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Generation of stable *Drosophila* cell lines using multicistronic vectors

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Insect cell culture is becoming increasingly important for applications including recombinant protein production and cell-based screening with chemical or RNAi libraries. While stable mammalian cell lines expressing a protein of interest can be efficiently prepared using IRES-based vectors or viral-based approaches, options for stable insect cell lines are more limited. Here, we describe pAc5-STABLEs, new vectors for use in *Drosophila* cell culture to facilitate stable transformation. We show that viral-derived 2A-like (or "CHYSEL") peptides function in *Drosophila* cells and can mediate the multicistronic expression of two or three proteins of interest under control of the Actin5C constitutive promoter. The current vectors allow mCherry and/or GFP fusions to be generated for positive selection by G418 resistance in cells and should serve as a flexible platform for future applications.

Since the first chicken embryo explants by Roux in 1885[1], cultured cells have been used as a tool to study biological processes for more than a century. A vast number of techniques have been developed for the analysis of mammalian cultured cells, including the establishment of stable cell lines that express DNAs, RNAs and proteins of interest. In recent years, approaches using retroviral and lentiviral-derived constructs to generate stable cell lines have been increasingly common[2]. The most extensive application of insect cell lines is in protein production, in which recombinant baculoviruses are used to infect lepidopteran cell lines to achieve high transient expression levels of proteins of interest[3]. In *Drosophila*, cell culture is an often-overlooked complement to the powerful genetic tools available in this research model. The utility of cell-based assays coupled with genome-wide RNAi or chemical library screening[4,5] and the promising potential of using *Drosophila* S2 cells for recombinant protein production[6,7] underlines the need for improved vector systems for working with *Drosophila* cells.

A number of strategies have been used to generate stably transformed *Drosophila* cell lines[8]. Cotransfection of the expression plasmid of interest with a second plasmid encoding drug resistance has been used[9–13], as well as single-vector strategies with two separate expression cassettes[14,15]. P-element-mediated transformation has also been used to incorporate selectable markers in cell lines[16]. In all cases, however, drug resistance can be preferentially selected over expression of the protein of interest, often necessitating single-cell cloning and screening of multiple stable clones. Viral-derived internal ribosome entry sites (IRES) have been used to develop multicistronic vectors in mammalian cells[17,18], and although IRES sequences have been described in insect systems[19–21], a similar approach has not been adopted to *Drosophila* cell culture vectors. Moreover, the use of IRES sequences to achieve multicistronic expression can also lead to variable levels of the expressed proteins[22,23].

"2A-like" sequences, also called CHYSEL (*cis*-acting hydrolase element) peptides, are found in viral polyproteins and serve as signals to direct apparent self-cleavage of the polyprotein into individual proteins. The sequences act on the ribosome peptidyltransferase center, causing release of the nascent chain while sense codons are still present, described as "ribosome skipping"[24–27]. The 2A-like sequences contain a canonical Asp-Val/Ile-Glu-X-Asn-Pro-Gly$^{(2A)}$Pro$^{(2B)}$ motif, which results in separation between the Gly$^{(2A)}$ and Pro$^{(2B)}$[28]. These sequences have been used for the generation of polycistronic vectors expressing multiple genes of interest in mammalian cells and transgenic animals, avoiding the use of internal ribosomal entries (IRES) or multiple promoters[29–33].

Here, we show that T2A, a 2A-like sequence from the insect virus *Thosea asigna*, is correctly processed in *Drosophila* cells, and describe new vectors (pAc5-STABLEs) that use T2A for the efficient production of stable cell lines, opening new possibilities for insect cell culture applications.

Results

New vectors for stable selection in *Drosophila* cells. To obtain strong constitutive expression, we chose the *pAc5.1* vector (Invitrogen), which contains the *Drosophila* Actin5c promoter, as a backbone for our new vectors. As a negative control for antibiotic selection, we used *pAc5–GFP* (Fig 1A), which expresses GFP, but does not contain a selectable marker. Several 2A-like peptides have been used successfully in polycistronic vectors for mammalian cells, but we reasoned that for insect cells, the best candidate was the T2A peptide sequence, derived from the insect cell virus *Thosea asigna*[26]. We generated *pAc5-STABLE1-Neo* (Fig. 1B), in which the sequence encoding the T2A peptide was cloned in frame between the GFP and the Neo^R genes. The Neo^R protein confers cellular resistance to the aminoglycoside antibiotic G418. Cells that survive antibiotic selection should therefore express the protein of interest in an obligate manner.

For cases in which there are two proteins of interest to be expressed at the same time, we generated *pAc5-STABLE2-Neo* (Fig. 1C). As *pAc5-STABLE1-Neo*, it contains the T2A peptide in frame between GFP and Neo^R. It also contains a sequence encoding a FLAG epitope-tagged version of mCherry fluorescent protein[34] fused C-terminal to the FLAG tag. This is separated from GFP by an additional T2A peptide, encoded by degenerate nucleotide sequence to prevent vector recombination. The vectors were designed for flexibility, to allow replacement of FLAG-Cherry, GFP, or Neo^R with any gene of interest, or to allow the generation of N-terminal or C-terminal fusions to the fluorescent proteins. To demonstrate the utility of the vector with a novel protein, the coding sequence of *Drosophila* CG6522 (ortholog of the focal adhesion protein TES,[35,36]; hereafter called dTES) was cloned at the N-terminus of GFP in pAc5-STABLE1-Neo (Fig. 1D).

