An RNAi Screen for Genes Required for Growth of Drosophila Wing Tissue

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ABSTRACT Cell division and tissue growth must be coordinated with development. Defects in these processes are the basis for a number of diseases, including developmental malformations and cancer. We have conducted an unbiased RNAi screen for genes that are required for growth in the Drosophila wing, using GAL4-inducible short hairpin RNA (shRNA) fly strains made by the Drosophila RNAi Screening Center. shRNA expression down the center of the larval wing disc using dpp-GAL4, and the central region of the adult wing was then scored for tissue growth and wing hair morphology. Out of 4,753 shRNA crosses that survived to adulthood, 18 had impaired wing growth. FlyBase and the new Alliance of Genome Resources knowledgebases were used to determine the known or predicted functions of these genes and the association of their human orthologs with disease. The function of eight of the genes identified has not been previously defined in Drosophila. The genes identified included those with known or predicted functions in cell cycle, chromosome segregation, morphogenesis, metabolism, steroid processing, transcription, and translation. All but one of the genes are similar to those in humans, and many are associated with disease. Knockdown of lin-52, a subunit of the Myb-MuvB transcription factor, or βNACtes6, a gene involved in protein folding and trafficking, resulted in a switch from cell proliferation to an endoreplication growth program through which wing tissue grew by an increase in cell size (hypertrophy). It is anticipated that further analysis of the genes that we have identified will reveal new mechanisms that regulate tissue growth during development.

KEYWORDS Drosophila wing disc tissue growth polyploid endoreplication

Tissues must grow to a specific size and shape for proper development. This process is regulated by signals that coordinate cell division, cell growth, and cell death across tissues in both time and space (Vollmer et al. 2017). Perturbations in these tissue growth programs are known causes of developmental malformations and cancer (Hanahan and Weinberg 2011; Khetarpal et al. 2016; Parvy et al. 2018). While many tissues grow through an increase in cell number by mitotic cell proliferation, others grow by an increase in cell size through alternative polyploid endoreplication cycles (Øvrebø and Edgar 2018; Gjelsvik et al. 2019). Much remains unknown, however, about how tissue growth is regulated to achieve normal organ size and shape. To identify genes that participate in this process, we have conducted an RNAi screen in the Drosophila wing.

The Drosophila wing disc has been an important model for developmental regulation of tissue growth and patterning (Hariharan and Serras 2017; Vollmer et al. 2017). Wing discs originate as a group of ~30-50 cells during embryogenesis, and then grow by cell proliferation during larval stages, ultimately reaching a size of ~30,000-50,000 cells (Worley et al. 2013). During larval stages, the developmental axes of the wing disc and the fates of different cells are progressively patterned by developmental signaling pathways (Ruiz-Losada et al. 2018). During subsequent pupal stages, cell proliferation ceases and the wing disc tissue
differentiates and everts to form different parts of the wing, wing hinge, and notum of the fly thorax (Aldaz and Escudero 2010). Early experiments using genetic and surgical manipulation of wing discs revealed fundamental principles of growth, patterning, and regeneration (Garcia-Bellido et al. 1973; Bryant 1975; Kiehle and Schubiger 1985; Maves and Schubiger 2003; Neto-Silva et al. 2009). Wing discs have continued to be important models for the discovery of conserved pathways that control tissue patterning and growth, including those that regulate the compensatory proliferation of cells in response to tissue damage (Neufeld et al. 1998; De La Cova et al. 2004; Hariharan and Serras 2017).

To identify genes that are important for tissue growth, we have screened a collection of GAL4-inducible short hairpin RNA (shRNA) strains for their effect on the Drosophila wing (Ni et al. 2011; Heigwer et al. 2018). We recently conducted a candidate shRNA screen of 240 genes, which RNA-Seq had shown are expressed at lower levels in endoreplicating cells in culture. This candidate screen showed that knockdown of genes in a CycA – Myb – Aurora B pathway induces cells in the wing and other tissues to switch to an alternative endoreplication growth program (Rotelli et al. 2019). Here, we report the results of a random screen of 5,260 additional shRNA strains, which has identified 18 genes whose knockdown impairs wing growth. The function of eight of the genes recovered in this screen has not been previously defined in Drosophila. The human orthologs of some of these genes are associated with disease, including those that manifest as tissue undergrowth or cancer. Immunofluorescent analysis of wing discs showed that knockdown of two genes induced a switch from mitotic cell divisions to polyploid endoreplication cycles, providing an inroad to understanding the regulation of these alternative growth programs.

**MATERIALS AND METHODS**

**Drosophila genetics**

*Drosophila* were raised on BDSC standard cornmeal medium at 25°. The TRiP UAS-shRNA Drosophila strains were made by the Drosophila RNAi Screening Center (DRSC) (Ni et al. 2011), and were obtained from the Bloomington Stock Center (Bloomington, IN) (Cook et al. 2010). The P GAL4-dpp.bkl1[40C.6 / TM3 Sb Ser and P GAL4-dpp.bkl1[40C.6 UAS-mRP / TM6 Tb strains were constructed from the Bloomington stock P GAL4-dpp.bkl1[40C.6 / TM6 Tb (#1551). See Table S1 for a complete list of strains and stock numbers.

**Adult wing screen**

The UAS-shRNA strains were crossed to P(GAL4-dpp.bkl1)[40C.6 / TM3 Sb Ser, and the wings of adult UAS-shRNA / + ; dpp-GAL4 / + progeny were scored for reduced growth of the region between longitudinal wing veins 3 (L3) and 4 (L4), a region that is also known as the first posterior compartment (FPC) (Ferris 1950), although it emanates from the anterior lineage compartment of the wing disc (Figure 1). The shRNA / + ; TM3 Sb Ser siblings from this cross served as internal negative controls. The shRNA strains found to affect wing growth / hair morphology in the primary screen were retested and scored for expressivity and penetrance. Adult wings were dry mounted with coverslips and imaged under bright field on a Leica DMRA2 microscope (Figure 2).

