© 2017. Published by The Company of Biologists Ltd | Development (2017) 144, 2529-2538 doi:10.1242/dev.147538

TECHNIQUES AND RESOURCES

RESEARCH ARTICLE

Oxygenation and adenosine deaminase support growth and proliferation of *ex vivo* cultured *Drosophila* wing imaginal discs

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ABSTRACT

The *Drosophila* wing imaginal disc has been an important model system over past decades for discovering novel biology related to development, signaling and epithelial morphogenesis. Novel experimental approaches have been enabled using a culture setup that allows *ex vivo* cultures of wing discs. Current setups, however, are not able to sustain both growth and cell-cycle progression of wing discs *ex vivo*. We discover here a setup that requires both oxygenation of the tissue and adenosine deaminase activity in the medium, and supports both growth and proliferation of wing discs for 9 h. Nonetheless, further work will be required to extend the duration of the culturing and to enable live imaging of the cultured discs in the future.

KEY WORDS: *Drosophila*, Development, Wing discs, Culture, Adenosine deaminase, Oxygenation

INTRODUCTION

The *Drosophila* wing imaginal disc has been an important model system over past decades for discovering novel biology related to a plethora of topics, including tissue growth control, pattern formation, epithelial morphogenesis, inter-cellular communication and signaling, and the biophysical interaction of cells (Buchmann et al., 2014; Garcia-Bellido, 2009; Kornberg and Guha, 2007; Restrepo et al., 2014; Worley et al., 2012). Most of this work has been carried out by dissecting, fixing and immunostaining discs from individual animals, either at different stages of development or at varying times after induction of genetic manipulations. These snapshots have been used to reconstruct the temporal progression of events and to deduce cause-effect relationships, but methods to follow the development of individual discs over time are lacking. One such method recently reported is to repeatedly image a wing disc through the larval cuticle at different points of development (Kanca et al., 2014; Nienhaus et al., 2012). Novel approaches would become possible, however, if wing imaginal discs could be cultured *ex vivo* for live imaging. Indeed, several recent studies have developed methods for live imaging of explanted wing discs (Aldaz et al., 2010; Gibson et al., 2006; Kicheva et al., 2007; Landsberg et al., 2009; Mao et al., 2011; Ohsawa et al., 2012; Tsao et al., 2016). Although these studies have found solutions to immobilize and image wing discs, the culture medium has been a key limiting factor because current media only support growth and/or proliferation of wing discs for a few hours.

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Received 25 November 2016; Accepted 11 May 2017

Culture conditions that allow wing discs to remain in a growing and proliferating state *ex vivo* have been sought after for many decades. In 1969, James Robb identified culture conditions that allow wing disc cells to stay alive and metabolically active (producing RNA and protein), but these conditions did not permit cell proliferation (Robb, 1969). In the past few years this topic has received renewed interest. Three recent studies have advanced our understanding and ability to culture wing imaginal discs *ex vivo* (Handke et al., 2014; Tsao et al., 2016; Zartman et al., 2013). Zartman, Restrepo and Basler used response-surface methods to optimize culture conditions so that cells in explanted discs can progress through mitosis for several hours (Zartman et al., 2013). Although progression through mitosis is maintained, under these conditions wing disc cells rapidly lose the ability to enter S phase. Indeed, this was also the key finding by Handke and colleagues, thereby pinpointing entry into S phase as one key challenge remaining to be solved (Handke et al., 2014). A recent report from Henry Sun's lab made a significant step forward, discovering a setup that supports both M-phase and S-phase progression of imaginal discs for 12 h (Tsao et al., 2016). This setup, however, does not support disc growth (Tsao et al., 2016).

We report here culture conditions that allow both growth and proliferation of wing imaginal discs *ex vivo* for 9 h. The combination of growth and proliferation requires oxygenation of the discs, as well as adenosine deaminase activity in the medium. Although the ability to maintain both growth and proliferation is a step forward compared with previously discovered culturing methods, additional work and further improvements will be required to allow longer term cultures as well as live imaging.

