenic lobes
Selection
of hosts
Haemocytes
intact
(GFP-tagged)
Haemocytes
ablated
hmlGAL4,UAS-GFP, UAS-hid
T0, haemocyte-depleted
Tumor
dissociation
500 cells
counted
T1, haemocyte-depleted
T1, "full" haemocyte
e
RFP scoring
Tumor Immergence
Survival
Tumor Malignancy
Crytome Sectioning
Tumor Distribution

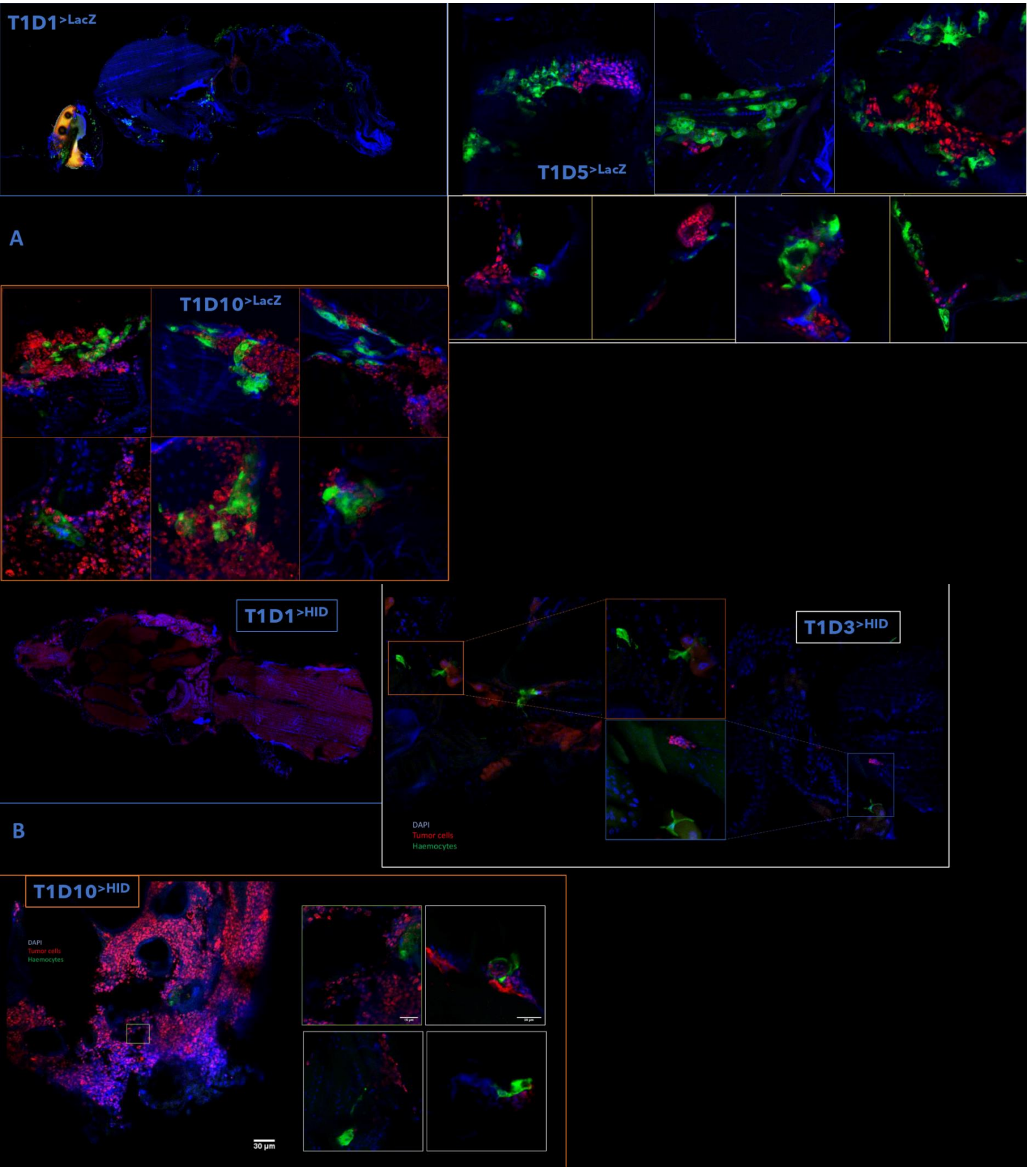
3.4.2 Spatiotemporal Distribution of Tumors in T1 Allografted Hosts

In the endeavor to illuminate the temporal dynamics of allografted (T1) cancer cells within hosts we utilized the RFP^+ expression of tumors (Fig. 7B) in cryosections of fixed adult flies (Fig.8). Since this method allows us to explore spatially the distribution patterns of cancer cells we isolated flies on the 1st, 3rd, 5th and 10th day after the injection. On the 3rd day we could already observe mesoscopically fluorescent (RFP^+) puncta in specific tissues of adult allografted flies. On the 10th day flies would often succumb to tumor lethality and die immediately after. By incorporating the earliest days of expansion and the final days of host lifespan, respectively, we hoped to unravel new insights during tumor progression *in vivo*. Despite the inability of this method to provide us with any information other than spatial distribution of tumor cells, we performed cryosections in both haemocyte-intact (hml>LacZ) and haemocyte-ablated (hml>Hid) T1 adult hosts (Fig. 7B).