This L3-L4 intervein region was also scored for aberrant wing hair (trichome) morphology, spacing, and planar patterning relative to the other areas of the same wing. Relative number of wing hairs per area (hair density) was measured using ImageJ (v1.5e) (https://imagej.nih.gov/ij/) with the FijiWings plugin (v2.2) (Dobens and Dobens 2013). For three separate wings, the trichome density from four selected areas of the L3-L4 intervein region was...
Figure 2. Adult wing phenotypes of shRNA strains that impaired growth. (A–S) Bright field images of adult wings from a wild type dpp-GAL4/+ control (A) or after expression of a UAS-shRNA targeting the indicated gene (B–S). Insets are higher magnifications to show wing hair phenotypes. Shown are the dorsal sides of the wings with anterior up. Scale bars are 150 μm for main panels and 75 μm for insets. (T, U) The length and number of wing hairs per unit area (hair density) were measured using the ImageJ plug in Fiji-wing. (T) Number of wing hairs per area from the L3-L4 intervein region divided by that in the L2-L3 + L4-L5 intervein regions of the same wings (N = three wings, with four L3-L4 areas and two L2-L3 + two L4-L5 areas per wing, "*" P ≤ 0.01, "**" P ≤ 0.05). (U) Length of wing hairs in the L3-L4 intervein region divided by that in the L2-L3 + L4-L5 intervein regions of the same wings (N = three wings with n = 20 hairs for L3-4 and 10 hairs for L2-L3 + 10 hairs for L4-L5 per wing. "*" = P ≤ 0.01, "**" = P ≤ 0.05, n.s. = not significant, by Student’s t-test).
compared to the average of two selections of the same size from the L2-L3 intervein region and two selections from the L4-L5 intervein region. The average length of twenty wing hairs from the L3-L4 intervein region were compared to that of ten wing hairs from the L2-L3 intervein region and ten wing hairs from the L4-L5 intervein region, for three separate wings. Relative wing hair density and relative length values were plotted using GraphPad Prism (version 7.04).

Antibody labeling and immunofluorescent microscopy
For quantification of ploidy in Figure 3, wing imaginal discs from Tb+ 3rd instar larvae were dissected and fixed as described (Schwed et al. 2002). Discs were labeled with rabbit anti-dsRed (Clontech, 632496) (1:400) and secondary anti-rabbit Alexa Fluor 568 (1:500) (Invitrogen), and stained with DAPI (0.5μg/ml). Discs were imaged on a Leica SP5 confocal and Leica DMR2 widefield epifluorescence microscope. ImageJ was used to quantify nuclear area and total DAPI fluorescence. The nuclear area and DAPI intensity of cells within the RFP+ dpp expressing stripe were normalized to cells outside of the stripe in the same wing discs (RFP+ cells / RFP- cells in Figure 3). The cells in the wing pouch area of each wing disc were scored, excluding the zone of non-proliferating cells, which are arrested in G1 and G2 phases of the cell cycle (Johnston and Edgar 1998).

Statistical Analysis
For Figures 2T and 2U, statistical significance was determined by two-tailed Student’s t-tests using Microsoft Excel (version 15.0.4753.1000). For Figure 3E, the mean and standard deviation for nuclear size and DNA ploidy were measured for wild type and each shRNA knockdown. The significance of the difference between each shRNA knockdown and the wild type control was assessed by a two-tailed Student’s t-test with cut off of P # 0.01.

Data Availability
A list of fly strains screened can be found in Table S1. All fly strains are publicly available from the BDSC. All fly strains and reagents generated in this study will be made freely available upon request.
Table 1 Summary of screen

| Total crosses¹ | Lethal² | Semi-Lethal³ | Viable⁴ | Mutant wing phenotype |
|----------------|---------|--------------|---------|----------------------|
| 5,260          | 507     | 113          | 4,640   | 18                   |

1: See Table S1 for a complete list of strains.  
2: The number of crosses in which Sb+, dpp-GAL4 offspring died before adulthood whereas Sb- siblings without dpp-GAL4 survived.  
3: The number of crosses in which only 25% of expected Sb+, dpp-GAL4 offspring survived to adulthood.  
4: The number of crosses in which the Sb+, dpp-GAL4 offspring survived to adulthood.

Supplemental material available at FigShare: https://doi.org/10.25387/g3.8309594.

