An efficient and multiple target transgenic RNAi technique with low toxicity in Drosophila

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Being relatively simple and practical, Drosophila transgenic RNAi is the technique of top priority choice to quickly study genes with pleiotropic functions. However, drawbacks have emerged over time, such as high level of false positive and negative results. To overcome these shortcomings and increase efficiency, specificity and versatility, we develop a next generation transgenic RNAi system. With this system, the leaky expression of the basal promoter is significantly reduced, as well as the heterozygous ratio of transgenic RNAi flies. In addition, it has been first achieved to precisely and efficiently modulate highly expressed genes. Furthermore, we increase versatility which can simultaneously knock down multiple genes in one step. A case illustration is provided of how this system can be used to study the synthetic developmental effect of histone acetyltransferases. Finally, we have generated a collection of transgenic RNAi lines for those genes that are highly homologous to human disease genes.
The continued creation of unique and ingenious genetic tools by many drosophilists over the past century has transformed *Drosophila* into a powerful model organism for diverse topics in biomedical research. In the past decade, we have witnessed the development and optimization of the transgenic RNA interference (RNAi) approach, which uses the Gal4/UAS system to control the expression of RNAi constructs, thereby depleting the transcripts of interest in a tissue- or developmental stage-specific manner.1–5. Due to its relative simplicity and versatility compared to the classical mutants, several large *Drosophila* transgenic RNAi collections have been generated, and their applications have made a major contribution to our understanding of the functions and regulation of genes in developmental and physiological contexts.1–3. These applications by many fly laboratories have also revealed the intrinsic limitations of these transgenic RNAi lines, such as false negative results for certain highly expressed genes because of poor efficiency, false positive results due to either off-target effects or the leaky expression of the basal promotor, and the unsolved challenge to simultaneously target multiple genes.1,6–12. Therefore, there is heightened interest in optimizing this transgenic RNAi approach to further increase its efficiency, specificity, and versatility.

Here, we report the development and characterization of a transgenic RNAi system based on a vector named pNP. This system works both in soma and germline with significantly reduced basal level transcription, thereby avoiding potential developmental defects due to leaky hairpin expression. We compare this system with the transgenic RNAi system based on the pVALIUM20 vector which has been widely used by the fly community.4,12. Using the same Gal4 lines, we observe that the hairpins expressed in the pVALIUM20 system resulted in no obvious phenotypes or lethality before adulthood, but using the pNP system to drive the expression of the same hairpins led to strong and specific tissue phenotypes. Moreover, the pNP transgenic RNAi system can efficiently target genes that are highly expressed and produce expected phenotypes. These observations support the greater efficiency of this pNP system compared to the pVALIUM20-based transgenic RNAi system.

This transgenic RNAi system can simultaneously deplete multiple genes, which allows us to study the functions of redundant genes or protein complexes. Moreover, the loss-of-function phenotypes produced by targeting two genes simultaneously are more robust than the traditional method to express hairpins using one recombinant carrying two UAS cassettes. To provide a case illustration of this system, we focus on the histone acetyltransferases (HATs), since depleting individual HATs in the *Drosophila* eye using RNAi fails to generate any phenotypes. Surprisingly, simultaneously knocking down the transcripts of three HATs, *chameau* (*chm*), *Tip60*, and *Gcn5*, by the pNP system generated phenotypes with severe eye defects. Our subsequent analyses revealed that these HATs work together in modulating Wnt signaling.

Finally, we generate a resource of reagents against the genes that are highly homologous to human disease genes. We anticipate that this transgenic RNAi system and the relevant resource will further aid the developmental genetic analysis of the functions and regulation of *Drosophila* genes in the future.

**Results**

**The pNP system works efficiently in soma and germline.** The conditional transgenic RNAi systems in *Drosophila* can temporally and spatially knock down target genes with the binary Gal4/UAS system. Without Gal4, the hairpin encoded by the DNA sequence downstream of the UAS would not be expressed, enabling us to generate and maintain thousands of transgenic RNAi lines based on the pVALIUM vectors.2–4. However, we noticed that 15.8% of the transgenic RNAi lines based on the pVALIUM20 system in the Tsinghua Fly center (THFC) cannot maintain homozygosity (Supplementary Data 1). Meanwhile, 8.5% of the ΔC31 and long double-stranded RNA (dsRNA) hairpin based transgenic RNAi library (KK collection) in the Vienna *Drosophila* Resource Center (VDRC) are heterozygous, and 17.9% of the pVALIUM20 system based transgenic RNAi lines in the VDRC are heterozygous (Supplementary Data 1). All these vectors used in the previously developed transgenic RNAi systems are based on the pUAST vector, whose promotor consists of tandem UAS repeats followed by the TATA box from the *Drosophila* hsp70 gene.4,14. Given that the hsp70 basal promotor can direct transcription without Gal4 induction in certain tissues at the standard 25°C,9, we speculated that the lethality of these homozygous transgenic RNAi lines could be due to the leaky activation of the hsp70 basal promotor.

To test this possibility, we replaced the hsp70 basal promotor in the pVALIUM20 vector with a *Drosophila* synthesized core promotor (DSCP).13. Since the hsp70 basal promotor is longer than DSCP, we added a 100 bp spacer sequence upstream of the UAS to ensure the appropriate working distance between the promotor and gypsy insulators. To achieve precise processing and expression of multiple shRNAs,14, we amplified an intergenic linker between miR-2a-1 and miR-2b-2 and cloned it downstream of the miR1 scaffold. With these optimizations, we designated the resulting construct as the pNP vector (Supplementary Fig. 1).