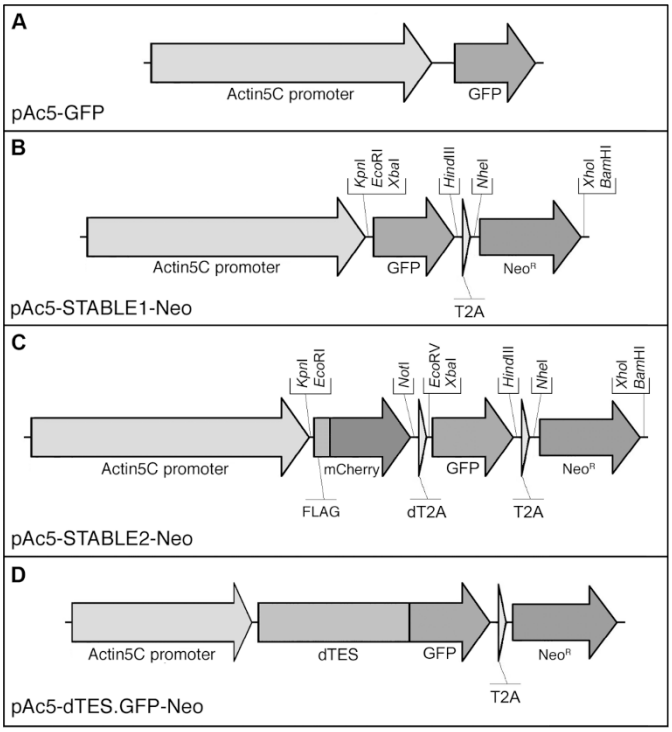

Figure 1 | Schematic representation of the p*Ac5-STABLE* vectors. All constructs were based on the vector backbone of *pAc5.1* (Invitrogen), with expression driven by the Actin5C promoter. (A) In *pAc5-GFP*, GFP alone served as a negative control for selection experiments. (B) In *pAc5-STABLE1-Neo*, designed for bicistronic expression, GFP and Neo^R are separated by the T2A sequence. Unique sites are shown. (C) *pAc5-STABLE2-Neo*, designed for tricistronic expression, features FLAG-tagged mCherry, GFP, and Neo^R, each separated by a T2A peptide. In the case of dT2A, it has identical peptide sequence to T2A, but degenerated sequence at the nucleotide level. Unique sites are shown. (D) *pAc5-dTES.GFP-Neo* was constructed by cloning *Drosophila* CG6522 into the *Eco*RI-*Xba*I sites of *pAc5-STABLE1-Neo*, generating an N-terminal fusion to GFP.

Generation of stable transformants using the *pAc5–STABLE1-Neo* vector. To test the vectors, we chose the cell line S2R+[37], an isolate of embryo-derived Schneider's line 2[38], since it is generally more adherent to cell culture vessels than parental S2 cells and has been used successfully in numerous RNAi screenings. We tested *pAc5-STABLE1-Neo* for positive selection against G418 compared to *pAc5–GFP* or non-transfected cells as controls. Equal quantities of the three different vectors were introduced into the cells, which were treated with 0 (Fig. 2A, D, G), 600 (Fig. 2B, E, H), or 2000 μg/ml (Fig. 2C, F, I) of G418 during four weeks. While very few control cells survived G418 selection for this period, cells transfected with *pAc5-STABLE1-Neo* continued growing even in the highest concentration (Fig. 2I). As anticipated, most of selected cells also expressed GFP, distributed homogenously throughout the nucleus/cytoplasm. Using image quantitation (which may underestimate weak expressors; see Methods), more than 68% of cells surviving in 2000μg/ml G418 also expressed GFP (Fig. 2L). This proportion was higher than non-selected cells or those with intermediate G418 selection (Fig. 2J–K). GFP intensity levels between cells were variable as detected by fluorescence microscopy, perhaps reflecting copy number of integrated vectors. Higher concentrations of G418 were not tested, and could potentially lead to more homogenous GFP levels. We conclude that vector *pAc5-STABLE1-Neo* can effectively confer G418 resistance to the GFP-expressing cells and that it can be used for the generation of stable cell lines.

Positive selection shows that Neo^R is correctly expressed and functionally active, but it could be functional even if expressed as an unprocessed GFP-T2A-Neo^R fusion protein. To discard this possibility and confirm that the T2A peptide was functioning, transfected cells were analysed by Western blot (Fig. 3). Using anti-GFP antibodies, the proteins show the right size of 27–30 kDa when expressed from both vectors *pAc5-GFP* and *pAc5-STABLE1-Neo*. Due to cloning strategy, GFP from *pAc5-GFP* contains extra amino acids at the C-terminus and migrates slightly slower than the GFP encoded by *pAc5-STABLE1-Neo*. No upper band corresponding to unprocessed GFP-T2A-Neo^R was observed, even in longer exposures (data not shown). These results indicate that the T2A sequence functions as expected and undergoes apparent self-cleavage in *Drosophila* S2R+ cells.

To confirm that the effect was not specific to S2R+ cells, we also tested *pAc5-STABLE1-Neo* in an additional *Drosophila* cell line, Kc167[39]. This line is derived from the Kc line, established from 6–12 hr female embryos. As before, we tested *pAc5-STABLE1-Neo* for positive selection against G418 compared to *pAc5–GFP* or non-transfected cells as controls. The Kc167 cells were treated with 0 (Fig. 4A, D, G), 600 (Fig. 4B, E, H), or 2000 μg/ml (Fig. 4C, F, I) of G418 during four weeks. Similar to our results with S2R+ cells, cells transfected with *pAc5-STABLE1-Neo* survived to a greater extent than those transfected with the control plasmids (more than 60%; Fig. 4L). This indicates that both GFP and Neo^R are functional and suggesting that T2A apparent self-cleavage is properly achieved in Kc167 cells as well.