RESULTS AND DISCUSSION

Overview of screen strategy and results

Our goal was to identify genes that are required for cell proliferation and growth. To do this, we expressed a collection of GAL4-inducible short hairpin RNA (shRNA) strains to knockdown genes and score the effect on wing growth. This collection of shRNA fly strains was made by the Drosophila RNAi Screening Center (DRSC) and obtained from the Bloomington Drosophila Stock Center (BDSC) (Cook et al. 2010; Ni et al. 2011). We screened a subset of the latest generation of inducible shRNA strains (TRIP VALIUM 20 vector) because they are more efficient and have fewer off targets than the previous generation of strains that expressed longer dsRNAs (Table S1) (Ni et al. 2011; Heigwer et al. 2018). We used a dpp-GAL4 driver to express these UAS-shRNAs in a stripe of cells along the anterior-posterior compartment boundary of the larval wing disc beginning during 2nd instar (Figure 1) (Posakony et al. 1990; Staehling-Hampton et al. 1999; Matsuda and Affolter 2017). These dpp-expressing cells are fated to form most of a central region of the adult wing between longitudinal veins 3 (L3) and 4 (L4), which we scored for reduced area relative to their previous generation of strains that expressed longer dsRNAs (Table S1) (Ni et al. 2011; Heigwer et al. 2018). We crossed 5,260 UAS-shRNA strains to dpp-GAL4 / TM3 Sb Ser, and scored adult wing of the shRNA / + ; dpp-GAL4 / + progeny for L3-L4 intervein region size and wing hair morphology, with the Sb- siblings lacking dpp-GAL4 serving as internal controls (Figure 1, Table S1). Among these 5,260 crosses, 507 resulted in lethality before adulthood specifically for the Sb+ progeny, indicating that knockdown of those genes in the dpp-GAL4 pattern was not compatible with life (Table 1, Table S1). In 113 crosses, less than 25% of the expected dpp-GAL4 (Sb+) class survived to adulthood, which we termed semi-lethal (Table 1, Table S1). Given that dpp-GAL4 expression is not restricted to the wing disc, it is unclear in which tissues knockdown caused lethality. Among the 4,753 shRNA crosses with adult Sb+ progeny, 18 had reproducible effects on the central part of the wing (Figure 2B-S, Table1, Table 2, Table S1). All these shRNA strains reduced the area of the L3-L4 intervein region to varying extents relative to sibling controls, which we categorized as mild (class I), moderate (class II), or severe (class III) effects on tissue growth (Figure 2, Table 2). Although each had reproducible and clear effects on tissue mass in the adult wing, the relative severity of these different shRNA phenotypes should be interpreted with caution given that the strength of RNAi knockdown could differ among them. Nonetheless, it is clear that expression of these 18 shRNA strains compromised the growth of wing tissue in the central dpp-GAL4 expression domain.

In addition to effects on tissue growth, knockdown of most of these genes affected the organization of the hairs on the surface on the wing (Figure 2, Table 2). This phenotype included disruption of the planar polarity of the hairs, but the orientation of the hairs appeared random, and it remains unclear whether this phenotype is a direct result of a disruption of planar cell polarity, or the indirect result of aberrant tissue growth and morphogenesis (Figure 2, Table 2).

Table 2 Genes required for growth¹

| Symbol | Name | Growth defect² | Wing hairs³ |
|--------|------|----------------|-------------|
| stg    | string | III | widely spaced, disordered |
| Cdc6   | Cdc6 | I - III | disordered |
| eco    | Establishment of cohesion | I - III | mild disorder, a few enlarged |
| fil     | fala fel | II | mild disorder |
| lin-52 | lin-52 | II | widely spaced, longer, disordered |
| pita   | pita | III | enlarged girth of base |
| smog   | smog | II | mild disorder |
| Rap1   | Rap1 GTPase | I | altered planar polarity |
| Cisd2  | CDGSH iron sulfur domain 2 | I - II | slightly longer, hair tufts |
| βNACtes6 | Nascent-associated complex β-subunit-like, testis 6 | II - III | widely spaced, longer, hair tufts |
| CG3568 | — | I | mild altered planar polarity |
| CG4459 | — | I - III | enlarged girth of base, disordered |
| CG8132 | — | I - II | mild disorder, hair tufts |
| CG9547 | — | II | mild disorder, denser anterior |
| CG12171 | — | II - III | mild disorder |
| CG34174 | — | II - III | disorder |
| CG34177 | — | I - III | Mild disorder |
| CG42516 | — | I - II | Mild disorder, hair tufts |

1: See Table S1 for FlyBase gene numbers and dsRNA stock numbers.  
2: Size of first posterior wing cell between veins L3 and L4. Class I = mild growth defect, Class II = intermediate growth defect; Class III = severe growth defect.  
3: Size, shape, and planar cell polarity of wing hairs.
Knockdown of two genes, \textit{lin-52} and \textit{\textbeta}Nact6, compromised growth of the L3-L4 intervein region and resulted in longer wing hairs, suggesting that their knockdown may induce a switch to an endoreplication growth program (Figure 2F, K, T, U Table 2).

Below, we discuss the genes recovered in this screen, their known or predicted function, their orthologs, and their disease associations. We took advantage of the newest online tools that are available through FlyBase, including the Gene to Function (G2F) application that is based on the DRSC Integrative Ortholog Prediction Tool (DIOPT) (Hu et al. 2011; Thurmond et al. 2019). We also mined information for orthologs and links to human disease using the Alliance of Genome Resources (Alliance) site, which is a new collaborative effort to harmonize data from six model organism knowledgebases and the Gene Ontology (GO) consortium (Howe et al. 2018).

\textbf{Cell Cycle and Chromosome Segregation Genes}  
\textit{string (stg)}: Knockdown of \textit{stg} resulted in severe undergrowth of wing tissue and more widely spaced wing hairs, with a disordered wing hair polarity in the L3-L4 intervein region of the adult wing (Figure 2B, T Table 2). While some wing hairs were longer, the average length was not significantly different from controls (Figure 2B, U). The \textit{stg} gene encodes one of two \textit{Drosophila} orthologs of the Cdc25 phosphatase, which dephosphorylates and activates Cdk1 kinase to promote mitotic entry (Edgar and O’farrell 1990). There are three orthologs of Cdc25 in humans whose increased function and expression have been associated with oncogenesis (Table 3) (Sur and Agrawal 2016). Given these known functions, the undergrowth after \textit{stg} knockdown is likely a manifestation of impaired mitotic entry and cell proliferation (Figure 2B, Table 2).

\textit{Cdc6}: Knockdown of \textit{Cdc6} resulted in a variably expressive phenotype of mild to severe undergrowth of wing tissue with disordered polarity of wing hairs (Figure 2C, Table 2). \textit{Cdc6} is a subunit of the pre-replicative complex, which binds origin DNA and is required for the initiation of DNA replication from yeast to human (Parker et al. 2017). Although Cdc6 protein is essential for DNA replication, its knockdown resulted in viable adults with reduced wing tissue, likely because of partial knockdown (Crevel et al. 2005) (Figure 2C, Table 2). This result is analogous to the phenotype of mild, hypomorphic alleles of human CDC6, which cause a heritable microcephalic primordial dwarfism known as Meier-Gorlin syndrome (Bicknell et al. 2011) (Table 3). Thus, similar to humans, partial impairment of \textit{Cdc6} function in \textit{Drosophila} results in undergrowth of tissues during development.