We designed and generated stable transgenic lines using this system to express shRNAs targeting a set of protein-coding genes with known loss-of-function phenotypes in the wing, eye, neurons, intestine, testis and ovarian stem cell system. Firstly, knockdown of *egfr, Hpla1, kis, Notch* in the wing driven by wing specific Gal4s generated severe wing defect (Fig. 1a), which is consistent with previous description.6,15–18. Furthermore, targeting of *white, light, hedgehog* (hh) using the ey-Gal4 produced the same eye phenotype as previous reports (Fig. 1b)19–21, suggesting the pNP transgenic RNAi system can efficiently deplete genes in soma. We also chose several genes with known loss-of-function phenotypes to analyze the efficiency of pNP system in the neurons. Consistent with previous reports,1,22,23 all of them caused lethality when driven by elav-Gal4, indicating that pNP works efficiently in neurons (Supplementary Fig. 2a). Next, we tested the efficiency of the pNP system in intestine, testis and ovarian stem cell system. We used the germ cell specific nos-Gal4 to knock down *piwi* or *bam*, which severely reduced ovary size compared with the control (Fig. 1c), and resulted in flies that did not lay eggs (Fig. 1d). In addition, RNAi of *bam* increased the number of germline stem cells (GSCs) as previous reports,24 while knocking down of *punt* in testis by nos-Gal4 showed no GSCs and less germ cells,25, and depletion of *Notch* in intestine by esg-Gal4 increased the number of intestinal stem cells and enteroendocrine cells (Supplementary Fig. 2).26. Taken together, these results show that the pNP transgenic RNAi system can efficiently and specifically knock down target genes in not only soma, but also neurons, intestine, testis and ovarian stem cell system.
similar to those of the wild-type animals at all developmental stages analyzed, but their levels are all dramatically increased in pVALIUM20-luciferase animals after the embryonic stage. To test the potential tissue-specific differences, we dissected different tissues from the third instar larvae and performed the luciferase assays. Similar luciferase intensities were observed in the muscle and the central nervous system (brain and nerve cord) with both vectors (Fig. 2b). However, we observed the significantly elevated basal expression of luciferase in salivary gland, male gonad, female gonad, fat body, and wing disc from the pVALIUM20 system, being 685, 41, 42, 167, and 4 fold higher than in the corresponding tissues from the pNP system (Fig. 2b).

To validate these observations based on the luciferase reporter, we compared the levels of heterochromatin protein 1 (HP1) by immunostaining using the polytene chromosomes from the pVALIUM20-Hp1a and pNP-Hp1a larvae without Gal4. Compared with the control and pNP-Hp1a, the levels of HP1a on polytene chromosomes from the pVALIUM20-Hp1a larvae were significantly decreased (Fig. 2c), and the relative HP1a intensity at the chromocenter was down to 20% (Fig. 2d), suggesting the leaky expression of shRNA in pVALIUM20-Hp1a. Consistent with this observation, we used the qRT-PCR assay and observed that the Hp1a mRNA levels in the salivary gland of pVALIUM20-Hp1a larvae were also significantly reduced compared to the control, while no differences in Hp1a mRNA levels were observed between pNP-Hp1a larvae and the control larvae at the same developmental stage (Fig. 2e). Taken together, these data support that, compared with the pVALIUM20 vector, the pNP system had significantly reduced the leaky expression.

To further test whether the leaky expression from the pVALIUM20 vector is deleterious to fly development thereby increasing the heterozygous rate of the transgenic animals, we built pVALIUM20 and pNP based transgenic lines against more than 300 genes, respectively (Supplementary Data 2). For each target gene, same shRNA was cloned into pVALIUM20 or pNP vector, respectively, and they were inserted in the same chromosome locus. When comparing the heterozygous rate of these transgenic lines, we observed that almost 15% of the pVALIUM20 system based transgenic lines were heterozygous, which is consistent with the rate in existing stock centers, while the pNP system based transgenic lines exhibited only 0.6% heterozygosity (Fig. 2f). These results demonstrate that the leaky
Fig. 2 Significantly reduced leaky expression from basal promoter. 

**a** Relative luciferase expression levels at different developmental stages of pVALIUM20-luciferase and pNP-luciferase flies without the Gal4 driver (n = 5, mean ± s.d.). 

**b** Relative luciferase expression levels in different tissues of pVALIUM20-luciferase and pNP-luciferase flies at larval stage without the Gal4 driver (n = 3, mean ± s.d.). 

**c** Immunostaining of polytene chromosome from pVALIUM20-Hp1a and pNP-Hp1a without the induction of Gal4. DNA was visualized with DAPI and HP1a was stained with mouse monoclonal anti-HP1a (HP1a C1A9). Dotted circles indicate chromocenter. Scale bars, 20 μm. 

**d** Quantification of the relative HP1a intensity at chromocenter of polytene chromosome in pVALIUM20-Hp1a or pNP-Hp1a without the Gal4 driver (n = 10, mean ± s.d.). Data are evaluated with one-tailed Student’s t-test. 

**e** Relative expression of HP1a in control, pVALIUM20-Hp1a or pNP-Hp1a salivary gland without the driving of Gal4 (n = 3, mean ± s.d.). Data are evaluated using one-tailed Student’s t-test. 

**f** The heterozygous percentage of pVALIUM20 system based transgenic lines and pNP system based transgenic lines.
expression is significantly reduced in the pNP system, also decreases the side effect shown by the pVALIUM20 system.

The pNP system is more efficient than the pVALIUM20 system. The pVALIUM20 system works well in somatic cells and female germline for many genes; however, shRNAs targeting some of the functional genes in development generated no obvious or only weak phenotypes. To validate whether the pNP system is more efficient than the pVALIUM20 system, we first tested the expression of luciferase in different tissues of pNP-luciferase and pVALIUM20-luciferase transgenic flies using different Gal4 drivers, including elav-Gal4, MS1096-Gal4, Nub-Gal4, MTD-Gal4, nos-Gal4, GMR-Gal4, and esg-Gal4. As shown in Supplementary Fig. 3, both pNP-luciferase and pVALIUM20-luciferase transgenic flies showed highly expressed luciferase comparing with control. Furthermore, the luciferase intensities of pNP-luciferase transgenic flies are all significantly higher than pVALIUM20-luciferase animals using these drivers, indicating the pNP system is more efficient. Then we chose the same shRNAs, targeting genes encoding proteins known for their critical functions during the development of eye, wing or ovary, and generated transgenic lines using the pNP system. The genes targeted were E2F1, Upf1, aTub67C, and H2B.

