Figures and figure supplements

The asymmetrically segregating lncRNA cherub is required for transforming stem cells into malignant cells

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Figure 1. *brat* tumor neuroblasts possess increased proliferation potential. (A) Cartoon depicting a *Drosophila* larval brain (OL optic lobe, VNC ventral nerve cord) harboring different neuroblast populations: mushroom body NBs (grey), type I (NB1, green) and II (NBII, orange) neuroblasts. Close-up shows a NBII lineage (iINP - immature intermediate neural progenitor, mINP - mature INP, GMC - ganglion mother cell) and the typical arrangement of cell types in a NBII clone (left). Proteins (blue) are asymmetrically segregated in NBII and mINP to ensure lineage directionality (right). (B) In *brat* mutants, the smaller daughter cell fails to differentiate and after a transient cell cycle block regrows into an ectopic neuroblast (tNB - tumor neuroblast). *brat* tumors continue to grow upon transplantation. (C) Representative images of adult host flies injected with FACS-sorted control NBs (GFP+) and *brat* RNAi tNBs (RFP+) from third instar larvae. Transplantations of brain pieces served as controls.

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Figure 2. brat tumors form independently of common DNA alterations. (A) DNA content analysis of brat\textsuperscript{ko6028} mutant and control brains showing non-dividing (2n) and mitotic cells (4n). DNA content for each genotype is shown separately in small boxes. (B–F) Genome sequencing of brat\textsuperscript{ko6028} brains.
and abdominal tissue (control). Note that Drosophila brain cells are diploid while the control samples harbor endo-replicating tissue and follicle cells with increased DNA copy number. (B) A representative coverage plot of chromosome arm 2L of one tumor is shown (top row), together with datasets indicating under-replicated (blue) and amplified (magenta) genomic regions. (C) Close-ups of boxes marked in (B) showing steady tumor coverage (red) and a drop (under-replication) or increase (amplification) in the coverage of the control sample (green). (D) Genes with identified somatic point mutations (coverage tumor >14 and control >8, allelic frequency ≥0.1) of three tumors do not overlap. (E) Affected genomic regions of identified small InDels. (F) In tumor 2 and 3, one small InDel each was identified in an exon with low allelic frequency. Affected genes are indicated.

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Figure 3. The lncRNA CR43283 is upregulated in brat tumor neuroblasts. (A) Cartoon illustrating the strategy to isolate NBII and tNBs. (B) Schematic overview of DigiTAG. cDNA fragments of the sequencing library harbor random 8-mer index tags (barcodes) unique for each individual molecule. After sequencing, reads are mapped and barcodes are assigned to each read. Reads with identical barcodes are removed to avoid amplification biases. (C) Scatter plot showing the expression in tNBs and NBII of genes detected by DigiTAG. Genes significantly up- or downregulated are shown in black (FDR 0.01, p<0.01), genes unchanged in expression levels in grey. CR43283 (cherub) is highlighted in red. (D) Protein coding genes show positive CSF scores, CR43283 has a negative CSF score (non-coding) similar to well known lncRNAs (ROX1, CRG). CSF score for each isoform of a gene is depicted. DOI: https://doi.org/10.7554/eLife.31347.005
Figure 3—figure supplement 1. Confirmation of up- and downregulated genes in brat tumor neuroblasts identified by DigiTAG. (A–C) Expression of indicated genes of FACS-sorted NBIs and tNBs expressing UAS-Stinger:RFP under the control of UAS-dcr2; wor-GAL4, ase-GAL80 was measured by qPCR. Data are mean ±SD. Student’s t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (A) brat mRNA levels are significantly reduced upon the
Figure 3—figure supplement 1 continued

expression of brat RNAi. n = 3 biological replicates. Expression of genes identified to be up (B) or downregulated (C) in brat RNAi tNBs compared to control NBIs are confirmed by qPCR. (B, C) For all tested genes n = 4 biological replicates.