RESULTS

A secreted protein promotes S-phase progression in cultured wing discs

To optimize *ex vivo* culture conditions for *Drosophila* wing discs, we first tested whether wing discs become hypoxic when placed into culture medium. We inverted larvae, kept wing discs attached to the carcass but removed other tissues, and placed these into a glass well containing 300 µl of medium. After 3 h of culturing, wing discs showed a marked upregulation of expression of the hypoxia-induced genes *LDH* (*ImpL3*) and *CG11652* (Fig. 1A-A′) (Li et al., 2013), suggesting the wing discs were hypoxic. This was significantly improved by either rocking the dish during incubation, or by placing the tissue and medium into a 2 ml tube rotating head-over-head (Fig. 1A,A′). As tubes rotating head-over-head produced the best suppression of hypoxia, we used this condition for the next experiments presented in Figs 1 and 2.

To find conditions that sustain both wing disc proliferation and growth, we used two separate read-outs for our culture optimization. As a read-out for proliferation, we measured EdU incorporation. Fresh discs show prominent EdU incorporation, whereas simple

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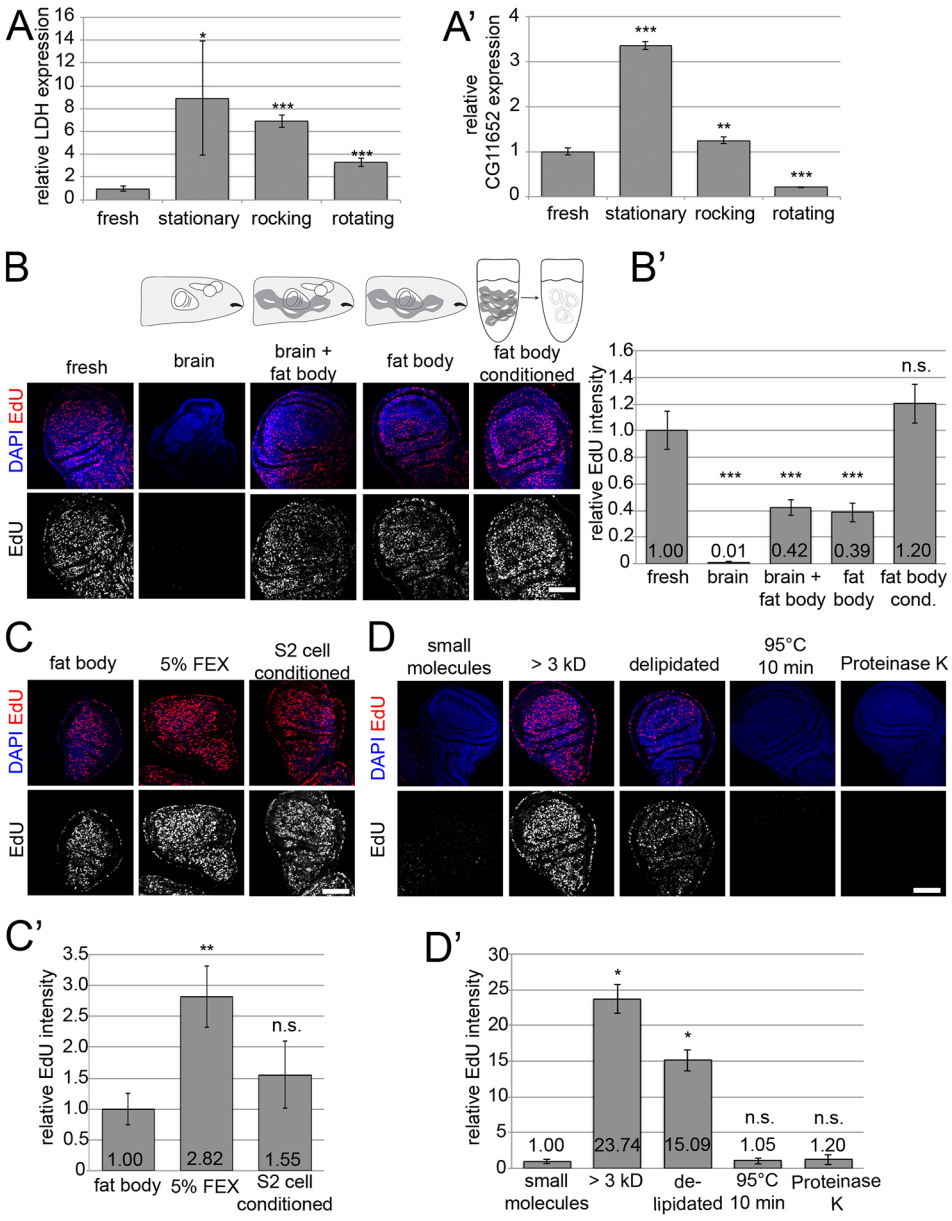