In our first example, we tested E2F1, the key transcription factor involved in regulating the G1-S phase transition of the cell cycle.27,28 Depletion of E2F1 in the eye using ey-Gal4 in the pVALIUM20-E2F1 system slightly reduced eye size; in contrast, the pNP-E2F1 system combined with ey-Gal4 generated a much smaller eye (Fig. 3a). In our second case study, we analyzed Upf1, an important protein involved in nonsense mRNA mediated decay,29 since knocking down Upf1 using MS1096-Gal4 and the pNP-Upf1 system did not generate obvious phenotypes. However, we found that depletion of Upf1 using pNP-Upf1 driven by MS1096-Gal4 led to small wings with disrupted anterior cross vein (a-cv) (Fig. 3b). Similarly, depleting aTub67C in the wing using the pVALIUM20-aTub67C system and Nub-Gal4 failed to generate any phenotypes, presumably due to the high abundance of this transcript in cells. Nevertheless, a similar experiment using the pNP-aTub67C system led to extremely small wings (Fig. 3c). These results suggest that the pNP system produces stronger phenotypes than pVALIUM20 in somatic tissues.

To compare the knockdown efficiency of these two systems in the female germline, we crossed the nos-Gal4 line with pNP-egg and pVALIUM20-egg. As shown in Fig. 3d, the ovaries from pVALIUM20-egg females were smaller than controls, but the ovaries from the pNP-egg females were further reduced in size (Fig. 3d). Consequently, as compared with a few eggs produced from the pVALIUM20-egg females, we did not obtain any eggs from the pNP-egg females (Fig. 3e). These results suggest that the pNP system also works more efficiently than the pVALIUM20 system in the female germline. Moreover, to exclude the possibility of off-target effect and rescue the phenotype generated in Fig. 3, we overexpressed the target genes that are insensitive to the hairpins in the RNAi flies driven by specific Gal4 lines. As shown in Supplementary Fig. 4, both the pVALIUM20-induced phenotype and the pNP-induced phenotypes were all significantly rescued, respectively, compared with the RNAi phenotypes alone, suggesting the specificity of these RNAi. Taken together, these results support the efficiency and specificity of this pNP system.

To further validate the notion that the pNP system is more efficient in depleting genes of interest than the pVALIUM20 system, we quantified the mRNA levels using the qRT-PCR assay. Although the pVALIUM20 system significantly reduced the transcripts of E2F1, Upf1, and egg compared to the control, greater depletion of these mRNAs was achieved sing the pNP system (Fig. 3f). For the aTub67C gene, the pVALIUM20 system did not affect mRNA level, but significant reduction was observed in wing discs using the pNP system (Fig. 3f). These observations are consistent with the aforementioned phenotypic assays. Remarkably, as a member of the tubulin family, aTub67C is highly expressed in cells; the pVALIUM20 system failed to knock down aTub67C transcripts (Fig. 3f), implying the weakness of the pVALIUM20 system in depleting highly expressed genes. Taken together, these results suggest that the pNP system is more efficient than the pVALIUM20 system in both soma and female germline.

The pNP system efficiently modulates high expression genes. To efficiently deplete genes that are highly expressed in cells has been a challenge in the field. The observations we made with the aTub67C gene prompted us to test additional cases to compare the pNP system with the pVALIUM20 system. For this, we chose a set of multiple-copy genes, histone H1, H2A, H2B, H3, and H4, all highly expressed in cells. Using the same hairpins for each histone gene, we constructed transgenic RNAi flies based on the pNP system or the pVALIUM20 system. When driven by MS1096-Gal4, knock down of all the histones using the pNP system generated severe wing defects (Supplementary Fig. 5), consistent with the critical role of histones in cell viability. Surprisingly, depleting H1 or H3 using the pVALIUM20 system generated weak or no aberrant phenotypes, while knocking down H2A, H2B, or H4 in this system caused lethality before the pupal stage (Supplementary Fig. 5). Given that the pNP system is more efficient but less leaky than the pVALIUM20 system, we suspected that the leaky expression of pVALIUM20-H2A, H2B, and H4 plus the MS1096-Gal4 induced RNAi in early developmental stages may cause the lethality of these pVALIUM20 transgenic lines.

To test this idea, we selected the Nub-Gal4; tub-Gal80ts system to compare the wing phenotypes using pNP and pVALIUM20. Gal80ts is a temperature-sensitive repressor of Gal4, under the control of the tubulin promoter.30,31 The crossed flies were first maintained under the permissive temperature 18°C which allows Gal80 to repress Gal4 activities. The third instar larvae were then shifted to 29°C, which inactivates Gal80 thereby allowing Gal4-dependent expression to occur normally and deplete the histone genes. As shown in Fig. 4a, we observed the severely disrupted wing phenotype for all the histones depleted using the pNP system, similar to the RNAi phenotypes driven by MS1096-Gal4 (Supplementary Fig. 5). In contrast, the pVALIUM20 system showed no obvious phenotype for any of the depleted histones (Fig. 4a). The qRT-PCR result also confirmed that the pNP system was more efficient than the pVALIUM20 system (Fig. 4b), supporting the pNP system could efficiently modulate high expression genes with low false positive results.