Haemocyte "involvement" was observed in all cases of both haemocyte-full and haemocyte-ablated flies (hml>hid-driven apoptosis of haemocytes was not absolute). Evidently, the few haemocytes that survived (Fig 8B) retained GFP^+ expression and overlapped with tumor masses. On day 1 (Fig. 8A,B) we could rarely observe single RFP^+ cells in any of our hosts. On the 3rd day (Fig. 8B) small clusters of tumor cells started aggregating in, presumambly, random regions of the host's body cavity, with a preference for populating the head region of the fly (data not shown). On day 5th these clusters increased both in frequency and size expanding into many different tissues and organs of the host (Fig. 8A,B). On the 10th day, almost every body cavity in both hosts was filled with tumor cells irrespective of haemocyte presence.

FIG. 8. TIME LAPSE OF THE SPATIAL DISTRIBUTION OF TUMOR CELLS IN THE 1ST ALLOGRAFT (T1) OF HAEMOCYTE-ABLATED & HAEMOCYTE-FULL HOSTS.

(A-B) Cryosections (40-50μm) of T1 tumor-bearing hosts (hml>LacZ and hml>Hid) at different time points (D1-10) post-injection. (A) One day post-injection (T1D1). We seldomly observed RFP^+ (single) cells, if any. Three days post-injection (T1D3) in haemocyte-ablated hosts. Notice the GFP^+-expressing haemocytes (hml>Gal4, UAS-GFP) even in the Hid-driven ablation of plasmatocytes. On this day, small clusters of tumor cells start accumulating in different tissues of the hosts. (C) Five days post-injection in haemocyte-full hosts. Haemocytes exhibit clustering towards expanded tumor masses, perhaps by "crawling" or adhering to tissues of the host. From our data, we could observe close interaction with the tracheal system of the adult flies (respiratory epithelia autofluorescence in UV wavelengths-blue fluorescence) both during tumor association as well as in their motile behavior (extended membrane protrusions). (D-E) Expansion of tumor cells in all regions within the body cavity of hosts. Haemocytes encapsulate tumor cells and expand their membranes, seemingly, to phagocytose individual or clusters of RFP^+ tumor cells. This behavior is present in both genotypes of our T1 hosts.

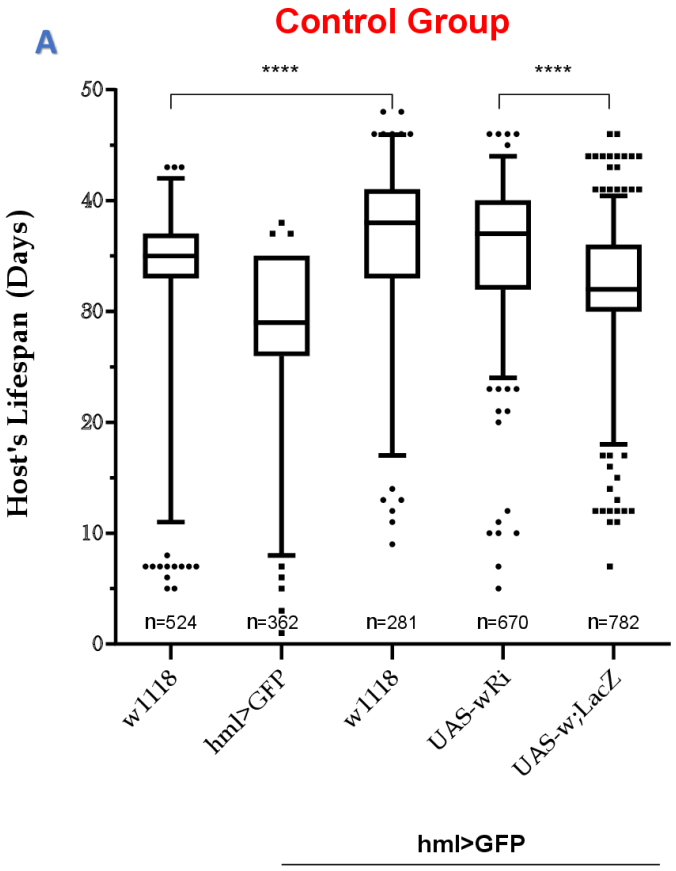

T1D1>LacZ
T1D5>LacZ
A
T1D10>LacZ
T1D1>HID
T1D3>HID
B
DAPI
Tumor cells
Haemocytes
T1D10>HID
DAPI
Tumor cells
Haemocytes
30 µm