Fig. 1. A secreted protein from the larval fat body, adult flies and S2 cells promotes S-phase progression in cultured oxygenated wing discs. (A,A′) Explanted wing discs become hypoxic unless oxygenated by rocking or rotating the culture, assayed via induction of the hypoxia-response genes *LDH* (A) and *CG11652* (A′). mRNA levels were detected by quantitative RT-PCR, normalized to rp49. Wing discs were cultured for 3 h in 300 µl SFM in a glass well that was either 'stationary' or agitated by gentle 'rocking', or placed in a 2 ml tube that was 'rotating' head-over-head ($n\geq3$). (B,B′) A molecule from the larval fat body stimulates S-phase progression in oxygenated cultured wing discs. Wing discs attached to the larval body wall were cultured by rotating in M3 medium containing 0.1 µM 20E for 5 h in the presence of brain only, fat body only or brain and fat body together, or for 3 h in medium that was conditioned with fat body for 3 h. Wing discs were stained with DAPI (nuclei) and for EdU incorporation (cells in S phase). Representative images in B are quantified in B′ ($n\geq3$). (C,C′) The S-phase-promoting fat body-derived signal is also expressed in adult flies and S2 cells. Wing discs were cultured by rotating in M3 medium containing 0.1 µM 20E with larval fat body for 3 h, with 5% fly extract (FEX) for 3 h or in S2 cell-conditioned medium for 4 h. High levels of EdU incorporation were detected in all three conditions. Representative images in C are quantified in C′ ($n\geq3$). (D,D′) The S-phase-stimulating factor is probably a protein. EdU incorporation in wing discs was assayed after 3 h of rotating culture in M3 medium containing 0.1 µM 20E to which 5% FEX was added that had been either fractionated or inactivated as follows: centrifuged through a protein concentrator column with a 3 kDa cutoff and either the flow-through ('small molecules') or the retentate ('>3 kD') was tested; delipidated by organic extraction to remove lipids; heat inactivated (95°C for 10 min); or proteinase K treated followed by phenol-chloroform extraction. Representative images in D are quantified in D′ ($n\geq3$). Scale bars: 75 µm. Error bars indicate s.d. *P<0.05, **P<0.01, ***P<0.001 using Student's t-test.

culture conditions usually lead to barely detectable levels of EdU incorporation (Fig. S1A,A′). We decided to optimize culture conditions using EdU incorporation as a proliferation readout for two reasons. First, we noticed that many explant conditions that cause wing discs to become negative for EdU incorporation (Fig. S1A) are capable of keeping the discs phospho-Histone 3 (PH3) positive (Fig. S1B). Hence, it appears it is more difficult *ex vivo* to maintain progression through S phase than progression through mitosis, as also observed by others (Handke et al., 2014). Second, unlike phospho-Histone which is a 'static' readout, EdU incorporation not only indicates that cells are in S phase, but also that they are progressing through S phase because they are actively replicating DNA and