Generation of stable cell population using the *pAc5-STABLE2-Neo*. Multiple self-cleavage peptides can be processed in the same cell, with published examples of up to 4 functional proteins being generated from a single polyprotein containing three 2A-like sequences[29,40]. To test whether more than two proteins can be processed from a multicistronic vector in insect cells, we generated the vector *pAc5-STABLE2-Neo*. Again, we used the *Thosea asigna* T2A peptide to separate the proteins. S2R+ cells were transfected

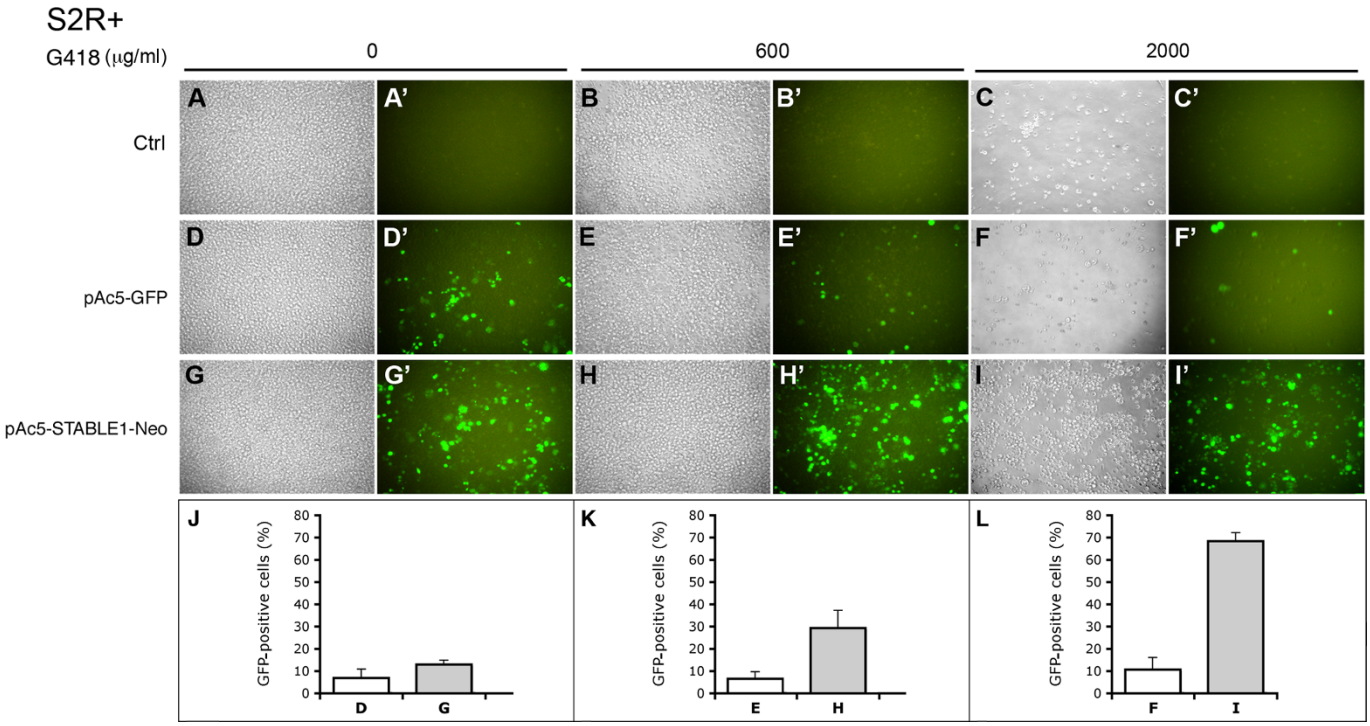

Figure 2 | *pAc5-STABLE1-Neo* confers G418 resistance to S2R+ cells. (A–I) Bright field images of S2R+ *Drosophila* cells that were mock-transfected (Ctrl; A–C), transfected with *pAc5-GFP* (D–F) or with *pAc5-STABLE1-Neo* (G–I). Three days after transfection, G418 was added to the media at concentrations of 0 (A, D, G), 600 (B, E, H) or 2000 μg/ml (C, F, I). (A'–I') Fluorescent images of the same cells from panels A to I showing GFP expression. (J–L) Percentage of surviving cells that are positive for GFP. Letters correspond to panels/treatments directly above each graph. All images were taken after 30 days of treatment with G418.

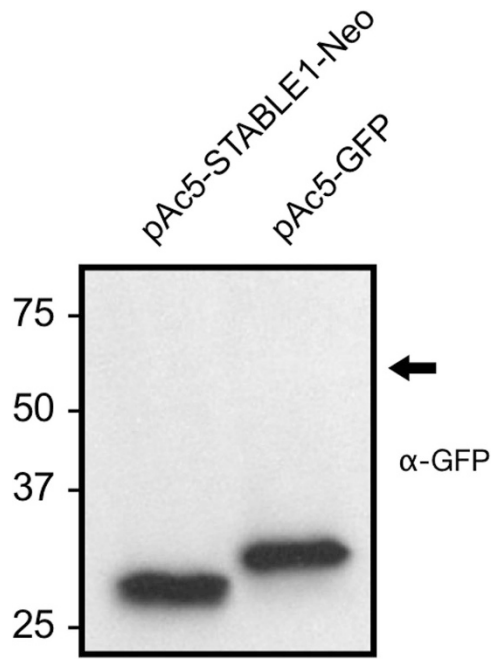

Figure 3 | T2A is correctly processed in *Drosophila* cultured cells. Western blot analysis of S2R+ cells transfected either with pAc5-STABLE1-Neo or pAc5-GFP vectors, probed with anti-GFP antibodies. Expected sized of non-processed GFP-T2A-neo is shown (58kDa; arrow). The slight difference in size between the GFP proteins derived from the two plasmids is caused by additional polylinker-derived amino acids in the case of pAc5-GFP. Molecular weights in kDa are indicated to the left.

with *pAc5-STABLE2-Neo* and compared to *pAc5-GFP* or mock-transfected cells as controls. Cells were treated with 0 (Fig. 5A, D, G), 600 (Fig. 5B, E, H), or 2000 μg/ml (Fig. 5C, F, I) of G418 during four weeks. Cells transfected with *pAc5-STABLE2-Neo* survived G418 treatment at the highest concentration and all surviving cells had detectable levels of GFP and FLAG-mCherry after four weeks of treatment. As expected, a high degree of overlap of the GFP/mCherry signals was observed (Fig 5I insets), although relative intensities also varied. These results suggested that the three proteins were processed correctly.

To verify that T2A processing was efficient, we checked the FLAG-mCherry and GFP products by Western blot analysis (Fig. 6). The GFP proteins encoded by both *pAc5-STABLE1-Neo* and *pAc5-STABLE2-Neo* were similar (27 kDa). The FLAG-mCherry protein was detected by anti-FLAG antibodies and also exhibited the predicted size (30 kDa). No upper bands corresponding to unprocessed fusion peptides were observed. These results demonstrate that dT2A and T2A are processed correctly in *Drosophila* cells and that *pAc5-STABLE2-Neo* can be used to generate stable cell lines that express two independent proteins of choice by selection with G418.