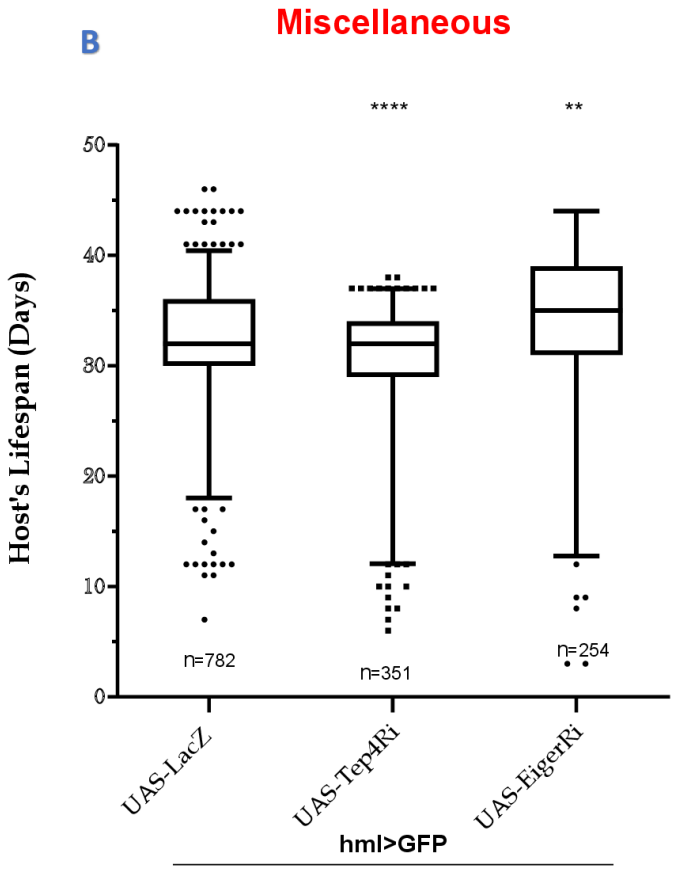
3.6. Addressing the role of Haemocytes in Tumorigenesis

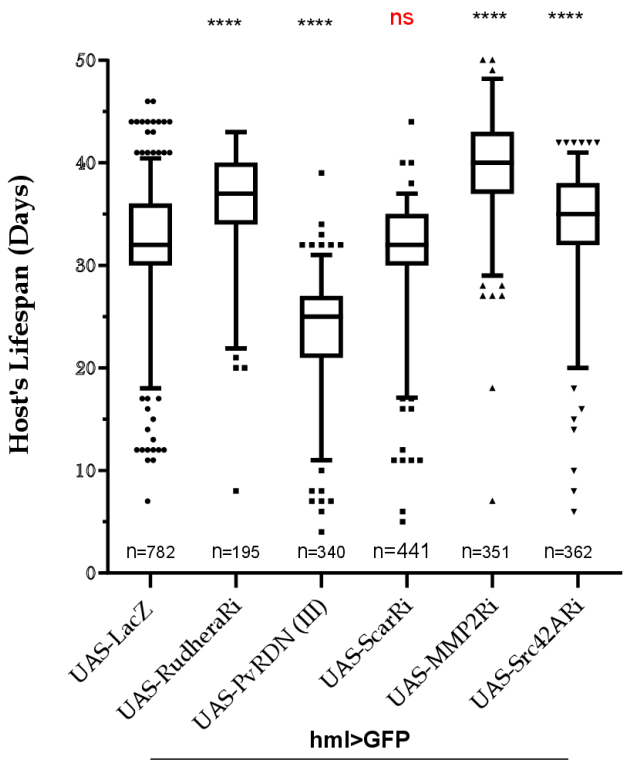
Finally, we set out to explore how perturbations in the function of plasmatocytes as cellular mediators of innate responses in the adult fly translate to host homeostasis. By incorporating the binary expression system UAS/GAL4 and transgenic lines for RNA-interference (RNAi) we generated a multitude of transgenic animals to knocking-down the expression of genes (one at a time) that have been shown to contribute to haemocyte function. GAL4 expression, together with an integrated coding sequence of GFP as a reporter, was driven by a *hemolectin* enhancer specifically expressed in all plasmatocytes (and a minor population of crystal cells)[75]. Each transgenic line carried hairpin sequences for a particular target mRNA of interest under the control of the UAS promoter. Progeny of each cross (RNAi line with the GAL4 reporter system) were incubated at 29°C right before eclosion (late pupal stages) to enhance the Gal4 efficacy. We performed survival assays for all of our transgenic animals to address the effect of perturbing haemocyte function on adult viability (Figure 7).