The false positive results in pVALIUM20-H2A, H2B and H4 flies were likely from the combination of the leaky expression and MS1096-Gal4 induced RNAi in early developmental stage. In our analysis, pVALIUM20-H2A, H2B and H4 driven by MS1096-Gal4 were all lethal (Supplementary Fig. 5), but were viable and generated weak wing phenotypes using Nub-Gal4; tub-Gal80ts, which prevented RNAi in early developmental stage (Fig. 4a). However, these hairpins driven by MS1096-Gal4 in the more efficient pNP system (also with low leaky expression) were viable (Supplementary Fig. 5) and gave the same severe phenotypes as Nub-Gal4; tub-Gal80ts generated (Fig. 4a). Taken together, these results suggest that the leaky expression of the pVALIUM20 system plus MS1096-Gal4 induced RNAi in early developmental stage can cause false positive results.

These observations indicated that the pVALIUM20 system may cause lethality because of leaky and non-specific expression,
**Fig. 3** The pNP system is superior to the pVALIUM20 system.  

**a** Knock down of *E2F1* in the eye using the pVALIUM20 system or the pNP system with the same shRNA driven by ey-Gal4. Scale bars, 200 μm.  

**b** RNAi of *Upf1* in the wing using MS1096-Gal4. Scale bars, 500 μm.  

**c** Targeting of αTub67C with the pVALIUM20 system or the pNP system driven by Nub-Gal4. Scale bars, 500 μm.  

**d** Knock down of egg in the germline controlled by nos-Gal4. Scale bars, 300 μm.  

**e** Fertility rates of control, pVALIUM20-egg and pNP-egg female flies using the nos-Gal4 driver (n = 10, mean ± s.d.). Data are evaluated with one-tailed Student’s t-test.  

**f** qRT-PCR analysis of the RNAi efficiency of *E2F1*, *Upf1*, αTub67C, and egg, respectively (n = 3, mean ± s.d.). Data are evaluated using one-tailed Student’s t-test (*p < 0.05, **p < 0.01, ***p < 0.001)
The pNP system can simultaneously target multiple genes. Proteins encoded by two or more genes in *Drosophila* can have partially overlapping or redundant functions, and thus depleting one of them may result in little or no effect on functional or phenotypic analyses. In addition, depletion of a single subunit of a protein complex may result in the formation of partial protein complexes, which can have dominant-negative or neomorphic effects in vivo. Thus it is advantageous to simultaneously deplete multiple genes. However, existing transgenic RNAi vectors can only accept a single shRNA; therefore, to simultaneously target two or more genes cumbersome genetic crosses are required. Another problem is that genetic recombination may reduce RNAi efficiency because of Gal4 dilution, as well as that unhealthy recombinants frequently occur.

![Image](https://example.com/image.png)

**Fig. 4** The pNP system could efficiently modulate high expression genes. 

*The Nub-Gal4; tub-Gal80ts system was used for conditional knock down of histones in the pNP and pVALIUM20 systems, with the flies first incubated at 18 °C and then shifted to 29 °C at third larval stage. Scale bars, 500 μm. B The act-Gal4; tub-Gal80ts system was used for qRT-PCR analysis of the RNAi efficiency, targeting H1, H2A, H2B, H3, and H4 (n = 3, mean ± s.d.). Data are evaluated using one-tailed Student’s t-test (\( p < 0.05, \^p < 0.01, \^\^p < 0.001 \)) which can be problematic particularly when applying this system for large-scale genetic screens. For example, we randomly selected 20 genes for which shRNAs in pNP gave wing defect phenotypes if crossed with wing-specific Gal4; three of them were lethal if the same hairpins were used in pVALIUM20, further supporting the high tissue specificity of the pNP system (Supplementary Data 3). Taken together, these results show that the pNP system is more efficient than the pVALIUM20 system in knocking down genes of interest, including those with high levels of expression, and that it produces the expected strong phenotypes without obvious leaky expression that often observed in the pVALIUM20 system.*
line and the pNP-E2F1-Ci line (Fig. 5c). To compare the efficiency of multiple shRNA knockdown from the pNP system with traditional genetic recombination, pNP-Ci and pNP-E2F1 were genetically recombined first and then crossed with the Nub-Gal4 line. Interestingly, only 2.47% of the pNP-Ci; pNP-E2F1 flies showed both Ci KD and E2F1 KD phenotypes; most of them generated either Ci KD or E2F1 KD phenotype alone, while 6.81% in fact produced no obvious phenotype (Fig. 5d, e). This variable result illustrates another drawback of using traditional genetic recombination to knockdown multiple genes, presumably due to competition for Gal4 binding by the two UAS cassettes. Moreover, the flies with the integrated line of pNP-Ci; pNP-E2F1 appeared weak as homozygosity was lethal before adulthood, likely due to too many exogenous genetic components. These

### Table: Phenotype of Nub-Gal4+ pNP-E2F1; pNP-Ci KD flies

| Phenotype                        | Number | Percentage |
|----------------------------------|--------|------------|
| No phenotype                     | 33     | 6.81%      |
| Ci KD phenotype                  | 238    | 49.07%     |
| E2F1 KD phenotype                | 202    | 41.65%     |
| Ci & E2F1 KD phenotype           | 12     | 2.47%      |
| **Total**                        | 485    | 100%       |
observations further support the notion that the pNP system is more robust and versatile than traditional recombination to target multiple genes.

To further validate the ability of pNP in depleting multiple genes simultaneously, we applied this system to Polycomb repressive complex 1 (PRC1), which function as transcriptional repressor complexes essential for normal development.32 Simultaneous knockdown of four core proteins of PRC1 generated severe eye defects, while depleting of them individually did not cause any defects in the eye (Supplementary Fig. 6a). The RNAi efficiency of tetradic-gene-KD is also comparable with single-gene -KD tested by qRT-PCR assay (Supplementary Fig. 6b). Taken together, these results demonstrate the remarkable effectiveness of the pNP system to simultaneously knock down multiple genes.