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Figure 4. cherub is dispensable for brain development. (A) Assessment of female fecundity. For each genotype n = 3 independent crosses. Data are mean ±SD. One-way ANOVA. Not significant (n.s.). (B) Fertility and viability of cherub mutants shown as percentage of collected eggs giving rise to adult flies. Per genotype n = 10 replicates each consisting of 100 collected eggs. Data are mean ±SD. One-way ANOVA. Not significant (n.s.). (C) Quantification of the negative geotaxis behavior in cherub mutants and control. For each genotype n = 10 replicates, each consisting of 10 adult flies. Data are mean ±SD. One-way ANOVA, not significant (n.s.). (D) NBII lineages (outline) of one brain lobe. NBII marked by Dpn. Dividing NBII show cytoplasmic Dpn staining (arrows). Scale bars 50 μm. (E) Close-ups of control and cherub mutant NBII lineages (outlined) show one single big Dpn⁺ NBII (arrow), Dpn⁻ Ase⁻ iINPs (arrowhead) and Dpn⁺ Ase⁺ mINPs (open arrowheads). Scale bars 10 μm. (D, E) UAS-dcr2; insc-GAL4, UAS-CD8::GFP was used to outline NB lineages. (F) Figure 4 continued on next page
Figure 4 continued

Quantifications of large Dpn+ NBII (n = 4 brain lobes from four different brains), Ase Dpn+ iINPs and Ase+ Dpn+ mINPs in control (n = 16 lineages from four brain lobes) and cherub mutant (n = 20 lineages from four brain lobes) L3 brains. Error bars show mean ±SD. Student’s t-test. n.s. not significant.

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Figure 4—figure supplement 1. Analysis of cherub mutants. (A) Overview of the cherub gene locus. Deletions of cherub mutants and target site of cherub RNAi are indicated (red). (B) Overviews of brain lobes (outlined) showing the absence (cherub DEL) or strong reduction (cherub promDEL) of cherub FISH signal in cherub mutants. Scale bars 50 μm. Each close-up shows one NB with surrounding cells. (C) Quantification of cherub transcript levels using qPCR on whole larval brain samples. n = 3 biological replicates. Data are mean ±SD. (D) cherub RNA levels are reduced by expressing cherub RNAi under the control of UAS-dcr2; insc-GAL4, UAS-CD8::GFP. Scale bars 20 μm.

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Figure 5. cherub is required for brat tumorigenesis. (A, B) Brain lobes (outlined) stained for Dpn (NB marker) and Ase (differentiating cells). OL, optic lobe. Scale bars 50 μm. Driver line was UAS-dcr2; insc-GAL4, UAS-CD8::GFP. (C) Percentage of hatched adult flies. brat RNAi flies were crossed to insc-GAL4/+; CyO. Flies with brat RNAi that inherit the balancer CyO hatch, but those with insc-GAL4 die. cherub mutants rescue the survival of insc-GAL4 + brat RNAi expressing flies. n = 2 independent experiments per genotype. For each genotype per experiment ≥50 flies were counted. Data are mean ±SD.

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Figure 5—figure supplement 1. cherub reduces tumor growth in brat mutants. (A) Representative images of brat and brat cherub mutant larval brains (NB marker Dpn, neuron marker Elav). Scale bars 100 µm. (B) Quantification of brat mutant (n = 17 brain lobes) and brat cherub double mutant (n = 19 brain lobes) tumor volume. (C) Percentage of adult survivors. (D) Representative images of control, cherub DEL^−/−, and cherub DEL^−/− + genomic cherub. Scale bars 100 µm.
Figure 5—figure supplement 1 continued