\textit{establishment of cohesion (eco)}: Knockdown of \textit{eco} had an intermediate to severe effect on tissue growth in the wing L3-L4 intervein region (Figure 2D, Table 2). The \textit{eco} gene encodes an acetyltransferase that has a conserved function in eukaryotes to establish sister chromatid cohesion during S phase (Wang and Christman 2001; Rudra and Skibbens 2013). It associates with the replication fork and acetylates the SMC3 subunit of the Cohesin Complex to promote sister chromatid cohesion of newly replicated DNA behind the fork (Ivanov et al. 2002; Zhang et al. 2008). Its putative human orthologs are the \textit{ESCO1,2} genes (Table 3). Mutations in \textit{ESCO2} cause Robert’s Syndrome, a heritable undergrowth syndrome characterized by reductions in limb size and craniofacial abnormalities, among other pleiotropic phenotypes (Gordillo et al. 1993; Vega et al. 2005). The undergrowth of the \textit{Drosophila} wing after \textit{eco} knockdown may be the result of increased chromosome instability and reduced cell proliferation (Figure 2D, Table 2).

\textbf{Chromatin regulation}  
\textit{pita (pita)}: Knockdown of \textit{pita} severely impaired wing tissue growth (Figure 2G, Table 2). The \textit{pita} gene encodes a zinc finger protein that is a subunit of a chromatin insulator complex (Maksimenko et al. 2015). The two most similar human proteins are the Zn-finger transcription factors ZNF121 (Amino Acid (AA) Identity (I) = 33%, Similarity (S) = 53%) and FEZF1 (AA I = 28%, S = 40%), although the DIOPT score for orthology is low (1/15) (Table 3). Loss of function alleles of \textit{FEZF1} cause Kallmann Syndrome, which is characterized by defects in development of the hypothalamic-pituitary-gonadal (HPG) axis, resulting in the impairment of gonadal development and the sense of smell (hypogonadotropic hypogonadism-22 with anosmia) (Kotan et al. 2014; Topaloglu and Kotan 2016) (Table 3). In \textit{Drosophila}, \textit{pita} regulates gene transcription in part through mediating higher-order chromosome structure (Maksimenko et al. 2015; Kyrchanova et al. 2017). \textit{pita} mutants also have defects in S phase and reduced expression of the replication protein Orc4 (Page et al. 2005). The tissue undergrowth after \textit{pita} knockdown may be a result of these cell cycle defects, but,
Table 3 Known or proposed functions, orthologs, and disease associations

| Symbol | Structure – Function | Human ortholog (DIOPT score) | Disease Associations |
|--------|----------------------|-------------------------------|---------------------|
| stg    | Phosphatase, activate Cdk1, Mitotic entry | Cdc25 (12) | Cancer |
| Cdc6   | Member pre-RC complex; Initiation of DNA replication | Cdc6 (12) | Meier-Gorlin Syndrome |
| eco    | Acetyltransferase; establishment of sister chromatic cohesion in S phase | ESCO1,2 (11) | Robert’s Syndrome |
| fli    | Regulatory subunit protein phosphatase 4; kinetochore integrity; chromosome segregation; morphogenesis | PPP4R3A (14) | Cisplatin sensitivity? |
| lin-52 | Subunit of Myb-MuvB / DREAM transcription factor complexes; cell cycle, development, et al. | LIN52 (13) | Tumor suppression |
| pita   | Chromatin insulator protein | | |
| smog   | G protein-coupled receptor; cell migration; cell shape; morphogenesis | GPR158 (5) | Prostate Cancer |
| Rap1   | Ras family GTPase; cell polarity, migration shape; developmental signaling; morphogenesis | RAP1A (14) | Ovarian cancer |
| Cisd2  | Iron-sulfur & zinc finger domains; Ca+ homeostasis, autophagy | CISD2 (13) | Wolfram Syndrome 2; Hepatocellular carcinoma |
| βNACtes6 | Transcription factor; co-translational chaperone; subcellular protein targeting | BTF3, BTF3L4 (1) | |
| CG3568 | | | |
| CG4459 | Organic ion transporter; drug / toxin metabolism; hormonal signaling; neurotransmission. | SLC22A15? (2) | |
| CG8132 | Omega Amidase; converts toxic oxoglutaramate to alpha-ketoglutarate | | |
| CG9547 | Glutaryl-CoA dehydrogenase; mitochondrial matrix; lysine and tryptophan metabolism | NIT2 (14) | Tumor Suppressor? |
| CG12171 | Steroid dehydrogenase | GCDH (15) | Glutaric Acidemia |
| CG34174 | Cdc7 and Cdk2 associated protein; DNA replication; ATR checkpoint signaling; transcription factor | | |
| CG34177 | Von Willebrand factor type C domain: predicted secreted protein; | MSMB (1) | Prostate cancer |
| CG42516 | TFIIIC complex; pol III transcription | GTF3C1 (3) | |

1: Protein domains and known or predicted function in Drosophila and/or other organisms.
2: Human ortholog predictions from DIOPT and Gene to Function (G2F), with match score in parentheses from 1 (weakest) to 15 (strongest).
3: Disease Associations curated by FlyBase, Alliance of Genome Resources, and OMIM.

given its role in global chromatin architecture, could also be the result of other pleiotropic effects on gene expression.