**HATs regulate eye development through Wnt signaling pathways.** In an effort to test this transgenic RNAi system for studying proteins with partially redundant functions, we chose to study a family of histone acetyltransferases (HATs), the enzymes that mainly acetylate lysine residuals on histones. Given that the HAT family of enzymes regulate transcriptional activation, DNA repair, and the cell cycle, dysregulation of their activities is often linked to epigenetic alterations in many developmental disorders and diseases such as cancer.33 Transcriptional activation requires multiple HATs, and the functions of some HATs are generally redundant; thus the depletion of one HAT is insufficient to produce phenotypes. We focused our analyses on a set of HATs belonging to the MYST or GNAT families, including chm, Tip60, and Gcn5. After generating the transgenic RNAi lines against chm, Tip60, Gcn5, and the combinations of chm-Tip60 and chm-Tip60-Gcn5, we crossed these lines with the eye-specific GMR-Gal4 line. We observed that the pNP-chm, pNP-Tip60, pNP-Gcn5, and pNP-chm-Tip60 did not produce any defects in the eye (Fig. 6a). However, simultaneously depleting all three HATs in the eye using the pNP-chm-Tip60-Gcn5 line led to a severely defective eye phenotype, characterized by the overproduction of eye pigment, necrotic cell death, and slightly reduced eye size (Fig. 6a) This is unlikely to be caused by off-target effects, as depletion of any one of them produced no obvious defects.

To confirm that this phenotype is indeed caused by depletion of chm, Tip60, and Gcn5, we performed a qRT-PCR assay to measure the transcripts of these genes in eye discs after RNAi triggered by GMR-Gal4. Compared with the control, the transcripts from individual RNAi lines and the triple RNAi line were all significantly reduced (Fig. 6b). Notably, the efficiency of RNAi depletion in the triple RNAi line was comparable to that of the individual RNAi lines, further demonstrating the power of the pNP system to simultaneously target multiple genes. To further exclude the possibility of off-target effects, we overexpressed chm-T2A-Tip60-T2A-Gcn5 that insensitive to the hairpins by using the T2A self-cleaving peptide in the chm-Tip60-Gcn5 triple KD flies, and the KD phenotype was fully rescued (Supplementary Fig. 7b), supporting the specificity of this RNAi experiment. Furthermore, we also generated a transgenic activation line that targeting these three genes simultaneously using the flySAM system we developed recently.34 As shown in Supplementary Fig. 7c, activation of these three genes also significantly rescued chm-Tip60-Gcn5 triple KD tumor-like phenotype, further supporting the specificity of this triple KD result.

Furthermore, to validate the enzyme activity of chm, Tip60, Gcn5 on histone acetylation, we performed immunostaining to detect the level of global H4 acetylation in polytene chromosomes after RNAi induced by salivary gland-specific 1824-Gal4. The intensity of immunostaining against H4 acetylation in polytene chromosomes from the pNP-chm, pNP-Tip60, pNP-Gcn5 lines was slightly lower compared to the control, whereas the H4 acetylation in polytene chromosomes from the pNP-chm-Tip60-Gcn5 line were almost undetectable (Supplementary Fig. 8). Taken together, these observations demonstrate the redundant roles of these HATs on epigenome, further supporting the ability of the pNP system to successfully modulate multiple genes.

To explore the molecular mechanism of the severe eye defect phenotype produced by triple RNAi against chm-Tip60-Gcn5, we detected the transcriptional level of genes downstream of several major signaling pathways, such as the JAK-STAT, EGFR, Hippo, JNK, dpp, Notch, and Wnt signaling pathways, because of their roles in regulating eye development. Specifically, we analyzed the expression of SOCS36E, aos, Yki, puc, dad, Su(H) and wg as the readouts for the activities of the aforementioned signaling pathways in eye discs with these HATs being depleted.35 As shown in Fig. 6c, wg, a target of Wnt signaling, was significantly up-regulated by more than five-fold of the control, while the representative target genes of the other signaling pathways tested were not significantly affected in HAT-depleted eye discs. These observations indicate that Wnt may be the major signaling pathway being affected by these HATs in eye discs.

To test the effects of depletion of the HATs on Wnt signaling, we generated the chm-Tip60-Gcn5 KD clones marked with GFP in wing discs and then analyzed the levels of Wg expression using immunostaining. As shown in Fig. 6d, the chm-Tip60-Gcn5 KD clones showed clearly elevated levels of Wg proteins. Meanwhile, we constructed wg transcriptional activation flies using the CRISPRa system,34 which showed exactly similar eye phenotype as the chm-Tip60-Gcn5 KD when driven by GMR-Gal4 (Fig. 6e). To further validate that the defective eye phenotype was indeed caused by elevated Wg signaling, we depleted wg in addition to the triple knocking down of chm, Tip60 and Gcn5. Compared to the chm-Tip60-Gcn5-GFP-RNAi control, which was the same as the chm-Tip60-Gcn5 KD phenotype, we observed that knocking down wg significantly rescued the eye phenotype caused by depletion of HATs. Similarly, simultaneous depletion of the three HATs and arm, encoding a key component of the Wg signaling pathway, dramatically rescued the defective eye phenotype caused by depletion of these HATs (Fig. 6e). Taken together, these analyses suggest that chm, Tip60 and Gcn5 synergistically play critical roles in regulating eye development, mainly through modulating the Wnt signaling pathway.