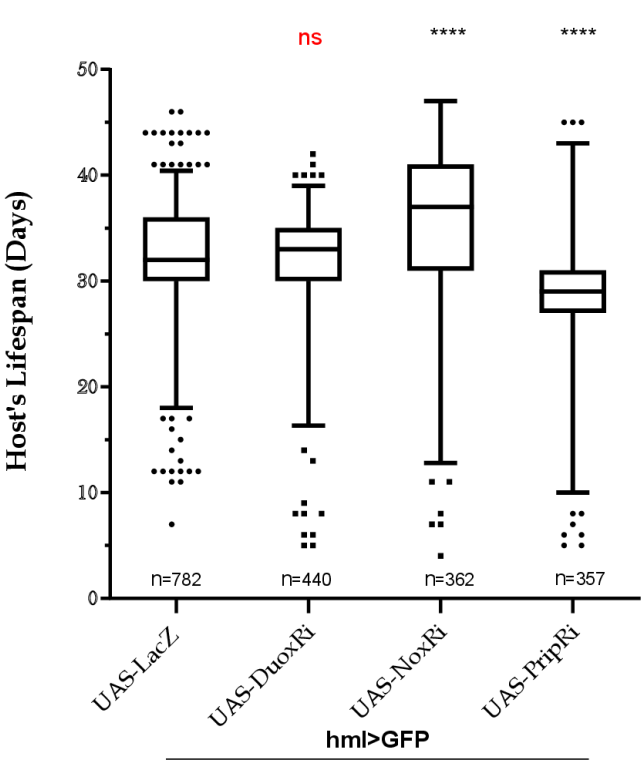
Intriguingly, only three transgenic lines failed to show alterations in longevity (ScarRi, DuoxRi and CroquemortRi) compared to our control group (UAS-lacZ). All other 17 transgenic animals (including different control genotypes such as w^{1118} and wRi) showed a large variability concerning the lifespan of the animals. Only three exhibited reductions in the longevity (PripRi, Tep4Ri and PvRRi), while the rest exhibited an enhanced lifespan that surpassed the median of our control group (33 days) by 2-5 days, depending on the target gene. It is worth noting that the total lifespan of a healthy and laboratory-raised fly averages between 6-to-8 weeks. These observed differences between our hosts may account for small discrepancies in the total longevity. However, when taking into account the total lifespan of this invertebrate organism, these alterations magnify into important indications of an irreversible consequence in host viability, indicating that lifespan is very sensitive to the genetic background. Interestingly, how and why slight enhances in longevity that originate from knocking-down genes essential for haemocyte function as cellular mediators of immuno-responses, is fascinating but fails to be addressed in this study in its totality. Partial ablation of haemocytes by driving the expression of two pro-apoptotic genes (Hid & the murine gene Bax) significantly impaired the longevity of the hosts (Figure 7E).