brain lobes) tumor volumes. Results are pooled from two independent experiments. Student’s $t$-test. ****$p<0.0001$. (C) The percentage of surviving adult escapers is increased in brat cherub double mutants compared to brat mutants. n = 4 independent crosses per genotype. For each genotype per experiment ≥76 flies were counted. Mean ±SD is shown. Student’s t-test. ****$p<0.0001$. (D) The cherub genomic rescue construct increases the number of Dpn+ tNBs in cherub DEL/−; brat $^{RNAi}$ brains. Brains are outlines. Scale bar 50 μm. Driver line was UAS-dcr2; insc-GAL4, UAS-CD8::GFP. DOI: https://doi.org/10.7554/eLife.31347.010
Figure 6. The RNA-binding protein Staufen segregates cherub asymmetrically into the INP upon cell division. (A) Close-up images of NB clones in interphase. In NBs (arrowhead) cherub FISH signal is detected as two dots and cortically enriched. Nearest daughter cells (arrows) show high cherub FISH signal. Figure 6 continued on next page.
levels in the cytoplasm. Scale bars 20 µm. (B) Close-up of a NBI and a NBII lineage. Cell types are defined by cell size and the marker combination of Dpn and Ase according to the cartoon. Newly born Dpn+iINPs (open arrowhead) have higher cherub levels than older Dpn− Ase⁺ mINPs (arrowhead). Mitotic INPs show uniform cytoplasmic cherub localization (arrow). (C) Close-up images of cherub localization in a mitotic NBII outlined by membrane-bound GFP in vivo. Scale bar 10 µm. (D) Representative image of a dividing NBII in vitro. 100% of mitotic NBIIIs show cherub crescents (E) opposite to the apical aPKC crescent as measured by the angle between aPKC and cherub crescents. An angle of 180° corresponds to crescents opposite each other. n = 23 NBIIIs. Scale bar 10 µm. (F) Expressing constitutively active Lgl (LglΔ3) leads to a uniformly cortical distribution while constitutively active aPKC (aPKCΔN) or miranda (mira) knockdown displaces cherub from the cell cortex. Scale bars 10 µm. (G) Ratios of cytoplasmic cherub intensity of INP (or daughter cell) and NBII are depicted. In control, asymmetric cell division leads to higher cherub levels in INPs (n = 11 NB-INP pairs from four different brains). Upon the expression of aPKCΔN (n = 11 NB-daughter cell pairs from four different brains) or mirandaRNAi (n = 12 NB-daughter cell pairs from four different brains), cherub levels are similar in NBIIIs and their recently born (closest) daughter cell. Data are mean ±SD. (H) cherub localization in control and upon staufen knockdown. Brain lobes (top) and NBII (bottom) are outlined. Scale bars 50 and 10 µm. Driver line was UAS-dcr2; insc-GAL4, UAS-CD8::GFP. (I) In contrast to control (n = 13 NBII-INP pairs of 4 different brains), staufen depletion leads to similar cytoplasmic cherub levels between INP and NBII (n = 15 NBII-INP pairs of 4 different brains). Data are mean ±SD. (J) RIP-qPCR analysis for cherub and the non-Staufen target RpL32. Note that brain lysates from brat1560238 mutants were used to enrich for NBs as cherub is only cortically localized in NBs. Data are mean ±SD. n = 3 independent RIP-qPCR experiments, Student’s t-test. *p<0.05. (K) Cartoon of a mitotic NB with apical and basal crescents. Close-up of the basal crescent (right) shows that the Staufen-Miranda complex localizes cherub to the plasma membrane.

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Figure 6—figure supplement 1. The expression and localization of cherub in the Drosophila CNS. (A) Overview of a larval brain showing cherub expression in the central brain (CB) and ventral nerve cord (VNC), but not in the optic lobe (OL). Scale bar 50 μm. (B) RT-PCR analysis on larval brain
and adult head samples showing similar cherub expression patterns between D. melanogaster, D. simulans and D. willistoni. (C, D) cherub expression in larval neuroblast (arrow) and daughter cells in D. simulans (C) and (D) larval brain lobe (outlined). Scale bars 10 μm (C) and 50 μm (D). (E) Overview of regions targeted by isoform-specific FISH probes. (F–H) Representative images and percentages of mitotic NBII with cherub crescents in vitro. Scale bars 10 μm. (I) Angle between cherub isoforms and aPKC crescents. Data are mean ± SD. (F–I) RA/RC n = 9 NBII, RB/RC n = 13 NBII, RC n = 11 NBII. (J, K) Localization of cherub isoforms in NBII and daughter cells in vivo.