**Discussion**

Armed with a variety of highly sophisticated genetic tools, Droso phila melanogaster is a powerful model organism for investigating the roles and regulation of genes in a broad spectrum of biological contexts. Loss-of-function and gain-of-function analyses provide the mechanistic insights into both the biological and the pathological processes. Compared to the gain-of-function approaches, loss-of-function analyses are less prone to potential artifacts, and many different techniques have been continuously developed to achieve this goal. A major breakthrough in recent years for loss-of-function analysis is the CRISPR-Cas9 genome editing system, which precisely generates mutation in the locus of interest using the endonuclease Cas9 guided by specific sgRNA. Despite the advantages of this technique, the mutant alleles generated by this method often produce strong biological consequences, such as lethality before adulthood or sterility if the gene product is required for gametogenesis. In addition, the CRISPR-Cas9 technique can have potential off-targets generated by mismatch between sgRNA and genome DNA, and bacterial Cas9 protein could be toxic, particularly in neurons. In fact, to be able to analyze a series of hypomorphic alleles or to achieve reduction of function, instead of complete loss of function of
interested genes is often beneficial to advance our knowledge about the spectrum of functions and regulation of proteins in development. Given the simplicity of its design, high efficiency in knocking down genes of interest, and high penetration in phenotypic consequences, we regard the Drosophila transgenic RNAi as a powerful complement to the classical null alleles or strong loss-of-function mutants generated by the CRISPR-Cas9 technique in deciphering the functions of genes. Transgenic RNAi technique is one of the most important genetic tools in Drosophila with the advantages of simplicity and high efficiency, and can knock down target genes at special developmental stage and particular tissues. In the past decade, several conditional

Fig. 6 Histone acetyltransferases regulate eye development through Wnt signaling pathways. a chm, Tip60, Gcn5 single knock down or chm-Tip60 double knock down exhibited wild-type phenotype driven by GMR-Gal4, while simultaneous knock down of chm-Tip60-Gcn5 using the pNP system produced severe eye defects. Scale bars, 200 μm. b qRT-PCR results show that chm, Tip60, Gcn5 are all reduced to comparable level in single gene knockdown or triple genes knockdown flies. (n = 3, mean ± s.d.). Data are evaluated using one-tailed Student’s t-test. c Wnt signaling pathway was significantly up-regulated as represented by Wg via qRT-PCR assay (n = 3, mean ± s.d.). Data are evaluated using one-tailed Student’s t-test. d Immunostaining result showed up-regulated Wg in GFP-marked chm-Tip60-Gcn5 KD clones. Dotted lines showed the GFP marked clone. Compared with control (indicated by long tail arrows), Wg signals were significantly increased in chm-Tip60-Gcn5 KD clone (indicated by short tail arrows). Scale bars, 50 μm. e Transcriptional activation of wg produced similar eye phenotype as chm-Tip60-Gcn5 KD flies. Knockdown of wg or arm at the background of chm-Tip60-Gcn5 triple RNAi clearly rescued the eye phenotype. Scale bars, 200 μm.
transgenic RNAi vectors have been developed, along with large-scale transgenic RNAi resources\textsuperscript{1,4}, which have greatly accelerated the progress of the Drosophila research community. However, there is still room for these existing transgenic RNAi systems to be further optimized. For example, it is puzzling to note that 15.8\% of the RNAi lines generated so far can only be maintained as heterozygous (Fig. 2I). These problems have made genetic analysis difficult, especially when combining these RNAi lines with different Gal4 lines or overexpression lines. In this study, we identified the key issue of this problem is the leaky expression from the basal promoter, even without the Gal4 protein. To solve this challenge, we replaced the hsp70 promoter with a DSCP promoter, thereby significantly reducing the rate of transgenic RNAi heterozygotes from 15\% down to 0.6\% (Fig. 2I).

Given that many gene products in Drosophila can have partially redundant functions in regulating different biological processes, thus depleting one type of these protein is often not effective to produce obvious phenotype. All the previous transgenic RNAi techniques can only carry a single shRNA; thus simultaneously targeting two or more genes requires multiple generations of genetic crosses to recombine them together, which can also result in unhealthy recombinants. In addition, genetic recombination may also reduce RNAi efficiency because of Gal4 dilution. Furthermore, combining three or more RNAi lines is not feasible in practice. The pNP vector developed in this work can carry multiple shRNAs and efficiently deplete different genes in parallel, thereby producing strong and additive phenotypes. Our case study of targeting three HATs illustrated the advantages of this transgenic RNAi system over the previous methods. Simultaneously depleting three HATs generated a severely defective eye phenotype, and our subsequent analyses revealed that hyperactive Wnt signaling caused by reduction of HATs accounts for these strong phenotypes (Fig. 6).

Although the transgenic RNAi technique is a powerful approach to studying gene function, caution is necessary to avoid potential false negative or false positive results. False negative results may be obtained particularly when performing genetic screens using the large collection of transgenic RNAi lines. One major factor that may contribute to this problem is the difficulty in depleting highly expressed genes to the threshold sufficient to cause phenotypic consequences. A key advantage of the pNP system, as shown in this work, is that it is much more efficient at knocking down highly expressed genes such as aTub67C than the previously developed pVALIUM20 system (Fig. 3c and Fig. 4). Furthermore, in the few cases that we tested, the pNP system yielded more clearly visible phenotypes than the pVALIUM20 system as one would expect (Fig. 3 and Fig. 4), suggesting that the RNAi from pNP system is more efficient and, therefore, the transgenic lines based on this system may help to reduce false negative rates.

Another question that needs caution is related to the false positive results. For example, some of the transgenic RNAi lines based on the pVALIUM20 system can cause lethality at different developmental stages, with or without combination with certain tissue-specific Gal4 lines\textsuperscript{4,9,10}. Although this phenomenon is usually interpreted as the consequence of transgenic RNAi against a developmentally essential gene, our comparisons between the pVALIUM20 and pNP systems, together with the temperature-sensitive Gal80 repressor, suggest that leaky and non-specific expression from the basal promoter in the pVALIUM20 vector plus Gal4 induced RNAi in early developmental stage can cause lethality. Our analyses with the pNP transgenic lines suggest that the pNP system is tighter, and therefore more specific in tissue-specific knockdown than the pVALIUM20 system. Thus, we expect that using the transgenic RNAi lines based on the pNP system may help to reduce false positive rates.