Development and Morphogenesis

**smog (smog):** Knockdown of smog had an intermediate effect on wing growth (Figure 2H, Table 2). smog encodes a G protein-coupled receptor that is required for a number of developmental processes (Kerridge et al. 2016). During embryogenesis, smog is required for embryonic cell migration and shape changes through its regulation of myosin II activity (Kerridge et al. 2016; Simões et al. 2017). Given these known functions, the reduced tissue mass in the adult wing after smog knockdown could be the result of defective cell shape changes and reorganization during wing growth and/or disc eversion and morphogenesis into the adult wing (Figure 2H, Table 2). The most similar human protein is the G protein-coupled receptor 158 (GPR158) (DIOPT 5/15), a broadly-expressed orphan receptor that participates in neurogenesis and has been associated with prostate development and cancer (Orlandi et al. 2015; Patel et al. 2015; Condomitti et al. 2018) (Table 3).

**Rap1 GTPase (Rap1):** Knockdown of Rap1 had a mild effect on wing growth (Figure 2I, Table 2). Rap1 is a member of the RAS superfamily of small GTPases, and regulates the actomyosin cytoskeleton for morphogenetic cell migration, apical-basal polarity, cell adhesion, and cell shape changes in a number of tissues (Knio and Brown 2002; Huelsmann et al. 2006; Boettner and Van Aelst 2007; Siekhaus et al. 2010; Wang et al. 2013). Thus, similar to smog, Rap1 knockdown may impair wing growth through altering actomyosin-mediated cell shape changes during growth and/or morphogenesis of the wing disc into the adult wing (Figure 2I, Table 2). However, Rap1 also regulates the hippo pathway (Chang et al. 2018), and is required for receptor tyrosine kinase signaling in the embryo, eye, and wing (Mishra et al. 2005; O’keefe et al. 2009; Mavromatakis and Tomlinson 2012), suggesting that disruption of these functions may also contribute to the observed wing phenotype after Rap1 knockdown. The planar cell polarity of the wing hairs was altered, consistent with previous reports that Rap1 has a function in this process (O’keefe et al. 2009) (Figure 2I, Table 2). The closest human protein is RAP1A, which evidence suggests also mediates cell shape, polarity, and migration in a variety of tissues, and is involved in ovarian cancer tumorigenesis and metastasis through stimulating cell proliferation, migration, and invasion (Pizón et al. 1988; Lu et al. 2016) (Table 3).

Metabolism and Physiology

**CDGSH iron sulfur domain 2 (Cisd2):** Knockdown of Cisd2 had a mild to intermediate effect on wing growth (Figure 2I, Table 2). Wing hairs were slightly longer and often grew in tufts of multiple hairs (Figure 2I, Table 2). As its name implies, the protein encoded by the Cisd2 gene has an iron-sulfur domain, and is 45% identical and 66% similar to human CISD2 protein, which localizes to the endoplasmic reticulum in human cells. Mutations in human CISD2 cause Wolfram Syndrome 2, a neurological disorder that presents with progressive blindness and deafness, and is associated with gastrointestinal ulcers and diabetes (Table 3) (Amr et al. 2007; Mozzillo et al. 2014). CISD2 is frequently deleted in hepatocellular carcinoma.
(HCC), and haploinsufficiency for Cisd2 in mice disrupts calcium homeostasis, causes fatty liver disease, and promotes HCC (Shen et al. 2017; Shen et al. 2018). A previous study of fly Cisd2 uncovered a genetic interaction with overexpressed Pimtail Protein Thioesterase (PPT1), a protein involved in protein degradation within the lysosome, and ceroid-lipofuscinosis, neuronal 3 (CLN3), whose ortholog is associated with lysosomal storage disease in humans (Jones et al. 2014). Jones and colleagues did not, however, find a mutant phenotype associated with Cisd2 on its own, using either a Cisd2 dsRNA transgene or animals homozygous for a transposon insertion allele of Cisd2 (Jones et al. 2014). Whether the wing undergrowth phenotype we observed is indeed caused by Cisd2 depletion will require further experimentation. If so, the wing phenotype is an entry point to further define the molecular mechanisms of Wolfram Syndrome 2 and hepatocellular carcinoma.

Translation and protein targeting

*Nascent-associated complex θ-subunit-Like, testis 6 (θNAC6s):* Knockdown of θNAC6s had an intermediate to severe effect on wing growth (Figure 2K, Table 2). It also reproducibly resulted in enlarged polyploid cells (Figure 2K, Table 2) (Katzenwing growth (Figure 2K, Table 2). It also reproducibly resulted in

paralogs in wandering larval 3rd instar imaginal discs, explaining how

However, examination of RNA-Seq data from the modENCODE

β

mutant phenotype associated with

paralogs in humans, Basic Transcription Factor 3 (BTF3) (DIOPT 1/15, AA I = 32% S = 47%) and Basic Transcription Factor 3Like 4 (BTF3L4) (DIOPT 1/15, AA I = 30%, S = 45%) (Table 3). As their name implies, these human proteins were initially defined as general transcription factors that bind the core promoter (Zheng et al. 1990). Subsequent studies showed that this eukaryotic family of proteins also regulates translation and are known as θNACs (Wiedmann et al. 1994). θNAC proteins bind θNAC proteins to form the hetero-
dimeric Nascent-Associated Complex (NAC), which associates with the ribosome where it acts as an ATP-dependent chaperone on actively translating proteins (Lauring et al. 1995; Deuerling et al. 2019). The NAC also regulates the cellular location of the ribosome, inhibiting the targeting of proteins to the ER and promoting targeting to mitochondria (Lauring et al. 1995; George et al. 1998). An absence of NAC function causes protein mislocalization and can result in cell death (Deuerling et al. 2019).