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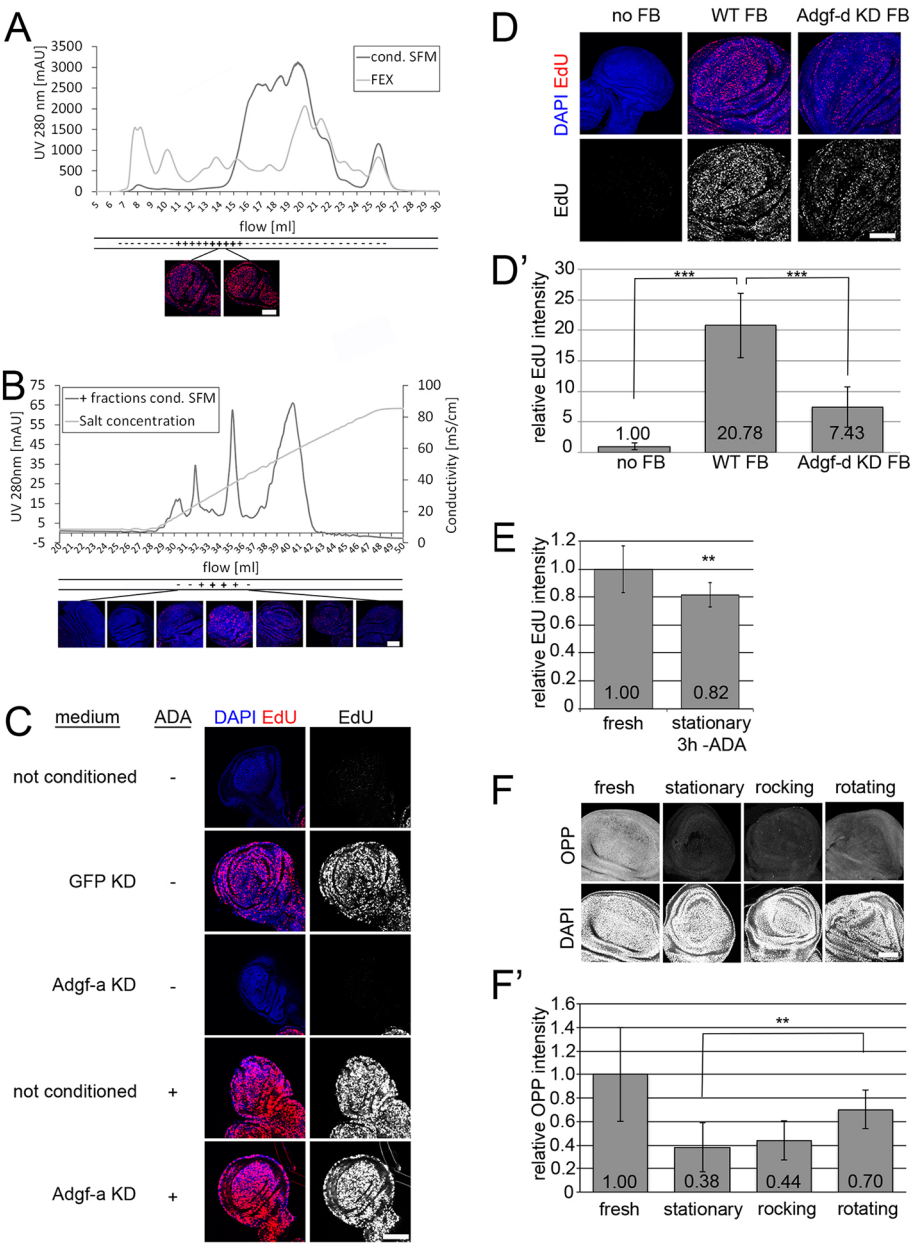