Generation of stable transformants expressing a GFP-fusion protein. As an additional example of the utility of these vectors, we generated a stable cell line that expressed a GFP-fusion with the novel protein CG6522, the *Drosophila* ortholog of the focal adhesion protein Testin. CG6522 (or dTes) contains a PET domain and three zinc-finger containing LIM domains, belonging to the superfamily of PET-LIM proteins. TES associates with cytoskeletal proteins such as Mena, zyxin, talin and actin[35,36] and has been shown to have tumor suppressor properties in mice[41]. By confocal microscopy, cells stably transfected with *pAc5-STABLE1-Neo* alone expressed GFP, which

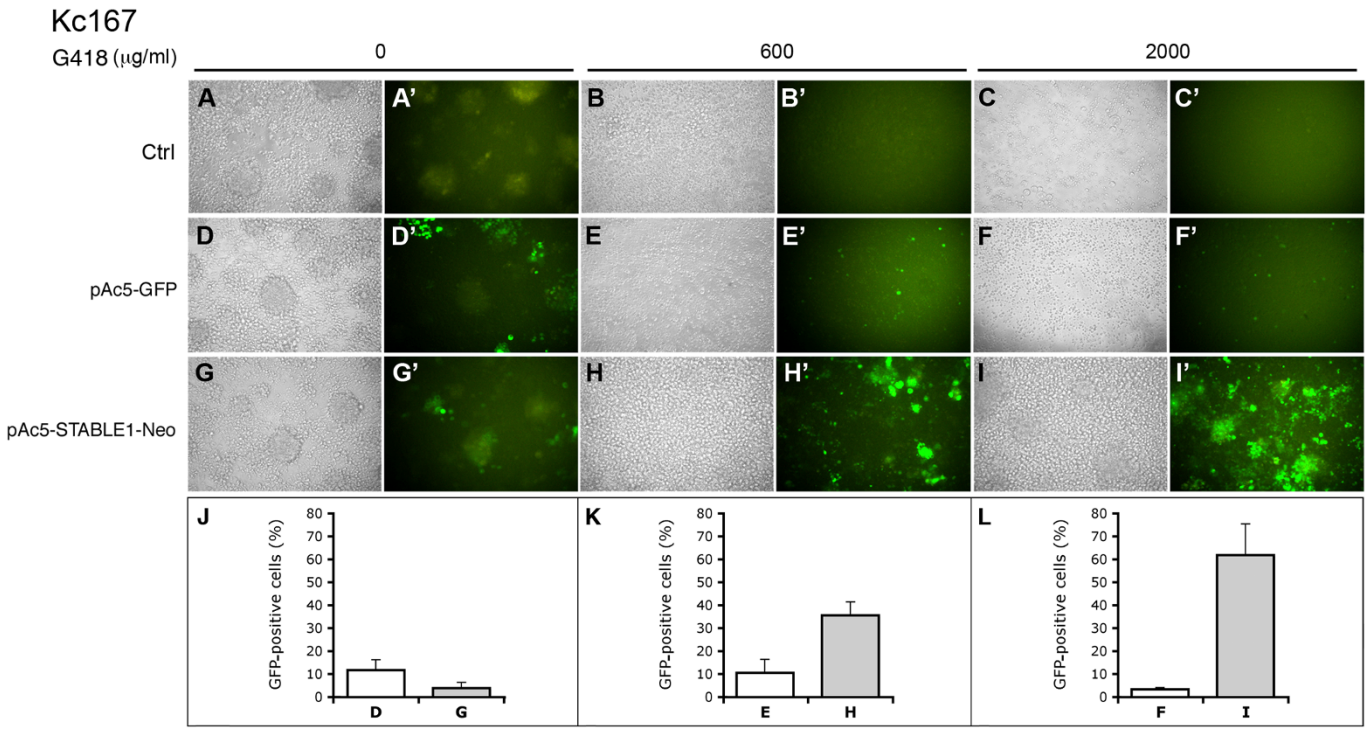

Figure 4 | *pAc5-STABLE1-Neo* confers G418 resistance in Kc167 cells. (A–I) Bright field images of *Drosophila* Kc167 cells that were mock-transfected (A–C), transfected with *pAc5-GFP* (D–F) or transfected with *pAc5-STABLE1-Neo* (G–I). Three days after transfection, G418 was added to the media at concentrations of 0 (A, D, G), 600 (B, E, H) or 2000 μg/ml (C, F, I). (A'–I') Fluorescent images of the same cells from panels A to I showing GFP expression. (J–L) Percentage of cells that are positive for GFP. Letters correspond to panels/treatments directly above each graph. All images were taken after 30 days of treatment with G418.

localised strongly in the nucleus in S2R+ cells, although staining is also visible in the cytoplasm. The cytoplasmic GFP expression did not colocalise extensively with F-actin (Fig. 7A, B). In contrast, stable cells generated with *pAc5-dTES.GFP-Neo* showed that dTes-GFP is highly enriched in the cytoplasm and showed extensive colocalisation with the F-actin cytoskeleton (Fig. 7C, D). Like human TES, this suggests that *Drosophila* dTes protein may also associate with actin. To confirm that the fusion protein is correctly processed, we analysed the cell extracts by Western blot (Fig. 7E). Anti-GFP antibodies revealed a band that corresponds to the predicted size of the dTES.GFP fusion protein (90 kDa from dTES plus 27 kDa from GFP), indicating that the T2A peptide is correctly processed.

Discussion

Here we have used viral-derived T2A sequences to create new vectors for establishing stable *Drosophila* cell lines. The vectors *pAc5-STABLE1-Neo* and *pAc5-STABLE2-Neo* produce dicistronic or tricistronic transcripts, respectively, that are efficiently processed in S2R+ and Kc167 cells. These vectors avoid the need to use cotransfection with a separate vector expressing an antibiotic-resistance gene or the need for a dual-promoter. Both vectors are versatile.