θNAC6s is one of six θNACs paralogs in Drosophila that are located in the middle of the arm of the X chromosome (five in cytogenetic region 12E and one in 13D). The names of these θNAC paralogs include the suffix testis (tes) because they were previously shown to be highly expressed in the D. melanogaster male germine during spermatogenesis where they associate with ribosomes (Kogan et al. 2017). However, examination of RNA-Seq data from the modENCODE project indicated that there is a pulse of expression of all six of these paralogs in wandering larval 3rd instar imaginal discs, explaining how knockdown of a gene named for its testis expression could impair growth of wing tissue (Graveley et al. 2011). All these paralogs are similar to human BTF3 and BTF3L4, but the protein encoded by the Drosophila bicoidal (bic) gene is much more similar to these human proteins (BTF3, DIOPT 12/15; AA I = 63%, S = 71%; BTF3L DIOPT 12/ 15 AA I = 67% S = 75%) (Markesich et al. 2000). Maternal Bic protein is localized to the anterior of the embryo where it establishes anterior identity by repressing the translation of the posterior determinant protein Nanos (Markesich et al. 2000). bic is widely expressed throughout development, suggesting that in most cells bic may be the principal B subunit of the Drosophila NAC, with the θNACs paralogs likely performing more specialized roles in the testis and imaginal discs (Graveley et al. 2011). Given the dual function of other θNAC proteins in transcription, however, it may be that the θNAC6s wing phenotype is the result of altered transcription. The reduced wing tissue and enlarged bristles after θNAC6s knockdown suggests that it is required for normal growth and may influence the choice between cell proliferation and endoreplication growth programs, a possibility that we explore further below.

Uncharacterized Drosophila genes

A number of the genes that were required for growth have not been extensively characterized in Drosophila, and, therefore, are known only by a Computed Gene (CG) number.

CG3568: Knockdown of CG3568 had a mild effect on wing growth and hair polarity (Figure 2L, Table 2). CG3568 is predicted to encode a 508 amino acid protein with no identifiable protein domains nor orthologs outside of other Dipteran species. In D. melanogaster, modENCODE RNA-Seq indicated that CG3568 is expressed in multiple tissues at multiple stages of Drosophila development (Graveley et al. 2011). It is perhaps interesting to note that the CG3568 protein, which has identifiable orthologs only in Diptera, begins with the amino acid sequence “MRSFLY.”

CG4459: Knockdown of CG4459 resulted in a variably expressible mild to severe undergrowth and wing hair polarity phenotypes (Figure 2M, Table 2). CG4459 encodes a widely expressed protein with a Major Facilitator Superfamily (MFS) domain characteristic of small solute transmembrane transporters in a variety of organisms (Table 3). The CG4459 protein is weakly similar to a large family of human Solute Carrier 22 (SLC22) paralogs in the human genome, the closest being SLC22A1, (AA I = 20%, S = 39%) (Gründemann et al. 1994). This family of human transmembrane proteins are organic cation transporters (OCTs) that mediate transport of various pharmaceuticals, toxins, hormones, neurotransmitters, metabolites, and other small molecules, and, therefore, play important roles in human physiology and pharmacology (Table 3) (Lozano et al. 2018; Nigam 2018). Further analysis of CG4459 may reveal new functions for this family of proteins in developing tissues.

CG8132: Knockdown of CG8132 resulted in severe defects in wing tissue growth, with some hairs growing in tufts (Figure 2N, Table 2). CG8132 is predicted to encode an omega-amidase that is highly similar to the human protein Nitralase Family Member 2 (Nit2) (DIOPT 14/15), which belongs to a family of enzymes that cleave carbon-nitrogen bonds (Lin et al. 2007) (Table 3). Evidence suggests that this omega-amidase removes potentially toxic intermediates by converting alpha-ketoglutarate and alpha-ketosuccinamate to biologically useful alpha-ketoglutarate and oxaloacetate, respectively, but the in vivo functions of this enzyme are controversial (Jaisson et al. 2009; Krasnikov et al. 2009). Other reports have shown that Nit2 has an effect on cell proliferation and may be a tumor suppressor (Lin et al. 2007; Zheng et al. 2015). A recent report showed that knockdown of CG8132 also strongly impaired growth and development of the Drosophila eye (Pletcher et al. 2019). Further characterization of the eye and wing phenotypes in flies will further define CG8132 / Nit2 cellular functions.

CG9547: Knockdown of CG9547 had an intermediate effect on wing growth (Figure 2O, Table 2). There was also a reproducible higher density of darkly pigmented wing hairs in the anterior part of the L3-L4 intervein region (Figure 2O, Table 2). The CG9547 protein is...
highly similar to human Glutaryl-CoA Dehydrogenase (GCDH) (DIOPT 15/15) (Table 3) (Goodman et al. 1995). This enzyme is a homotetramer that localizes to the mitochondrial matrix and is involved in lysine and tryptophan metabolic processes (Lenich and Goodman 1986; Goodman et al. 1995; Schmiesing et al. 2014). In a number of different human populations, alleles of GCDH cause the metabolic disorder glutaric acidemia type I, an early-onset neurodegenerative disorder (Table 3) (Goodman et al. 1995; Hedlund et al. 2006; Schmiesing et al. 2017; Schmiesing et al. 2018). In Drosophila, expression of CG9547 is upregulated in response to starvation and oxidative stress, and its knockdown altered eye growth (Fujikawa et al. 2009; Gruenewald et al. 2009; Pletcher et al. 2019).

**CG12171**: Knockdown of CG12171 had intermediate to severe effects on wing growth (Figure 2P, Table 2). The CG12171 protein is predicted to be a steroid dehydrogenase with similarity to the human steroid dehydrogenase called Hydroxysteroid 17-beta dehydrogenase 14 (HSD17B14) (DIOPT 4/15) (Table 3) (Lukacik et al. 2007; Letunic and Bork 2018; El-Gebali et al. 2019). Evidence suggests that HSD17B14 is involved in steroid catabolic processes and acts on a number of steroids including estradiol, testosterone, fatty acids and prostaglandins (Lukacik et al. 2007). High throughput protein interaction screens in flies showed that CG12171 protein physically interacts with proteins encoded by CG31549 and CG31548 genes, both of which are also predicted to have steroid dehydrogenase activity (Guruharsha et al. 2011). The protein products of these three genes are highly similar (69–81% pairwise amino acid identity), and the genes are clustered together at one locus on chromosome 3R, suggesting that they are paralogs that arose through gene duplication and have related functions in steroid biochemistry. Investigating the function of these three genes may reveal novel insights into how cell autonomous regulation of steroid biochemistry mediates tissue growth and differentiation (Figure 2P, Table 2).