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Figure 6—figure supplement 2. Staufen, similar to cherub, is asymmetrically segregated from the NBII into INPs. (A) Close-up images of Staufen localization in a dividing NBII marked by membrane-bound GFP. (B) Images of NBII and daughter cells marked by membrane-bound GFP showing Staufen enrichment in the most recent born iINPs (arrows), which are identified by the closest NBII proximity and the lack of Dpn. (C) Upon staufen depletion, cherub is not enriched at the basal cell pole (arrow) of in mitotic NB (dashed outline). (A–C) Scale bars 10 μm. Lineages marked with UAS-dcr2; insc-GAL4, UAS-CD8::GFP.
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cherub transcripts harbor conserved secondary RNA structures. (A) Overview of the genomic region of cherub. Two measurements of evolutionary conservation are depicted, PhyloP (Basewise conservation) and phastCons (Element conservation). Below, pairwise
alignments of 27 D. species with D. melanogaster. Darker grey values indicate higher levels of conservation as scored by phastCons. Ns in the gap region are marked in yellow, one or more unalignable bases in the gap region are shown as double line and a discontinuity is highlighted with a blue bar. A scheme of the phylogenetic tree depicts the relationship between different Drosophila species (left). Sequence repeats and evolutionarily conserved RNA structures are depicted. (B) Pairwise sequence identity of the cherub locus. Complete linkage clustering of the Euclidean distance of percent sequence identity between the genomic loci of different D. species. (C) RNAz-predicted stem structures (corresponding to red secondary structures in (A)) resemble Staufen binding sites. Shades of color indicate absence (dark) or presence (bright) of mutations preventing base pairing in some sequences of the alignment. For stem 4, multiple sequence alignments are shown.

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Figure 7. The asymmetrically segregated IncRNA cherub accumulates in brat tumor neuroblasts. (A) Intensity measurements of nascent cherub transcript in NBIs (n = 43 NBs) and brat tNBs (n = 43 tNBs) from three independent FISH experiments. Student’s t-test. Not significant (n.s.) p > 0.05. (B) Figure 7 continued on next page
cherub localization in a brat mutant brain lobe (left, scale bar 100 μm) and close-up of tNBs (right, scale bar 20 μm). (C) Quantification of the angle between aPKC and cherub crescents of mitotic tNBs in vitro. An angle of 180° corresponds to a basal cherub crescent. n = 23 mitotic tNBs. Mean ±SD is shown. (D) Representative image of a mitotic tNB in vitro. Scale bar 10 μm. Percentage of tNBs showing a cherub crescent during cell division is indicated. n = 82 mitotic tNBs. (E, F) cherub expression in NBII clones (outlined) induced with the TARGET system for 24 hr (E) or 72 hr (F). Arrowheads exemplify Dpn⁺ tNBs and the open arrowhead marks the NBII. Scale bars 20 μm. UAS transgenes are expressed by UAS-dcr2; wor-GAL4, ase-GAL80; UAS-CD8::GFP. (G) Quantification of cortical cherub intensity after 72 hr clone induction. Control n = 7 NBII and bratRNAi n = 16 tNBs. Data are mean ±SD. Student’s t-test and ****p<0.0001.

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Figure 7—figure supplement 1. Cortical cherub levels are increased in brat tNBs. (A) Representative images show NB linages marked by insc-GAL4; UAS-CD8::GFP. FISH signals from the probe set against cherub, including nuclear foci in NBs (arrows), were abolished upon RNase treatment. RNase H treatment was performed post-hybridization to allow DNA oligonucleotide probes to bind to its target RNAs, as RNase H specifically cleaves RNA/DNA duplexes. Scale bars 10 μm. (B) cherub intensity measurements across cells show higher cherub at the periphery (cortex) of tNBs than of NBII. For each condition $n = 10$ NBs.