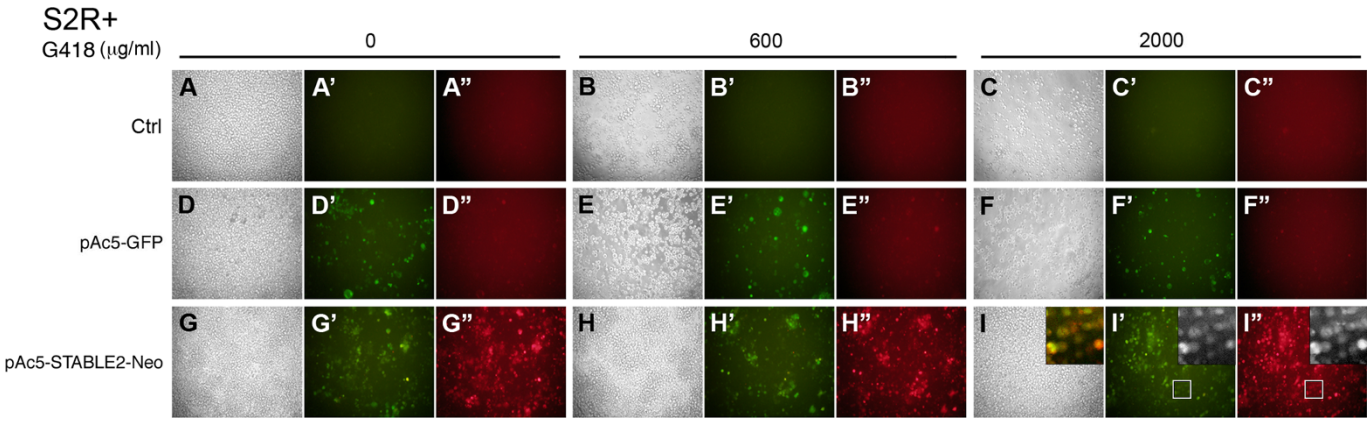

Figure 5 | Expression of two different proteins as well as a selectable marker from a single vector in *Drosophila* cultured cells. (A–I) Brightfield images of *Drosophila* S2R+ cells that were mock-transfected (Ctrl; A–C), transfected with *pAc5-GFP* (D–F) or transfected with *pAc5-STABLE2-Neo* (G–I). Three days after transfection, G418 was added to the media at concentrations of 0 (A, D, G), 600 (B, E, H) or 2000 μg/ml (C, F, I). Fluorescent images of the same cells from panels A–I showing GFP expression (A'–I') or FLAG-mCherry expression (A''–I''). White boxes mark zoomed region shown in inset (panel I, overlay). All images were taken after 30 days of treatment with G418.

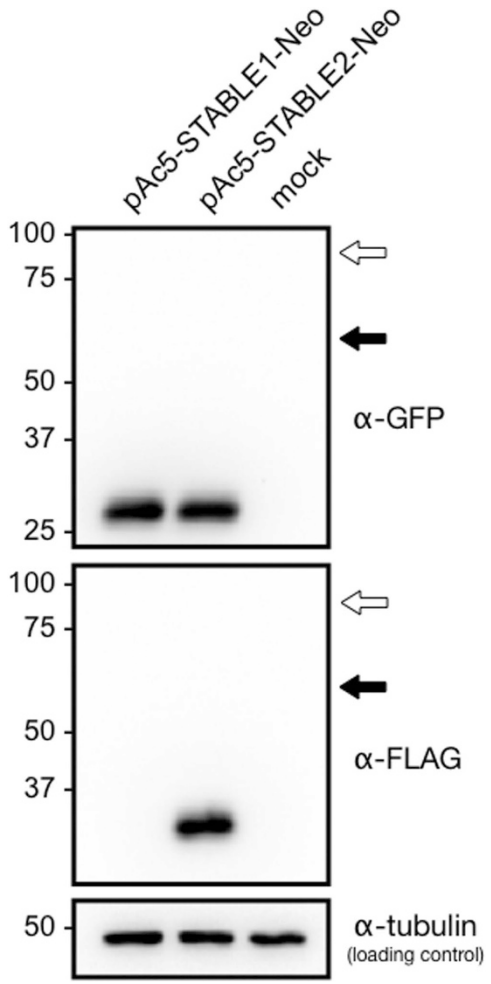

Figure 6 | T2A and dT2A are correctly processed in *Drosophila* cultured cells. Western blot analysis of S2R+ cells transfected either with pAc5-STABLE1-Neo or pAc5-STABLE2-Neo vectors, or only mock-transfected. Observed bands using antibodies against GFP and FLAG (to detect FLAG-Cherry) were consistent with correct processing of the T2A/dT2A sequences. Expected sizes of non-processed FLAG-Cherry-dT2A-GFP-T2A-neo (91kDa; white arrow) or GFP-T2A-neo (58kDa; black arrow) are depicted; none is detected in either case, even in long exposures. Molecular weights in kDa are indicated to the left.

Using *pAc5-STABLE1-Neo* one can choose between making an N-terminal or C-terminal GFP fusion protein or to substitute GFP by any protein of interest. *pAc5-STABLE2-Neo* allows the expression of distinct fluorescent protein fusions, or simultaneous expression of a fluorescent marker (GFP or mCherry) and a protein of interest (by substituting one of the fluorescent protein-encoding modules). This allows visual identification of cells expressing the protein of interest, without worry about the fluorescent protein interfering with protein function. Although all drug-selected cells should express the protein of interest, the co-expressed fluorescent protein may also allow discrimination between high- and low-expressors when coupled with fluorescence-activated cell sorting (FACS). Alternatively, ORFs with different tags could be substituted. Tags such as 6×HIS, GST, or others that facilitate protein purification will make these vectors useful for recombinant protein production for biochemical and structural biology purposes, especially where multi-protein complexes are being studied. Although T2A-processing appears efficient in both S2R+ and Kc167, it is highly recommended to confirm processing of polyproteins by Western blot. Actin 5C-based expression and G418 selection may also be compatible with other insect cell types[42,43], increasing the utility and potential applications of these vectors. Since we have shown that T2A-mediated multicistronic expression is feasible in *Drosophila* cultured cells, a similar strategy may be applied *in vivo* to expand the already powerful molecular genetic toolbox available through fly transgenesis.