**CG34174**: Knockdown of CG34174 had intermediate to severe effects on wing growth (Figure 2Q, Table 2). CG34174 encodes a small protein of 217 AA that is weakly similar to human Cdk2 Interacting Protein (CINP) (AA I = 23%, S = 40%) (Table 3). The human CINP protein was initially identified by virtue of binding to the essential S phase kinases Cdc7 and Cdk2 (Grishina and Lattes 2005). That study also provided evidence that CINP is phosphorylated by Cdc7 and physically associates with subunits of the origin recognition complex (ORC) and mini chromosome maintenance (MCM) complex, leading to the hypothesis that CINP has a direct role in DNA replication (Grishina and Lattes 2005). A subsequent study showed that CINP is required for the DNA damage response and G2 cell cycle arrest that is mediated by the ATR-ATRIP checkpoint kinase (Lovejoy et al. 2009). That study showed that CINP physically interacts with ATR-ATRIP but did not find evidence for a physical interaction between CINP and CDK2 or Cdc7 (Lovejoy et al. 2009). A recent study reported a physical interaction between CINP and the oncogene transcription factor Kruppel-like factor 5 (KLF5), and showed that CINP knockdown suppressed the transcriptional, cellular cycle, and tumor promoting effects of KLF5 overexpression, leading the authors to conclude that CINP is a KLF5 transcriptional coactivator (Wu et al. 2019). Thus, it is possible that the CINP protein moonlights in multiple cellular processes. Further analysis of CG34174 will inform which of these function(s) are important for cell proliferation and tissue growth in vivo.

**CG34177**: Knockdown of CG34177 had severe effects on tissue growth (Figure 2R, Table 2). It is predicted to encode a small protein of 107 AA with a von Willebrand factor C-domain that is often found in secreted proteins (Sheldon et al. 2007). The most similar protein in humans is the secreted protein Microsemioprotein beta (MSMB) (Mbikay et al. 1987), but the DIOPT score is low (1/15), with the fly and human proteins being 23% identical and 32% similar (Table 3). However, MSMB protein sequence is known to be rapidly evolving in primates, suggesting that CG34177 may indeed be an ortholog of it. Further, human MSMB protein is expressed in the prostate, while fly CG34177 protein is expressed in the accessory gland, the fly analog of the mammalian prostate, with both proteins being secreted into seminal fluid in flies and mammals (Mbikay et al. 1987; Sitnik et al. 2016). Lower levels of expression and allelic variants of the MSMB gene have been associated with prostate cancer (Harries et al. 2010; Waters et al. 2010; Lou et al. 2012; Peng et al. 2017; Bergström et al. 2018). MSMB and CG34177 are expressed in tissues other than the prostate and accessory gland, including larval imaginal discs, consistent with its knockdown reducing growth of the wing, but the functions of the human and fly proteins have not been defined (Ulvsbäck et al. 1989; Graveley et al. 2011).

**CG42516**: Knockdown of CG42516 resulted in a mild to intermediate wing undergrowth phenotype, with some hairs growing in tufts (Figure 2S, Table 2). CG42516 protein is weakly similar to human general transcription factor IIIC subunit 6 (GTF3C6) (DIOPT 3/15, AA I = 22%, S = 41%) (Table 3). GTF3C6 is a subunit of the small nuclear RNA (snRNA) activating protein complex, which is required to recruit RNA pol III to promoters of small nuclear RNA genes, including 5S RNAs and tRNAs (Dumay-Odelot et al. 2007). A cogent hypothesis, therefore, is that knockdown of CG42516 impairs growth because of reduced expression of small RNAs that participate in protein translation and other essential cellular processes.