Finally, inspired by the major advantages of the pNP system as characterized in this work, we have generated a collection of transgenic RNAi lines against the 1093 genes that are highly homologous to human disease genes, available from the Tsinghua Fly Center (Supplementary Data 4 and http://fly.redbux.cn/). We have performed a phenotypic analysis using MTD-Gal4, which induced RNAi depletion of these disease-associated genes specifically in germline, and then analyzed the effect on egg laying. We have analyzed 576 genes so far and 28.6\% of the screened genes showed abnormal egg laying, suggesting the efficiency of this pNP system and the association of these genes with reproductive disease (Supplementary Data 5). We anticipate that this RNAi approach, as well as the relevant resources, will aid the Drosophila community to advance our understanding of the regulatory networks underpin on the normal developmental and physiologic processes.

\section*{Methods}

\subsection*{Vector and DNA constructs.} pVALIUM20 was cut with SpeI to remove the gypsy, Loxp, UAS and hsp70 promoter, and then was ligated with an oligonucleotide fragment generated by annealing the two primers that contained EcoR I, Spe I, Bgl II, Avr II, Xba I, Nhe I sites (fwd: 5'-CTATGCTGAAACACTAGTCAAGTCG-3', rev: 5'-GTTCGGATCTGGATGATGATGATGATG-3'), which were mutated at EcoRV, BamHI, NheI, KpnI, SnaI, MfeI sites by AccuPrime Pfx DNA Polymerase (Invitrogen) (fwd: 5'-GTGACTCTCAGTTGACACTTGC-3', rev: 5'-TCAAGCCAGCTGACTGACGCA-3').

We named the vector V20-MCS1. The V20-MCS1 vector was cut with Xhol/KpnI and ligated with a small fragment cut from pGFPP-N1 with the same enzymes. The resulting vector was mutated at EcoRv, BamHI, Xhol, KpnI, SnaI, MfeI sites by AccuPrime Pfx DNA Polymerase (Invitrogen) (fwd: 5'-GTGACTCTCAGTTGACACTTGC-3', rev: 5'-TCAAGCCAGCTGACTGACGCA-3').

\subsection*{Generation of transgenic RNAi lines based on pNP.} All the previous transgenic RNAi techniques can only carry a single shRNA; thus simultaneously targeting two or more genes requires multiple generations of genetic crosses to recombine them together, which can also result in unhealthy recombinants. In addition, genetic recombination may also reduce RNAi efficiency because of Gal4 dilution. Furthermore, combining three or more RNAi lines is not feasible in practice. The pNP vector developed in this work can carry multiple shRNAs and efficiently deplete different genes in parallel, thereby producing strong and additive phenotypes. Our case study of targeting three HATs illustrated the advantages of this transgenic RNAi system over the previous methods. Simultaneously depleting three HATs generated a severely defective eye phenotype, and our subsequent analyses revealed that hyperactive Wnt signaling caused by reduction of HATs accounts for these strong phenotypes (Fig. 6).

Although the transgenic RNAi technique is a powerful approach to studying gene function, caution is necessary to avoid potential false negative or false positive results. False negative results may be obtained particularly when performing genetic screens using the large collection of transgenic RNAi lines. One major factor that may contribute to this problem is the difficulty in depleting highly expressed genes to the threshold sufficient to cause phenotypic consequences. A key advantage of the pNP system, as shown in this work, is that it is much more efficient at knocking down highly expressed genes such as aTub67C than the previously developed pVALIUM20 system (Fig. 3c and Fig. 4). Furthermore, in the few cases that we tested, the pNP system yielded more clearly visible phenotypes than the pVALIUM20 system as one would expect (Fig. 3 and Fig. 4), suggesting that the RNAi from pNP system is more efficient and, therefore, the transgenic lines based on this system may help to reduce false negative rates.

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Fly strains. GMR-Gal4, ey-Gal4 were used to drive expression in the eye. Nub-Gal4, MS1096-Gal4, C96-Gal4, Nub-Gal4; tub-Gal80 were used to drive expression in the wing. MTD-Gal4 and nos-Gal4 drove expression in the ovary and nos-Gal4 was also used in the tests. 1824-GAL4 (Bloomington #1824) drove expression in the salivary gland. eg-Gal4 and eg-Gal4, USP-GFP; tub-Gal80 were used to drive expression in the gut. elav-Gal4 was used to perform qRT-PCR assay of histone H1, H2A, H2B, H3, H4 lines, hs-FLP, act5C<STOP>Gal4, UASp-GFP/Cyo was used to perform chm-Tip60-Gcn5 triple KD clone assays. Detailed genetic information is available at FlyBase (http://flybase.org/).

Egg-laying assay. To perform egg-laying assay, we collected genetic females within 6 h of eclosion, and mated ten virgin females with eight w1118 males per vial. Later, every female was transferred to new vials, ten vials were scored for each group. The flies were transferred to new vials containing fresh food every day. Then we counted the number of eggs laid by individual female from day three to nine, and one time for one day. Approximately 10 vials were scored for each group.

qPCR assay. RNA that from salivary glands, wing discs, eye discs of L3 larvae, and ovary or entire flies in adult stage were isolated using TRIzol (Invitrogen). The total RNA was treated with DNaseI and 1 μg RNA subject to reverse transcription using the GoldScript cDNA Assay Kit (Invitrogen). qPCR reaction was performed by using SYBR Premix Ex Taq (TAKARA), and the results were analyzed with the iQ5 real-time PCR detection system (Bio-Rad). Calculation of steady state RNA levels was by the 2–ΔΔCT method. The primers used for qPCR are listed in Supplementary Data 6.

Luciferase assay. Luciferase activity was measured using the Steady-Glo Luciferase Assay Kit (Promega). 40 embryos, five wandering L3 larvae, five pupae or five female flies (2-day-old) were collected in 300 μL GLO lysis buffer for each sample; each luciferase assay contained five independent samples. Muscle and fat body of five L3 larvae, 20 brain discs, 40 wing discs, 40 gonads, 20 salivary glands of L3 larvae, and 10 guts from female flies were dissected in cold PBS and transferred to 90 μL GLO lysis buffer for each sample; three independent samples were used for each assay. Samples homogenized and then centrifuged at 12,000×g for 15 min, and then take 50 μL supernatant in 96-well plates. After incubation in the dark for 20 min, luminescence was measured on a luminometer (Thermo Scientific, VARIOSKAN FLASH).