G418-based selection is effective and takes 3–4 weeks to establish a stable population. Puromycin selection (mediated by the *pac* gene) has been used successfully for *Drosophila* cell selection when using co-transfection or dual promoter approaches[13,15]. Our earlier attempts to use puromycin selection in combination with the T2A sequence, or with a distinct 2A peptide derived from porcine teschovirus-1 (P2A), were problematic, linked to poor apparent cleavage efficiency (data not shown). Nevertheless, by using extended T2A sequences[44], distinct linker sequences between modules, or placing the Puro^R cassette in the first (5′), rather than last (3′), position, the use of puromycin selection may be possible. Since our vector design allows other selection cassettes to be substituted for Neo^R, using other selection strategies, such as those using hygromycin, or the faster-acting blasticidin[45,46], may give further flexibility and reduce the time to achieve stable populations.

In conclusion, the vectors *pAc5-STABLE1-Neo* and *pAc5-STABLE2-Neo* described here will allow the creation of stable *Drosophila* cultured cells in an efficient way and serve as a flexible platform for future insect-cell based applications.

Methods

DNA constructs. *pAc5.1-V5-His-A* (Invitrogen) and *pAc5.1B-EGFP* (called pAc5-GFP in text; obtained from Addgene; gift of E. Izaurralde[47]) were used in the construction of vectors described here. In *pAc5-STABLE1-Neo* (Fig. 1), GFP is followed by the T2A peptide sequence derived from *Thosea asigna* (EGRGSLLTCGDVEENPGP) and the neomycin resistance gene (Neo^R; confers resistance to G418 in eukaryotic cells; derived from pIRES-Neo) (Invitrogen). mCherry was synthesised (Geneart) and later PCR-amplified to add an N-terminal FLAG epitope and a C-terminal T2A sequence, which has degenerate sequence at the nucleotide level to minimise vector recombination (called dT2A in text). This cassette was cloned into *pAc5-STABLE1-Neo* as an *Eco*RI-*Xba*I fragment, generating *pAc5–STABLE2-Neo*. Organisation of unique restriction sites, ORFs and T2A sequences in pAc5-STABLEs is summarised in Figure 1. Unique sites allow replacement of FLAG-Cherry, GFP, and/or Neo^R, allowing flexibility for new applications. In most cases, PCR amplification and subcloning was used to generate plasmids. Primer sequences and cloning details are available upon request. All vectors were sequence-verified and DNA preparation was done using standard procedures.

Cell culture, transfection, antibiotic treatment and immunofluorescence. *Drosophila* S2R+ cells[37] and *Drosophila* Kc167[48,49] were obtained from the *Drosophila* Genome Resource Center (DGRC) and were cultured in *Drosophila* Schneider's medium (Invitrogen) supplemented with 10% of fetal bovine serum (FBS), 1% of penicillin/streptomycin at 25°C in a humidified incubator.

Cell transfection was performed using the non-liposomal reagent Effectene (Qiagen) following the manufacturer's instructions using a DNA:Enhancer ratio of 1 : 8 and DNA:Effectene ratio of 1 : 3. 25µl of the transfection solution was added to each well in a 96-well plate and 100 µl of medium containing 1×10^5 cells were dispensed per well. All transfections were performed in quadruplicate. After 72 hours, selective medium was added with different concentrations (0, 600, 2000 µg/ml) of neomycin/G418 (Sigma). Every 5–7 days cells were split if necessary and selective media was replaced. After 3 weeks, cells were transferred to 24-well plates. At 4 weeks, cells were visualised using a CKX41 fluorescence microscope using a 20× objective (NA0.40; Olympus) and images captured using a mounted camera (Olympus E330) and identical exposure times (2 sec.) Percentages of GFP-expressing cells among those detected by bright-field were calculated by analysis of 4 fields, using Photoshop and ImageJ software. Longer exposures confirmed that some weak expressors were not detected when using 2 sec. exposure (data not shown).

For immunofluorescence, after 48–72 hours, transfected cells were transferred to ConA-treated coverslips for attachment[50] and fixed with 4% paraformaldehyde, 0.1% Triton X-100 in PBS, and treated as previously described[51] using DAPI (Sigma) or Alexa568-phalloidin (Invitrogen). Confocal imaging was performed using a TCS-SP2 DM-IRE2 microscope with the 63× objective (HCX-PL-APO, NA1.4; Leica).

Western blot analysis. Cells were harvested in 2× Laemmli buffer and lysates were analysed by SDS-PAGE on 10% gels. Antibodies against GFP (Roche) or FLAG epitope (M2; Sigma) were used, followed by HRP-conjugated anti-mouse secondary antibodies (Jackson Immunoresearch). Signals were detected using Supersignal West Pico (Pierce) and the Chemidoc XRS system (BioRad).