**Knockdown of βNACtes6 and lin-52 induces a switch to the endoreplication growth program**

One motivation for the screen was to identify genes that influence the decision between mitotic cell proliferation and the polyploid endoreplication growth program. We therefore screened for more widely spaced and longer wing hairs, a phenotype associated with larger polyploid cells (Adler et al. 2000; Hanson et al. 2005; Olofsson and Axelrod 2014). Knockdown of *stg* resulted in more widely spaced wing hairs, while *lin-52, and βNACtes6*, resulted in both more widely spaced and longer wing hairs, suggesting that cells in these wings may have switched to an endoreplication growth program (Figure 2 B, F, K, Table 2). To address whether cells in these and the other 15 gene knockdowns switched to endoreplication, we measured the nuclear size and DNA content of cells in the late third instar larval wing discs. Specifically, we measured the nuclear area and total DAPI fluorescence intensity of wing disc cells in the central *dpp-GAL4; UAS-shRNA expression domain*, identified by co-expression of *UAS-mRFP* (RFP+), and normalized it to the average nuclear area and fluorescent intensity of control, mRFP-negative cells (RFP-) in the wing pouch region of the same wing disc. *UAS-RFP / +; dpp-GALA4 / +* control animals had nuclei that were of similar size and DNA content in the RFP+ and RFP- cells, and whose average we normalized to 1 (Figure 3A, A’, E). The range of DAPI intensity of wing disc cells in the central *dpp-GAL4; UAS-shRNA expression domain* (*P > 0.01 by t-test*) (Figure 3E).
Knockdown of \( \text{stg} \) resulted in a central stripe of wing cells with more widely spaced nuclei that appeared less brightly stained with DAPI (Figure 3B, B’). Quantification showed that \( \text{stg} \) knockdown did indeed increase nuclear size \((P < 0.01)\), but not total DNA content (measured on both widefield and confocal microscope platforms) (Figure 3E). This result is consistent with previous reports that \( \text{stg} \) mutant wing disc cells arrest at G2 / M and continue to grow in size without replicating their DNA (Neufeld et al. 1998). Our results are consistent with the hypothesis that continued cell growth during a G2/M arrest is associated with increasing size of the nucleus without DNA replication, explaining why the total DAPI intensity per nucleus did not increase, but the DAPI brightness / area was lower in these enlarged nuclei. Consistent with this hypothesis, most \( \text{stg} \) knockdown cells had a relative DAPI fluorescence of 1.5, which would correspond to cells in G2 with a 4C DNA content. These results suggest that an increase in nuclear and wing hair size can occur through cell growth without polyploidization. In contrast, \( \beta \text{NACtes6} \) or \( \text{lin-52} \) knockdown increased both nuclear size and DNA content (Figure 3C-D’, E). This result suggests that knockdown of these genes induces cells to switch from mitotic proliferation to a polyploid endoreplication program through which tissues grow by an increase in cell size (hypertrophy) rather than cell number, consistent with the observed enlarged wing hair phenotype in adults (Figure 2K, Table 2). It is known that the \( \text{Lin52} \) protein is required for the Myb subunit to associate with the core of the MMB transcription factor complex (Andrekja et al. 2011; Guiley et al. 2015; Guiley et al. 2018). This \( \text{lin-52} \) phenotype is, therefore, consistent with our previous finding that knockdown of \( \text{Myb} \) switches wing and other cells to endoreplication (Rotelli et al. 2019). Similar to \( \text{Myb} \) knockdown, it is likely that knockdown of \( \text{lin-52} \) impairs the induction of mitotic gene expression by the MMB and promotes a switch to endoreplication cycles that skip mitosis (Rotelli et al. 2019). Knockdown of \( \beta \text{NACtes6} \) or \( \text{lin-52} \) also reduced the area of the L3-L4 intervein region in adult wings, suggesting that tissue growth through an increase in cell size did not fully compensate for growth by cell proliferation (Figure 2F, K, Table 2).

**Conclusion**

We have identified 18 UAS-shRNA TRiP strains that compromise growth of the wing. Ten of the genes targeted by these UAS-shRNA strains have known functions in *Drosophila*, whereas eight genes have not been previously characterized. All but one of these 18 genes are similar to human genes, many of which have been associated with disease. Our results suggest that reduced expression of two genes, \( \beta \text{NACtes6} \) and \( \text{lin-52} \), promotes a switch to endoreplication growth program. A switch to endoreplication after \( \text{lin-52} \) knockdown is consistent with our recent finding that repression of a CycA – MMB – AurB pathway promotes endoreplication. \( \beta \text{NACtes6} \) has a conserved function to regulate translation and protein trafficking, but it is unclear how this is linked to the decision of tissues to grow through an increase in cell size or cell number. While the molecular function of most of the proteins encoded by the 18 genes recovered in this screen have either been described or can be inferred, many have not been fully evaluated for function in developing tissues. Among important questions that remain are how these genes affect cell division, cell death, differentiation, and the accumulation of tissue mass. Further analysis of these genes in *Drosophila* will be a model for defining their function in tissue growth, and how their dysfunction contributes to disease.

The genes identified in this screen fall into a number of broad functional classes, including cell cycle, chromosome segregation, morphogenesis, metabolism, steroid biochemistry, transcription, and translation. Not unexpectedly, five genes whose knockdown affected growth have functions in cell cycle and / or chromosome duplication / segregation \((\text{stg, eco, flh, cd66, CG34174})\). Further study of \( \text{CG34174} \) will help to sort out which of the many functions ascribed to its human ortholog, CINP (DNA replication, damage checkpoint, transcription) are relevant to its function in *in vivo* (Grishina and Lattes 2005; Lovejoy et al. 2009; Wu et al. 2019). Six of the genes fall into the broad class of metabolism and / or organismal physiology \((\text{Cisd2, CG12171, CG4459, CG8132, CG9547, CG4459})\). Notably, a recent candidate shRNA screen of genes with known or predicted metabolic function showed that \( \text{CG8132, CG9547, and CG4459} \) also influence growth of the *Drosophila* eye disc (Pletcher et al. 2019). The cellular activity of these metabolic genes *in vivo* remains incompletely defined, and an important question is whether their activity is similar among all cells or modulated in concert with the development and function of different cell types.

\( \beta \text{NACtes6} \) shRNA expression induced a switch to an endoreplication growth program. The \( \beta \text{NACtes6} \) shRNA is not predicted to affect the expression of the other \( \beta \text{NAC} \) paralogs (five \( \beta \text{NACtes} \) and \( \text{bic} \)) (Table S1). In addition to their high level of expression during spermatogenesis, all six \( \beta \text{NACtes} \) genes are expressed in imaginal discs, while two of them are also expressed during late embryogenesis (Roy et al. 2010). An important question is whether these different paralogs have tissue specific functions for regulating translation and protein trafficking. Future genetic analysis with loss of function alleles and molecular assays will be required to sort out the division of labor among these paralogs. Our findings lead us to hypothesize that at least \( \beta \text{NACtes6} \) regulates translation and / or trafficking of a protein that is required for mitotic cell cycles in imaginal discs, and that in the absence of this mitotic function cells switch to alternative endoreplication cycles. Given that the human \( \beta \text{NAC} \) orthologs are also transcription factors, it is possible that \( \beta \text{NACtes6} \) influences cell cycle choice by regulating transcription. Investigation of the function of \( \beta \text{NAC} \) proteins in *Drosophila* will provide new insights into the function of this family of proteins and their influence on alternative growth programs in development.

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