Fig. 2. Adenosine deaminase promotes EdU incorporation in cultured wing discs. (A,B) Fractionation of fly extract (FEX) and conditioned SFM identifies a peak with S-phase-promoting activity. (A) Fly extract and conditioned SFM were first fractionated on a Superdex75 size-exclusion column, yielding four fractions that promote high levels (bold '+') of EdU incorporation, six fractions with medium ('+') and all other tested fractions with low ('–') levels of EdU incorporation after 1.5 h of rotating wing disc culture in M3 medium containing 0.1 µM 20E. (B) The conditioned SFM fractions promoting high EdU incorporation were pooled and fractionated again on an ion-exchange column, eluted with increasing NaCl concentrations, identifying two fractions that promote high EdU incorporation levels. (C) Adgf-a secreted from S2 cells or purified adenosine deaminase from calf intestine (ADA) promotes S-phase progression in cultured wing discs. Discs were rotated for 5 h in either fresh M3 medium ('not conditioned') or M3 medium conditioned by S2 cells treated with either non-targeting GFP dsRNA ('GFP KD') or Adgf-a dsRNA ('Adgf-a KD'), either supplemented or not supplemented with purified adenosine deaminase (ADA). All media were supplemented with 0.1 µM 20E and 1× pen/strep. (D,D′) The fat body-derived signal that promotes EdU incorporation in cultured wing discs is Adgf-d. Discs were rotated for 3 h in 300 µl medium (M3+0.1 µM 20E+1× pen/strep) together with fat bodies from 30 control (wild type) or Adgf-d knockdown larvae (Adgf-d KD). (D) Representative images. (D′) Quantification of EdU intensity relative to a sample cultured in the absence of fat bodies ($n \geq 7$). (E) ADA is not required for wing disc S-phase progression in stationary cultures where the medium is not mixed. Quantification of EdU incorporation in freshly dissected wing discs or wing discs cultured stationary for 3 h in medium (Schneider's+10 µg/ml insulin+2.5 nM 20E+4× pen/strep) lacking ADA ($n \geq 8$). (F,F′) Mixing of the medium is required to maintain protein translation in cultured wing discs, assayed via incorporation of O-propargyl-puromycin (OPP). Wing discs were cultured when stationary, rocking or rotating for 18 h in Robb's medium containing 8.3 ng/ml ADA, 2.5 nM 20E, 10 µg/ml insulin and 4× pen/strep. (F) Representative images for each condition. (F′) Quantification of average signal intensity of multiple discs ($n \geq 8$). Scale bars: 75 µm. Error bars indicate s.d. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ by Student's *t*-test.

incorporating the EdU nucleotide. We quantified EdU staining by integrating the total EdU signal intensity in the wing and normalizing to area (Fig. S1A′); however, quantification of EdU area also produces similar results and the two measurements correlate very tightly (Fig. S1A″). As a real-time readout for growth, we used O-propargyl-puromycin (OPP) incorporation, which assays rates of ongoing translation via incorporation of a puromycin analog into nascent polypeptides (Fig. S1C,C′).

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To test whether oxygenation plus an appropriate basal medium are sufficient to allow S-phase progression in cultured wing discs, we used the rotating setup and cultured discs in different basal media for 3 h prior to EdU addition. Regardless of basal medium composition, wing discs in culture quickly lose EdU incorporation despite oxygenation (Fig. S1D).

We reasoned that one or more factors might be missing from our culture conditions to support S-phase progression. We tested whether larval tissues could provide this factor. We co-cultured discs attached to the body carcass together with the brain, the fat body, or the brain and the fat body simultaneously, and found that the fat body is potent at promoting wing disc S-phase progression (Fig. 1B). As the fat body and the wing discs are not physically attached in this setup, we tested whether the S-phase-promoting signal is secreted. To achieve this, we conditioned medium by isolating fat bodies from early 3rd instar larvae, incubating them for 3 h in the medium and then removing the fat bodies. Wing discs cultured in this fat body-conditioned medium showed prominent EdU incorporation (Fig. 1B,B′), suggesting that the S-phase-promoting signal is indeed secreted from the fat body and stable enough to allow conditioning.

We sought to perform a biochemical fractionation of the conditioned medium to identify the S-phase-promoting factor. As fat bodies are tedious to isolate, we searched for a more abundant source of this factor by testing adult female fly extract (FEX) as well as serum-free medium (SFM) conditioned by *Drosophila* S2 cells. Addition of either 5% FEX or S2 cell-conditioned SFM to the *ex vivo* cultures was able to promote wing disc EdU incorporation to levels similar to those of discs cultured in the presence of fat bodies (Fig. 1C,C′). This suggests that the S-phase stimulating factor is produced not only by the larval fat body, but also by adult female flies and cultured S2 cells.