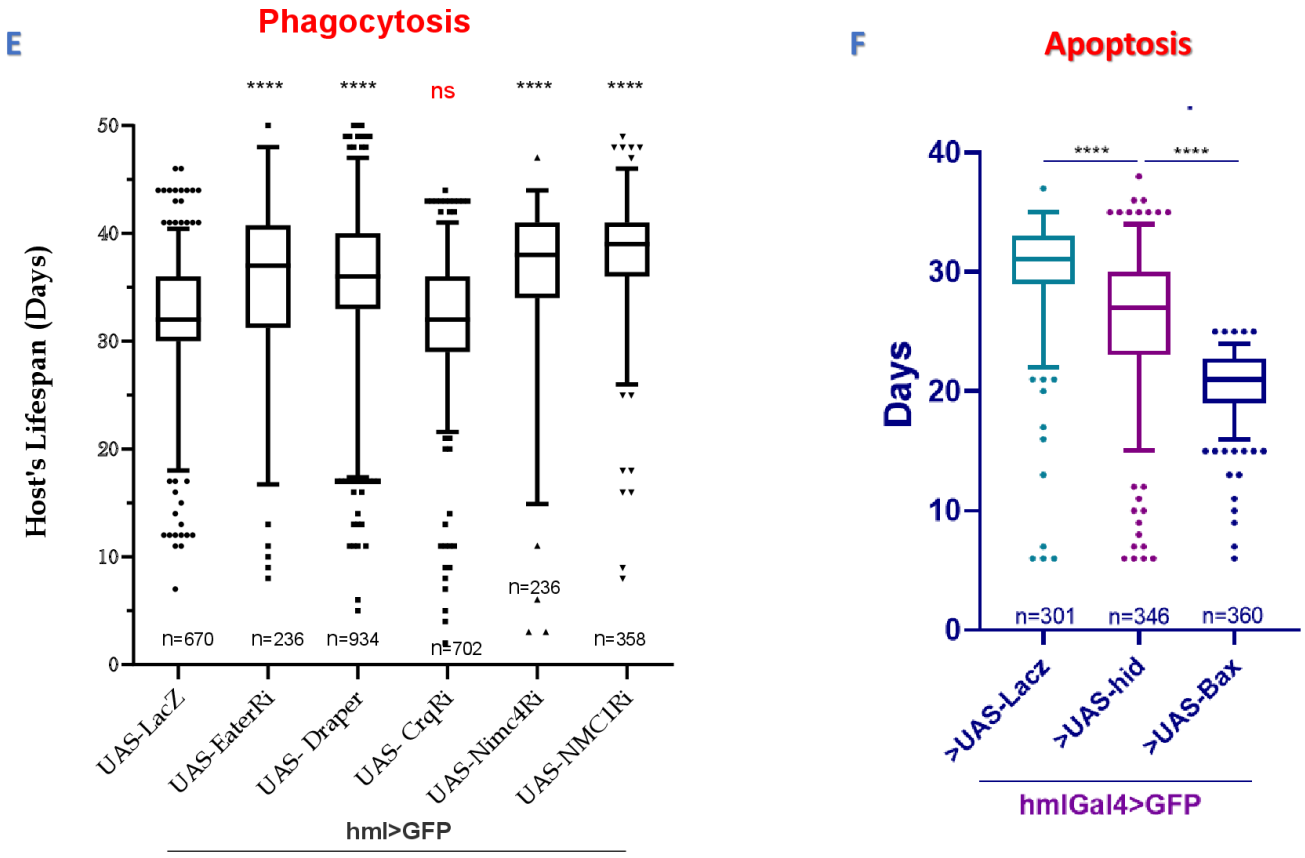
Fig. 9. Survival Assays of Transgenic (RNAi) Adult Flies for Haemocyte-specific Deficiencies
(A-F) Control and RNAi-mediated knock-down of individual genes specifically in the haemocyte population of adult flies. Our data were primarily grouped depending on overlapping functions of our target genes (when possible). (A) Control genotypes portraying variety in the longevity of adult flies. It is essential to properly select a control genotype when generating survival assays (see Discussion) and incubate each RNAi line with a control background during survivals. (B) Haemocyte-specific knock-down, the TNF-orthologue Eiger (inflammation-induced signaling) and the Tep4 (opsonization factor) [*Miscellaneous* Category]. Only Tep4Ri shows attenuation of longevity, whilst EigerRI-flies show increase in host lifespan. (C) Knock-down of the metalloprotease MMP2 (embryonic expression essential for haemocyte dispersal), PvR (Platelet-like growth factor/vascular endothelial factor receptor), Rudhera (cytoskeletal remodeling protein), Scar/WAVE (component of the actin-nucleation complex) and the Src42A (Lyn mammalian orthologue). ScarRi-flies exhibited identical lifespan with our control group (UAS-LacZ). PvRRi-flies showed a conserved and dramatic attenuation of host lifespan. Remaining genotypes showed enhanced lifespan. (D) Components of the redox-sensitive apparatus of haemocytes. Duox and Nox are transmembrane NADPH oxidases responsible for extracellular accumulation of H2O2. However, only the former showed no alterations in the lifespan of hosts. Consistently, Nox and increased lifespan. Loss of function of Prip (AQ1 mammalian orthologue), an aquaporin-like transporter of H2O2, significantly attenuated host longevity. (E) Knock-down of all four scavenger apoptotic receptors Eater, Croquemort (Crq), Draper, NimC4 (Nimrod Family) and phagocytic receptor NMC1 that are expressed in haemocytes. Only CrqRi-flies exhibited similar lifespan with our control group. Deficiency in any of the remaining receptors greatly increased lifespan of host flies. (F) Dramatic reduction in longevity is observed during ablation of haemocytes with either the proapoptotic gene Hid (head involution defective) or the murine gene Bax. Whiskers in each box plot represent 2,5 and 97,5 percentiles. Statistical analysis was performed with t-test: $p<0.05$ (*), $p<0.005$ (**), $p<0.001$(***) and $p<0.0001$ (****).


Control Group
A
Host's Lifespan (Days)
****
****
n=524
n=362
n=281
n=670
n=782
w1118
hml>GFP
w1118
UAS-wRi
UAS-w;LacZ
hml>GFP


Miscellaneous
B
Host's Lifespan (Days)
****
**
n=782
n=351
n=254
UAS-LacZ
UAS-Tep4Ri
UAS-EigerRi
hml>GFP


Actin/Migration
C
Host's Lifespan (Days)
****
****
ns
****
****
n=782
n=195
n=340
n=441
n=351
n=362
UAS-LacZ
UAS-RudheraRi
UAS-PvRDN(III)
UAS-ScarRi
UAS-MMP2Ri
UAS-Src42ARi
hml>GFP


Redox-sensitive Aparratus
D
Host's Lifespan (Days)
ns
****
****
n=782
n=440
n=362
n=357
UAS-LacZ
UAS-DuoxRi
UAS-NoxRi
UAS-PripRi
hml>GFP