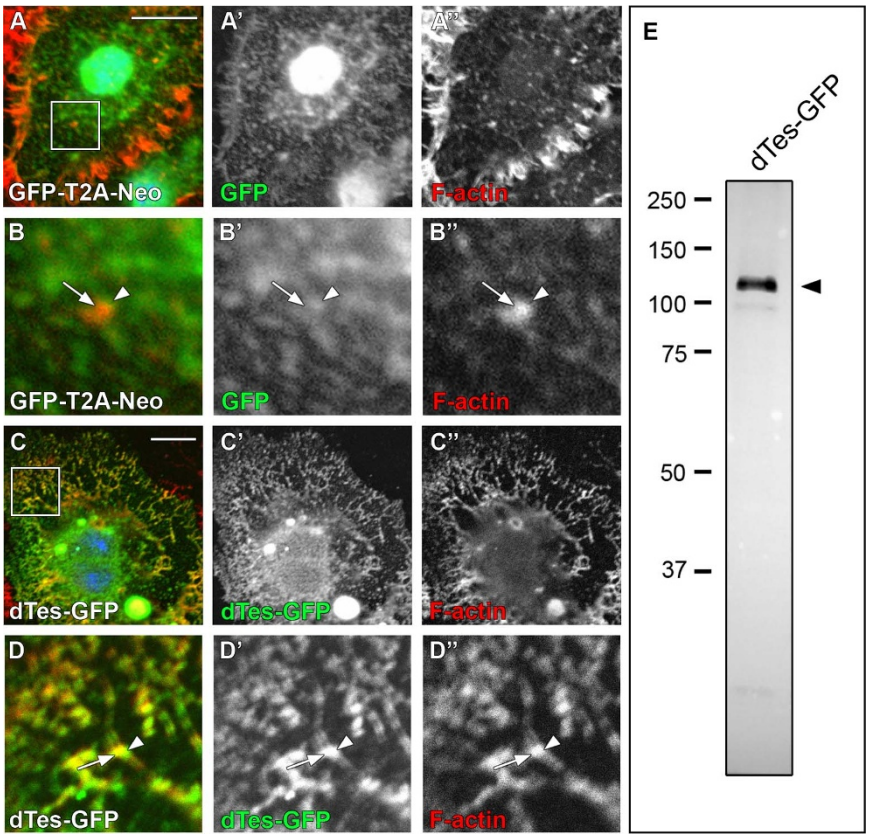

Figure 7 | dTes-GFP colocalises with actin cytoskeleton in S2R+ cells. (A–D) Confocal pictures showing the expression of GFP encoded by *pAc5-STABLE1-Neo* (GFP-T2A-neo; A, B) or the fusion protein encoded by *pAc5–dTes.GFP-Neo* (dTES-GFP; C, D). Cells are stained with Alexa568-Phalloidin (red) to show the F-actin cytoskeleton and with DAPI (blue) to show the nuclei. Grayscale images represent single green (A'–D') or single red channels (A''–D''). B and D show the region indicated in A or C, respectively. Arrowheads in B and D indicate dTes-GFP protein accumulation, while arrows show F-actin. Scale bars in A and C = 8 μm. (E) Western blot analysis of S2R+ cells transfected with *pAc5-dTES.GFP-Neo* and probed with anti-GFP. A single band of expected size is observed. Molecular weights in kDa are indicated to the left.

1. Hamburger, V. Wilhelm Roux: visionary with a blind spot. *J Hist Biol* **30**, 229–238 (1997).
2. Cockrell, A. S. & Kafri, T. Gene delivery by lentivirus vectors. *Mol Biotechnol* **36**, 184–204 (2007).
3. Jarvis, D. L. Baculovirus-insect cell expression systems. *Methods Enzymol* **463**, 191–222 (2009).
4. Mohr, S., Bakal, C. & Perrimon, N. Genomic screening with RNAi: results and challenges. *Annu Rev Biochem* **79**, 37–64 (2010).
5. Castoreno, A. B. *et al.* Small molecules discovered in a pathway screen target the Rho pathway in cytokinesis. *Nat Chem Biol* **6**, 457–463 (2010).
6. Sorensen, H. P. Towards universal systems for recombinant gene expression. *Microb Cell Fact* **9**, 27 (2010).
7. McCarroll, L. & King, L. A. Stable insect cell cultures for recombinant protein production. *Curr Opin Biotechnol* **8**, 590–594 (1997).
8. Cherbas, L., Moss, R. & Cherbas, P. Transformation techniques for *Drosophila* cell lines. *Methods Cell Biol* **44**, 161–179 (1994).
9. Van der Straten, A., Johansen, H., Rosenberg, M., Sweet, R. W. Introduction and constitutive expression of gene products in cultured *Drosophila* cells using hygromycin B selection. *Methods in Molecular and Cellular Biology* **1**, 1–8 (1989).
10. Rio, D. C. & Rubin, G. M. Transformation of cultured *Drosophila melanogaster* cells with a dominant selectable marker. *Mol Cell Biol* **5**, 1833–1838 (1985).
11. Bourouis, M. & Jarry, B. Vectors containing a prokaryotic dihydrofolate reductase gene transform *Drosophila* cells to methotrexate-resistance. *Embo J* **2**, 1099–1104 (1983).
12. Makridou, P., Burnett, C., Landy, T. & Howard, K. Hygromycin B-selected cell lines from GAL4-regulated pUAST constructs. *Genesis* **36**, 83–87 (2003).
13. Iwaki, T., Figuera, M., Ploplis, V. A. & Castellino, F. J. Rapid selection of *Drosophila* S2 cells with the puromycin resistance gene. *Biotechniques* **35**, 482–484, 486 (2003).
14. Koelle, M. R. *et al.* The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67**, 59–77 (1991).
15. Iwaki, T. & Castellino, F. J. A single plasmid transfection that offers a significant advantage associated with puromycin selection in *Drosophila* Schneider S2 cells expressing heterologous proteins. *Cytotechnology* **57**, 45–49 (2008).
16. Segal, D., Cherbas, L. & Cherbas, P. Genetic transformation of *Drosophila* cells in culture by P element-mediated transposition. *Somat Cell Mol Genet* **22**, 159–165 (1996).
17. Rees, S. *et al.* Bicistronic vector for the creation of stable mammalian cell lines that predisposes all antibiotic-resistant cells to express recombinant protein. *Biotechniques* **20**, 102–104, 106, 108–110 (1996).
18. Gurtu, V., Yan, G. & Zhang, G. IRES bicistronic expression vectors for efficient creation of stable mammalian cell lines. *Biochem Biophys Res Commun* **229**, 295–298 (1996).
19. Masoumi, A., Hanzlik, T. N. & Christian, P. D. Functionality of the 5′- and intergenic IRES elements of cricket paralysis virus in a range of insect cell lines, and its relationship with viral activities. *Virus Res* **94**, 113–120 (2003).
20. Cherry, S. & Perrimon, N. Entry is a rate-limiting step for viral infection in a *Drosophila melanogaster* model of pathogenesis. *Nat Immunol* **5**, 81–87 (2004).
21. Carter, J. R., Fraser, T. S. & Fraser, M. J., Jr. Examining the relative activity of several dicistrovirus intergenic internal ribosome entry site elements in uninfected insect and mammalian cell lines. *J Gen Virol* **89**, 3150–3155 (2008).
22. Mizuguchi, H., Xu, Z., Ishii-Watabe, A., Uchida, E. & Hayakawa, T. IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Mol Ther* **1**, 376–382 (2000).
23. Underhill, M. F., Smales, C. M., Naylor, L. H., Birch, J. R. & James, D. C. Transient gene expression levels from multigene expression vectors. *Biotechnol Prog* **23**, 435–443 (2007).
24. Ryan, M. D., King, A. M. & Thomas, G. P. Cleavage of foot-and-mouth disease virus polyprotein is mediated by residues located within a 19 amino acid sequence. *J Gen Virol* **72** (Pt 11), 2727–2732 (1991).
25. Donnelly, M. L. *et al.* Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. *J Gen Virol* **82**, 1013–1025 (2001).
26. Donnelly, M. L. *et al.* The 'cleavage' activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring '2A-like' sequences. *J Gen Virol* **82**, 1027–1041 (2001).
27. de Felipe, P. *et al.* E unum pluribus: multiple proteins from a self-processing polyprotein. *Trends Biotechnol* **24**, 68–75 (2006).