To characterize the biochemical features of this S-phase promoting factor, we either heat inactivated or fractionated FEX (Fig. 1D,D′). Using protein concentrator columns with a 3 kDa size cutoff, we separated FEX into fractions containing small (<3 kDa) or large (>3 kDa) molecules and found that the S-phase-promoting factor is larger than 3 kDa (Fig. 1D,D′). We excluded the possibility that the factor is a lipid because delipidated FEX could still promote EdU incorporation (Fig. 1D,D′). In contrast, both heat inactivation and proteinase K treatment abolished the activity of the factor, suggesting that the S-phase-stimulating factor is a protein (Fig. 1D,D′).

Adenosine deaminase promotes S-phase progression in cultured wing discs

FEX and S2 cell-conditioned SFM are complex mixtures of proteins. To isolate the S-phase-promoting protein, we fractionated FEX and conditioned SFM on a size exclusion Superdex 200 HR 10/30 column (Fig. 2A) and tested the fractions for the ability to promote wing disc EdU incorporation. This identified one peak with S-phase-promoting activity that eluted at the same volume in both the FEX and conditioned SFM fractionations (Fig. 2A). Mass spectrometry analysis of this peak, however, revealed that it still contained hundreds of proteins, so we pooled the active fractions and fractionated them again on a Mono QTM 5/50 Gl ion-exchange column (Fig. 2B). This again identified a single peak of activity, which was analyzed by mass spectrometry, yielding a list of ∼100 candidate S-phase-promoting proteins. We then searched for the responsible factor by knocking down candidate genes with dsRNA in S2 cells, and testing whether the knockdown abolished the ability of the cells to condition medium. This identified adenosine deaminase-related growth factors (Zurovec et al., 2002) as the responsible factor (adgf-a, which is expressed in S2 cells and adgf-d, which is expressed in fat body). Medium conditioned by S2 cells treated with a non-targeting GFP dsRNA was able to promote S-phase progression in cultured wing discs (‘GFP KD’, Fig. 2C), whereas medium conditioned with Adgf-a knockdown S2 cells was not (‘Adgf-a KD’, Fig. 2C). Likewise, wild-type fat body was able to stimulate S-phase progression of co-cultured wing discs (‘WT FB’, Fig. 2D,D′), whereas Adgf-d knockdown fat bodies had significantly reduced activity (Fig. 2D,D′). These experiments show that Adgfs are required to promote wing disc S-phase progression, but they do not test whether they are sufficient. To test this, we added commercially available adenosine deaminase (ADA, the mammalian functional homolog of Adgf) purified from calf intestine to non-conditioned culture medium. Purified ADA was sufficient to promote EdU incorporation of wing discs cultured *ex vivo* for 5 h either in non-conditioned medium, or in medium conditioned with Adgf-a knockdown S2 cells (Fig. 2C).

As both larval and imaginal tissues express endogenous Adgfs, we wondered why supplementation of the culture medium with ADA is necessary. One possibility is that by mixing the medium, the endogenously secreted Adgfs are diluted. To test this, we incubated wing discs in medium lacking ADA, and found that in the absence of rotation or mixing they remain EdU positive (Fig. 2E). In contrast to cell proliferation, however, we noticed that cell growth requires mixing of the medium. When discs are cultured in a stationary setup, protein translation, assayed via incorporation of O-propargyl-puromycin (OPP), is strongly impaired compared with a setup where the medium is mixed (Fig. 2F). In addition, most discs in a stationary setup have OPP incorporation that is not uniform throughout the disc (Fig. S2A), and this is also renormalized when the culture is mixed (Fig. S2A), so that OPP incorporation is uniform as in fresh discs. This may be due to nutrients becoming limited in the absence of mixing, and is also in agreement with findings by the Sun lab that in their setup the wing discs proliferate but do not grow (Tsao et al., 2016). In summary, mixing of the medium is required to promote OPP incorporation, and consequently supplementation with ADA is required to promote EdU incorporation. Hence, we use this setup for all subsequent experiments presented here.