The scientific idea behind this large survival screen was to establish a well-defined longevity outcome in each of our unchallenged adult flies (n>200 flies per genotype). These findings would establish the background for the next step of our experimental design, namely the exploration of each gene's significance upon tumor transplantation within our hosts. This larger screen is being carried out by Chrysanthi Voutyraki and is still in progress. For the purposes of the present thesis, we focused on the RNAi of two phagocytic genes, Draper and Crq. Upon establishing a link between hemocyte-specific genes and phenotypic outcome (longevity), we selected hosts from the same transgenic crosses and injected them at day 5~6 (maturation) with dissociated tumor cells (~500 cells per injection) from T0 tumor-filled abdomens of donor flies. This ensures a more reproducible tumour burden per host, as opposed to injecting primary brain lobes, which show a considerable variability in tNB content. Additionally, it minimizes the injury of transplantation, since a much finer needle is used to transfer the cell suspension than would be needed for a bulky brain lobe. In contrast to the FLIP-out system, we continued with the genetic approach described earlier (Fig.7,8) by temporally regulating the expression of NΔecd in all larval NBs upon temperature induction driven by a *grainyhead* enhancer-Gal4 line (*grh*, temporal effector expressed in all embryonic and larval NBs)[37]

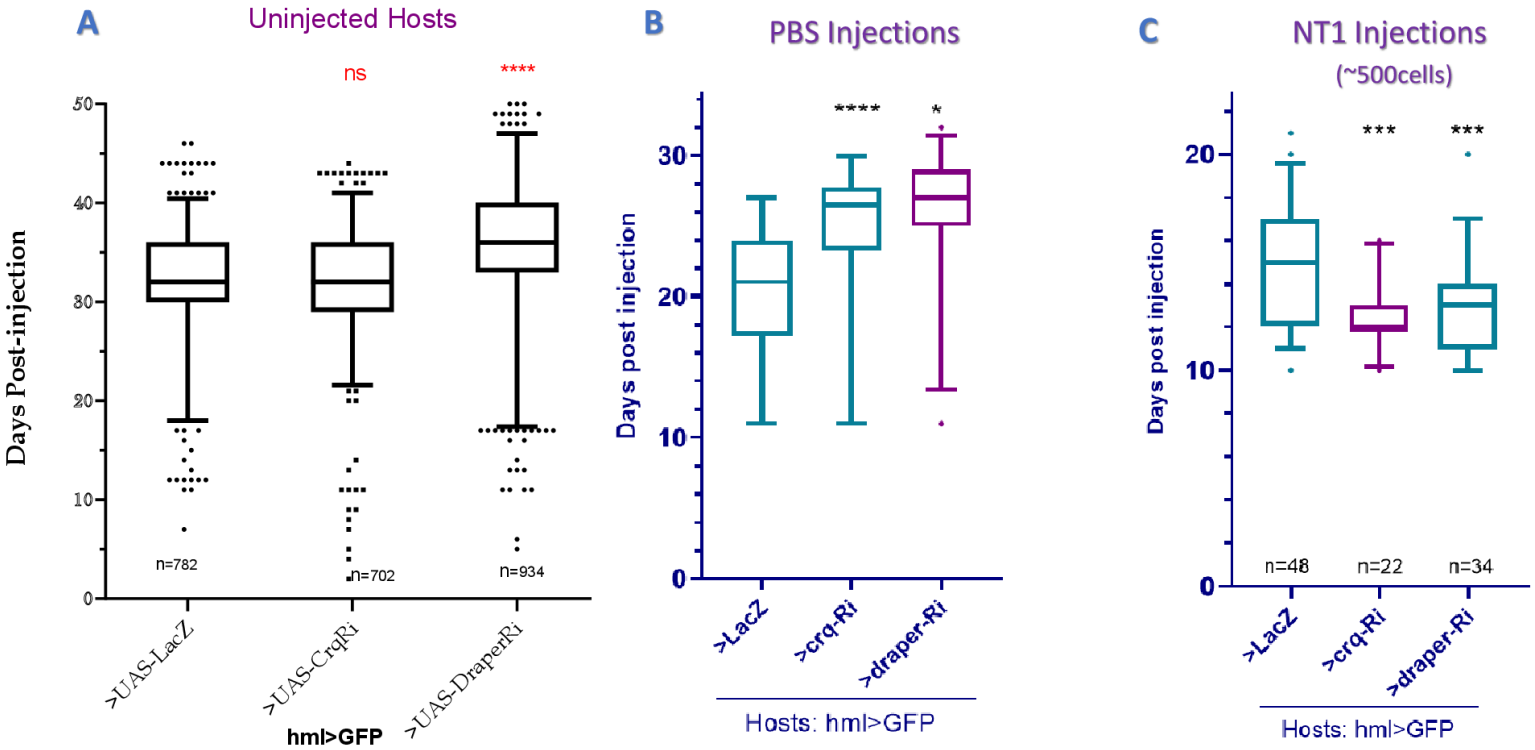


FIGURE 10 HAEMOCYTE-SPECIFIC DRAPER AND CROQUEMORT RECEPTORS PROMOTE HOST LIFESPAN UPON TUMORIGENESIS

(A) Survival assays of uninjected flies deficient for Crq and Draper expression within haemocytes. (B) Survival assays of the aforementioned hosts after PBS (1X) injections. CrqRi flies injected (n=10). DraperRi flies injected (n=31). (C) Survival assays of the same haemocyte-deficient flies after injection with tumor cells (n~500) dissected from the same tumor-bearing donor flies (T0). Whiskers in each box plot represent 2,5 and 97,5 percentiles. Statistical analysis was performed with t-test: $p<0.05$ (*), $p<0.005$ (**), $p<0.001$(***) and $p<0.0001$ (****).

4. Discussion

In this work, we have highlighted the role of aberrant Notch-signaling in formulating a hyperproliferative CNS by derailing the developmental trajectory of larval NB lineages. N-signaling is evolutionary paramount for proper design and specification of many different tissues and organs throughout the metazoans. Importantly, recent efforts have linked abnormal N-signaling with many diseases and cancers in vertebrate organisms. Many solid tumors[76] and leukemias[26,77] in humans originate or are maintained through the epigenetic and transcriptomic dictations of improper N-signaling.