Polytenic chromosome immunostaining. The third-instar larval polytene chromosomes was dissected and immunostained(ref). Chromosomes were incubated with mouse monoclonal anti-HPla (HP1a C19, 1:100) or rabbit polyclonal anti-acetylated-histone H4 Antibody (EMD Millipore, 06-866, 1:200). After incubation for 1 h at room temperature, chromosomes were washed three times in PBS and incubated with a goat anti-mouse antibody conjugated to FITC (DHSB, 1:100) and a goat anti-rabbit antibody conjugated to FITC (DHSB, 1:100) for 2 h (room temperature). After one PBS wash, the slides were incubated with 4,6-diamidino-2-phenylindole (Sigma, 1: 1000) to stain DNA. The slides were mounted in Fluoromount mounting media (Sigma, F4680), and the immunostaining images were performed by using a Nikon ECLIPSE Ti inverted microscope (×100 model). Adobe Photoshop software was used for measurements.

Wing disc immunostaining. Clones of RNAi cells in the wing disc were generated by FLP/FRT-mediated mitotic recombination(ref). To generate clones, y, hs-FLP; act5C<STOP>GAL4, UASp-GFP/Cyo was crossed with the UAS-Gcn5-Tip60-chm triple RNAi line at 25°C. The L1 stage larvae were heat-shocked at 37°C for 1 h. Then the larvae were cultured at 29°C until the L3 stage. The L3 stage larvae were dissected in PBS for immunostaining. Wing discs were incubated with rabbit monoclonal anti-GFP (Abcam, 1:1500) and mouse polyclonal anti-acetylated histone H3 Antibody (Santa Cruz Biotechnology, sc2010, 1:200), mouse monoclonal anti-ProS (DHSB, 1:200), rabbit monoclonal anti-GFP (Abcam, 1:1500). Images were obtained with Zeiss LSM780 fitted with an ultraviolet laser.

Construction of transgenic RNAI plasmids. Detailed information on primers can be found in Supplementary Data 7. For the large scale RNAi constructs, primers were ordered in a 96-well plate format and annealed at 95°C. The pNP vector was digested with EcoRI/NheI, and ligated with the annealed primers. Using PCR to select clones ( fwd: 5′-GCTGAGGACATGCTGTGATGATCCATACC-3′, rev: 5′-TAAATCGTGTTGATGTCATACC-3′), the correct clones were PCR for a 750 bp band and the wrong clones showed a 1000 bp band. Correct constructs were confirmed by DNA sequencing; the primer was 5′-GCTGAGGACATGCTGTGATGATCCATACC-3′.

For the more than one short hairpin constructs, we firstly cloned all short hairpins into the pNP vector as mentioned above; one resulting vector was linearized by digesting with SpeI and another vector was digested with SpeI and XbaI to get the fragment (300 bp) containing a short hairpin. The resulting constructs were purified (Axyprep DNA Gel Exraction Kit) and ligated each other. Following transformation, correct clones were selected by PCR ( fwd: 5′-GCTGAGGACATGCTGTGATGATCCATACC-3′, rev: 5′-TAAATCGTGTTGATGTCATACC-3′) and further confirmed by restriction enzyme digestion (SpeI and XbaI). The resulting vector could be cut with SpeI and ligated with another short hairpin fragment which was cut with SpeI and XbaI from another vector.

Construction of transgenic overexpression plasmids. To generate RNAI insensitive overexpression lines, we cloned the vector by using Gibson assembly method. First we the pVALIUM20 and pNP vector by EcoRI/NheI enzymes, PCR the coding sequences of E2F1, Upl, a Tub74C, egg, and chm-T2a-Tip60-T2a-Gcn5; then we used the Hei1 Clone Plus Multi One Step Cloning Kit (Yeasen, CAT:10912ES10) to assemble them together, finally we performed site directed mutagenesis by PCR. PCR primers for all can be found in Supplementary Data 8.

Construction of wg and chm-Tip60-Gcn5 activation vector. wg and chm-Tip60-Gcn5 activation were achieved by using the CRISPRa system, and the construct was generated as described in Jia et al. (2018) (ref). Primer sequences for sgRNAs are: wg: fwd: 5′-TTCGCGGAACTAGCATGGCAG-3′, rev: 5′-AACGCTGTGCATGGC-3′, chm: fwd: 5′-tcgGATGCATGGCAGGCGCC-3′, rev: 5′-aaacGAGGACTTTCCACTGTC-3′, Tip60- fwd: 5′-ctgGCACTTGTCTGATATCCTG-3′, rev: 5′-aaacGAGGACTTTCCACTGTC-3′, Gen5-fwd: 5′-tcgGTGACCTGCGCCTCTGGTC-3′, rev: 5′-aaacGAGGACTTTCCACTGTC-3′.

Statistics analysis. GraphPad Prism and Excel was used to calculate the mean and the s.d. for all the column diagram data, as well as the statistical analysis. For the n of each test, and detailed statistical analysis method is shown in the figure legend.

Data availability

The data that support the findings of this study are available from the corresponding author under reasonable request.

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Author contributions
J.-Q.N. designed the experiment. H.-Q.Q., R.-G.X., F.W., J.-Y.J., Q.L. Z.C., P.P., D.S. and L.-P.L. performed the experiments. H.-H.Q., R.-G.X., F.W., J.-S., R.Z., D.M., X.W., Y.J., P.P., D.S. and L.-P.L. performed the experiments. H.-H.Q., R.-G.X., F.W., J.-Y.J., Q.L. Z.C., G.W. and S.L. performed data analysis. J.-Q.N. wrote and revised the manuscript.

Additional information
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