A combination of oxygenation and ADA can maintain S-phase progression in wing discs cultured for 3 h

As head-over-head rotation of tubes containing the cultured tissues is quite harsh on the wing discs, we aimed to develop a setup that permits stirring and oxygenation of the medium without physically agitating the wing discs. To achieve this, we placed the culture medium in a small glass beaker with a stir bar, and suspended into the medium a ‘basket’ consisting of a 2 ml plastic tube where the bottom was cut off and replaced with a metal mesh (Fig. 3A). The inverted larvae carrying the wing discs were then placed into the basket, so that they are bathed in medium that is stirring and hence oxygenated, without being damaged. We confirmed that this setup adequately oxygenates the discs for up to 9 h by quantitative RT-PCR, assaying the expression of hypoxia-induced genes *LDH* (Fig. 3B) and *CG11652* (Fig. 3B′). If the stirring is turned off, wing discs quickly become hypoxic (3 h) and remain hypoxic for the rest of the *ex vivo* culturing (‘− stirring’, Fig. 3B,B′). In contrast, when the medium is mixed by turning on the stir plate underneath the culture setup, wing discs no longer induce expression of *LDH* or *CG11652*, even after 9 h of culturing (‘+ stirring’, Fig. 3B,B′).

We next confirmed that ADA is required for EdU incorporation in this ‘stirring’ setup, as it was in the head-over-head rotation setup. Indeed, as expected, in the absence of ADA, wing discs quickly

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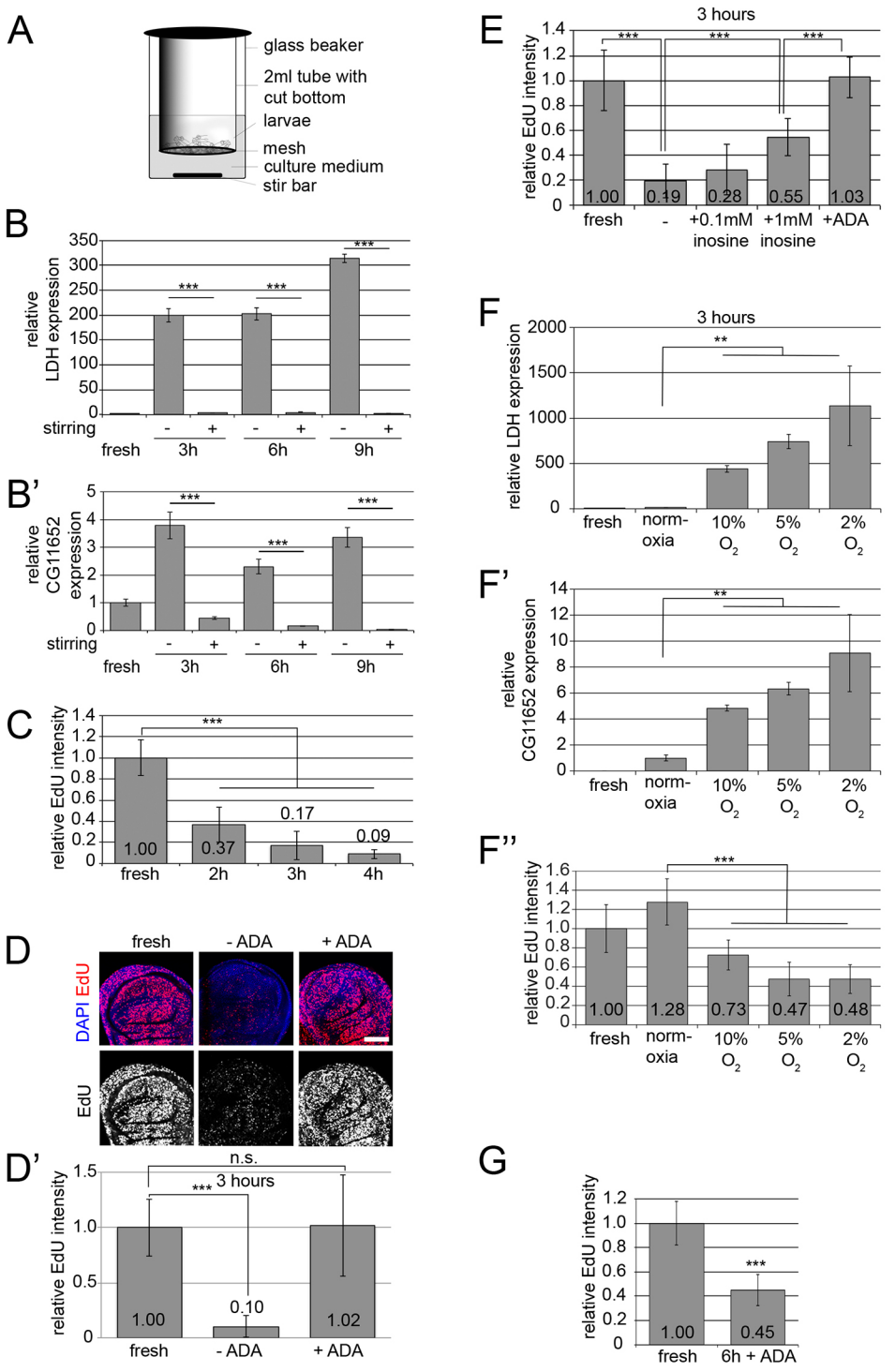