Focused on previous (unpublished) data, we decided to knock-down two major regulators of the self-renewal capacity of type I NBs. However, we failed to address the propensity of Type II NBs towards the observed tumorigenic potential of our allografted tumors. With our genetic approach we randomly induced hyperproliferation in Type II NBs (4A' and 4B'). This amounted to an order of magnitude higher ectopic NB production (data not shown). Despite exclusion of these gigantic lineages in our final quantifications, we have to take into account the bias behind our ascertainment of the *in vivo* tumor behavior. In the same manner, we failed to address the *in vivo* significance of our fragmented categories of (Type I) tNB lineages (Figure 3). It remains to be seen if Type II lineages actively participate or even guide the phenotypic outcome within hosts (GFP^+ emergence/Tumor Lethality), despite being outnumbered (8:90 Type II vs I NBs per brain lobe).

Additionally, concerning the allografted NΔ;dMycRi CNSs, our quantification method clearly showed a small fraction of the measured lineages to exhibit extreme hyperproliferative properties (>20NBs/clone) like in the majority of NΔ;wRi measured clones. Yet, upon injection we never observed within the lifespan of our laboratory-reared flies (6~8weeks) any sign of tumor emergence. Hypothetically, two scenarios can explain our conflicting, albeit exciting, observations; (1) The tumorigenic "potential" (prior to tumor initiation) outlived the lifespan of the adult flies. To that end, future directions could create allograft series from the abdomen of NΔ;dMycRi-injected (non-GFP^+, T0) aging flies. Ongoing work in our laboratory, has shown NΔ-tumors to respond differently to the host microenvironment and exhibit a rather aggressive evolution upon sequential allograft transplantation (~500cells). Tumorigenesis is based on aberrantly self-renewal stem progenitors, and this agrees with our quantifications. Alternatively, by utilizing the NB-specific driver *grainyhead*, we could express our UAS-NΔ;UAS-dMycRi transgenic cassette upon larval hatching. Temporal control through the incorporation of $GAL80^{ts}$ is imperative, since embryonic expression is lethal. Then, new allograft injections could be generated with these larval CNSs. (2) Opposing forces arise between the host's immune activation and tumor expansion. By the time we could detect the tumor, the blood cells of the adult fly have effectively exterminated the foreign tissue and, as such, GFP^+ fails to emerge. This scenario is more likely for the NΔ;ImpRi CNSs, which were enriched in the intermediate levels of hyperplastic lineages and that also translated to a retarded tumor progression *in vivo*. All in all, injected ImpRi-tumors exhibit discrepancies concerning the day of tumor initiation *in vivo.* Despite constituting part and parcel of identical genetic manipulations, these hyperplastic neural-derived tumors, also, progress with substantially different paces within our paradigm of host homeostasis.

Innate immunity encompasses the only defensive mechanism with which flies have been equipped to barricade against a multitude of invading forces. Especially, when considering that

larvae grow and feed within decaying organic matter. Notwithstanding its simplicity in its circulatory system or the cell mediators composing the fly's immuno surveillance system, important insights can be extrapolated from invertebrate-based insights. It is still not known if the fly's cellular and humoral immune responses can protect it against malignant cells and we have started a project in the lab to address this question.