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Figure 8. Temporal neuroblast identity controls brat tumor growth. (A) The expression of genes characteristic for a young NB identity are upregulated in tNBs compared to control NBIIs. Depicted is the log2 foldchange in expression of NBIIs versus tNBs. (B) Cartoon depicting the control of larval...
temporal NB identity by opposing RNA-binding protein (RBP) gradients (C) Expression of the early NB identity gene Imp in bratRNAi and bratRNAi-cherubDEL/promDEL tNBs. (D) Syncrip localization in bratRNAi and bratRNAi-cherubDEL/promDEL tNBs. (C, D) Scale bars 10 μm. Driver line UAS-dcr2; wor-GAL4, ase-GAL80, UAS-CD8::GFP. Membrane-bound GFP outlines tumor tissue. (E, F) Survival rates of adult flies bearing primary control tumors or rejuvenated (orange) or aged (purple) brat tumors. Student’s t-test. ****p<0.0001, n ≥ 4 independent survival experiments. 50% survival rate shown as mean ±5D. (G, H) Overview of brat brain lobes (outlined) stained with the NB marker Dpn upon downregulation of Imp (G) or Syp (H). Scale bars 50 μm. (I, J) Quantification of tumor volume from three independent experiments. Data are mean ±5D. Student’s t-test. ****p<0.0001. (I) Knockdown of Imp (n = 15 brain lobes) results in smaller tumors compared to control tumors (n = 13 brains lobes). (J) Tumors with reduced Syp levels (n = 15 brain lobes) are larger than control tumors (n = 14 brain lobes). (E–J) Transgenes were expressed using the NBII-specific driver line UAS-dcr2; wor-GAL4, ase-GAL80, UAS-CD8::GFP.
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Figure 8—figure supplement 1. brat tumor growth requires a young neuroblast identity. (A) Survival rates of adult flies bearing primary control tumors or svpRNAi (rejuvenated, orange) or svp overexpression (aged, purple) brat tumors. 50% survival rates shown as mean ±SD. Student's t-test. ****p<0.0001, n = 6 independent survival experiments. (B) Overview of brat brain lobes (outlined) stained with the NB marker Deadpan (Dpn) upon overexpression of svp. Scale bars 50 μm. (C) Overexpression of svp (n = 26 brain lobes) reduces brat tumor size compared to control tumors (n = 26 brain lobes). Quantification of tumor volume from three independent experiments. (A–C) Transgenes were expressed using the NBII-specific driver line UAS-dcr2; wor-GAL4, ase-GAL80; UAS-CD8::GFP.

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Figure 9. cherub facilitates the binding between Staufen and the late temporal factor Syncrip. (A) Syncrip (Syp) localization in a mitotic NB (outlined) in vivo. Scale bar 10 μm. (B) Representative image and percentages of mitotic NBIIIs in vitro showing Syp crescents. n = 17 mitotic NBIIIs. Scale bar 10 μm. (C) Angle measurement between Syp and apical aPKC crescents. n = 17 mitotic NBIIIs. Mean ± SD is shown. (D, E) Syp is delocalized in NBIIIs (open arrowhead) and cytoplasmic enrichment in newly born INPs (arrowhead) is lost in cherub mutants (D) or upon Staufen reduction (E). Scale bars 20 μm. (E) UAS-dcr2; insc-GAL4, UAS-CD8::GFP was used. (F) Immunoprecipitation of Staufen in the presence and absence of cherub. (G) Immunoprecipitation of expressed HA-tagged Syp in the presence and absence of RNase treatment. (H) RIP-qPCR analysis of cherub and the negative control RpL32 upon Syp-HA pull down. Student’s t-test, **p<0.01, n = 3 independent RIP-qPCR experiments, depicted as mean ± SD. (F–H) Brain lysates from brat RNAi tumors and brat RNAi cherub DEL-/ or brat RNAi UAS-Syp-HA were used to enrich for NBs. Driver line used was UAS-dcr2; wor-GAL4, ase-GAL80, UAS-CD8::GFP. (I) Cartoon summarizing the role of cherub in Miranda-Staufen-Syncrip complex formation.

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Figure 9—figure supplement 1. cherub, Staufen and Syncrip localize to the basal pole of mitotic NBII s. (A–C) Pairwise comparison of the localization of cherub, Staufen and Syncrip in mitotic NBII s in vitro. Scale bars 5 μm. (D–F) Representative plot of Li’s intensity correlation analysis of one NBII for Figure 9—figure supplement 1 continued on next page.
Figure 9—figure supplement 1 continued

the respective channels. Covariance of intensities is calculated as \((A_i-a)(B_i-b)\). \(A/B_i\) is the pixel intensity and \(a/b\) is the mean intensity of channel A or B. Colocalizing pixels are positive and adopt a C-shape. (G) Pearson’s coefficient of pairwise comparison of Staufen-cherub (\(n = 11\) NBIs), Syncrip-cherub (\(n = 8\) NBIs) and Staufen-Syncrip (\(n = 13\) NBIs). Mean ±SD are shown.

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