28. de Felipe, P. Skipping the co-expression problem: the new 2A "CHYSEL" technology. *Genet Vaccines Ther* **2**, 13 (2004).
29. Szymczak, A. L. *et al*. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat Biotechnol* **22**, 589–594 (2004).
30. Fang, J. *et al*. Stable antibody expression at therapeutic levels using the 2A peptide. *Nat Biotechnol* **23**, 584–590 (2005).
31. Osborn, M. J. *et al*. A picornaviral 2A-like sequence-based tricistronic vector allowing for high-level therapeutic gene expression coupled to a dual-reporter system. *Mol Ther* **12**, 569–574 (2005).
32. Provost, E., Rhee, J. & Leach, S. D. Viral 2A peptides allow expression of multiple proteins from a single ORF in transgenic zebrafish embryos. *Genesis* **45**, 625–629 (2007).
33. Trichas, G., Begbie, J. & Srinivas, S. Use of the viral 2A peptide for bicistronic expression in transgenic mice. *BMC Biol* **6**, 40 (2008).
34. Shaner, N. C. *et al*. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat Biotechnol* **22**, 1567–1572 (2004).
35. Coutts, A. S., MacKenzie, E., Griffith, E. & Black, D. M. TES is a novel focal adhesion protein with a role in cell spreading. *J Cell Sci* **116**, 897–906 (2003).
36. Garvalov, B. K. *et al*. The conformational state of Tes regulates its zyxin-dependent recruitment to focal adhesions. *J Cell Biol* **161**, 33–39 (2003).
37. Yanagawa, S., Lee, J. S. & Ishimoto, A. Identification and characterization of a novel line of *Drosophila* Schneider S2 cells that respond to wingless signaling. *J Biol Chem* **273**, 32353–32359 (1998).
38. Schneider, I. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. *J Embryol Exp Morphol* **27**, 353–365 (1972).
39. Cherbas, P., Cherbas, L., Lee, S. S. & Nakanishi, K. 26-[^{125}I]iodoponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors. *Proc Natl Acad Sci U S A* **85**, 2096–2100 (1988).
40. Carey, B. W. *et al*. Reprogramming of murine and human somatic cells using a single polycistronic vector. *Proc Natl Acad Sci U S A* **106**, 157–162 (2009).
41. Drusco, A. *et al*. Knockout mice reveal a tumor suppressor function for Testin. *Proc Natl Acad Sci U S A* **102**, 10947–10951 (2005).
42. Lycett, G. J. & Crampton, J. M. Stable transformation of mosquito cell lines using a hsp70:neo fusion gene. *Gene* **136**, 129–136 (1993).
43. Zhao, Y. G. & Eggleston, P. Comparative analysis of promoters for transient gene expression in cultured mosquito cells. *Insect Mol Biol* **8**, 31–38 (1999).
44. de Felipe, P., Luke, G. A., Brown, J. D. & Ryan, M. D. Inhibition of 2A-mediated 'cleavage' of certain artificial polyproteins bearing N-terminal signal sequences. *Biotechnol J* **5**, 213–223 (2010).
45. de la Luna, S., Soria, I., Pulido, D., Ortin, J. & Jimenez, A. Efficient transformation of mammalian cells with constructs containing a puromycin-resistance marker. *Gene* **62**, 121–126 (1988).
46. Kimura, M., Takatsuki, A. & Yamaguchi, I. Blasticidin S deaminase gene from Aspergillus terreus (BSD): a new drug resistance gene for transfection of mammalian cells. *Biochim Biophys Acta* **1219**, 653–659 (1994).
47. Eulalio, A., Huntzinger, E. & Izaurralde, E. GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat Struct Mol Biol* **15**, 346–353 (2008).
48. Cherbas, L., Koehler, M. M. & Cherbas, P. Effects of juvenile hormone on the ecdysone response of *Drosophila* Kc cells. *Dev Genet* **10**, 177–188 (1989).
49. Cherbas, L., Yonge, C. D., Cherbas, P. & Williams, C. M. The morphological response of Kc-H cells to ecdysteroids: Hormonal specificity. *Roux's Archives of Developmental Biology* **189**, 1 (1980).
50. Buster, D. W., Nye, J., Klebba, J. E. & Rogers, G. C. Preparation of *Drosophila* S2 cells for light microscopy. *J Vis Exp* (2010).
51. Sanchez, J. *et al*. Sumoylation modulates the activity of Spalt-like proteins during wing development in *Drosophila*. *J Biol Chem* **285**, 25841–25849 (2010).

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Author contributions

J.D.S. and R.B. conceived the project and wrote the manuscript; J.D.S., M.G. and R.B. planned the experiments and analysed the data; M.G., I.M.-R., S.J., and L.P. performed the experiments.

Additional information

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