During our survival assays of RNAi (uninjected) hosts we addressed the importance of using an appropriate control group for comparing the longevity between our different genotypes. We have also documented that the lifespan of adult flies is affected upon genetic manipulation specifically of adult haemocytes. By succeeding to produce many flies (n>>200) for each RNAi line from different technical and biological experiments we have a good estimate of the variation of our population samples across our comparisons. In light of a new study we excluded the white gene from our analysis seems it has been shown regulate the proliferative capacity of gut stem cells in *Drosophila*[78]. It is worth noting, that replicates and different RNAi lines should be performed simultaneously and always with a control population at hand, since, seasonal changes magnify the minor differences we observe during our longevity survivals and so any observation should be carefully curated. Interestingly, upon comparison of our survival assays we observed a great deal of variability in each genotype compared to our control (hml>UAS-GFP, UAS-LacZ). As we elaborated in Figure 9, only three RNAi lines did not show statistically significant changes in longevity, namely Crq, Duox and Scar. Fascinatingly, previous studies have demonstrated that Duox knock-down does not alter total host longevity[16]. These lines of evidence, partly, overlap with the results from our PBS (control) injections concerning Crq- and Draper-deficient haemocytes. When trauma is induced and haemocytes lack either one of these two prominent scavenger receptors, adult flies live longer -whereas in uninjected flies, lifespan was not strongly affected compared to the control. However, upon tumor injection, the host susceptibility is increased dramatically (reduced lifespan). In parallel, haemocyte-ablation driven by two different pro-apoptotic genes (Hid and Bax) reduces longevity in uninjected flies, illuminating an essential physiological role of blood cells for the routine homeostatic response of hosts.

To that end, we hypothesize that haemocytes are essential for the routine immunosurveillance of adult flies. Haemocyte abolishment in unharmed flies negatively impacts homeostasis. Conversely, upon wound induction, the response of haemocytes seems to generate a more inflammatory-like state that can be harmful to survival. Ablating components of the redox-sensitive system of haemocytes as shown by the previous study (ROS regulation) or the scavenger-mediated recruitment of circulating/tissue-resident haemocytes as shown in our study (indirectly bypassing ROS production) may indeed, independently, preserve host lifespan. Especially when we take into account that haemocyte-recruitment to the wound area is essential for any observed ROS accumulation and inter-tissue communication. The notion that haemocyte activation may be harmful to the organism is supported by another study that linked the secretion of Upd3 (Toll-mediated within haemocytes) with maturation defects[79].

Like in vertebrates, the equilibrium between pro- and anti-inflammatory responses is essential for organismal homeostasis. Despite the evolutionary conservation of signaling effectors, the primitive blood system of the fly might be more prone to perturbations as an indirect consequence of the observed rapid resolution of invading agents in larvae and adults. Finally, in our two RNAi lines (CrqRi and DraperRi) we observed dramatic reduction in host longevity following tumorigenesis. Necrotic and apoptotic cells might be a consequence of either tumor intrinsically-events (dead tumor cells) and/or the subsequent stress-induction of tissues/organs of

the host (apoptosis-mediated apoptosis). Failure to encapsulate these foreign bodies from receptor-deficient haemocytes may explain these observations.

Future directions can utilize our findings concerning the attenuation of tumor aggressiveness upon Imp or Myc downregulation. The observed retardation of both tumor initiation and lethality *in vivo*, are evident of a slower pace of ectopic tNB production upon aberrant N-signaling. It would be interesting to explore the role of each gene's overexpression during larval neurogenesis in normal NB lineages. Myc and Imp interact positively to promote the self-renewal state of "juvenile" NBs. In the same manner that N-signaling overactivation can reset the developmental clock of stage-specific NBs, we could hope to identify new insights in the stem-like transcriptional network that refines the aberrant developmental trajectory of tNBs. More importantly, we could highlight the importance of their interplay by combining LoF/GoF experiments between Imp and Myc or other temporal effectors to explore the hierarchical ordeal of lineage progression. We could then interpret our findings within our model of allograft series to establish a framework of cooperative forces that exacerbate or diminish tumorigenic potential of NΔ-tumors. Focused on the haemocyte role during tumorigenesis, we have identified two essential phagocytic receptors for attenuating tumor progression. By repeating our experimental method for every RNAi line in our arsenal, we could unlock new perspectives in the functional entity of plasmatocytes upon tumorigenesis in adult flies. Additionally, we could integrate different genotypes of tumors to perceive these deficiencies in plasmatocytes as absolute for host lifespan or non-obligatory depending on the dynamics of each tumor's genetic background. In our work, a future approach would be to inject ImpRi tumors in allografts of our Crq- or Draper-Ri hosts. Arguably, the handicapped phagocytic activity of haemocytes might allow an increased tumor progression as seen in control (wRi) NΔ-tumors. In the case of dMycRi tumors we could even hypothesize that the window between tumor initiation and haemocyte interaction could be postponed enough to allow formation of secondary tumors, an observation we never documented in our haemocyte-intact hosts upon injection. Finally, cryosections could pave the way for describing differences in the behavior of RNAi-expressing haemocytes as we have documented (Fig. 8). We could address basic processes such as tracheal- or tumor-association, active migration to the trauma or tumor masses and even characterize the dependency of tumor expansion to their genetic characteristics[80].

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