Fig. 3. Oxygenation and ADA maintain proliferation and growth of cultured wing discs for 3 h. (A) Illustration of the stirring culture setup used for all the following experiments. Six larvae are placed in a cage that is suspended in 3 ml of medium stirred with a stir bar. (B,B′) This 'stirring setup' allows oxygenation of discs for at least 9 h. mRNA levels of hypoxia-induced genes *LDH* (B) and *CG11652* (B′) were measured by quantitative RT-PCR, normalized to rp49, in wing discs that were either freshly dissected or cultured for indicated times in Schneider's medium containing 1× pen/strep, 10 µg/ml insulin and 2.5 nM 20E with the setup shown in A, with the stir bar either stirring or still. (C) Oxygenation is not sufficient for S-phase progression in wing discs cultured using the 'stirring' setup shown in A. Wing disc EdU incorporation was measured after indicated culture times in Schneider's medium containing 10 µg/ml insulin, 2.5 nM 20E and 4× pen/strep ($n \geq 8$). (D,D′) ADA is required for S-phase progression in a 3 h stirring disc culture, even if the tissue itself is not agitated. EdU incorporation in wing discs was measured after 3 h of stirring the culture in Schneider's containing 10 µg/ml insulin, 2.5 nM 20E, 4× pen/strep and 8.3 ng/ml ADA. (D) Confocal images of representative discs from each sample. (D′) Average EdU intensity quantified from ≥7 discs/sample. (E) Both inosine production and adenosine detoxification by ADA promote S-phase progression in cultured wing discs. Average EdU incorporation in discs cultured with stirring for 3 h in Schneider's medium containing 10 µg/ml insulin, 2.5 nM 20E, 4× pen/strep and indicated concentrations of inosine or 8.3 ng/ml ADA are shown. $n \geq 5$. (F-F″) Oxygenation is required for S-phase progression in cultured wing discs. Stirring cultures were placed in a hypoxia incubator to regulate atmospheric oxygen concentrations. mRNA levels of hypoxia-inducible genes *LDH* (F) and *CG11652* (F′), and EdU incorporation (F″) were analyzed after 3 h of stirring the culture at indicated oxygen concentrations in M3 medium containing 10 µg/ml insulin, 5 nM 20E, 1.6 nM JH, 1× pen/strep and 8.3 ng/ml ADA. $n \geq 8$. (G) Oxygenation and ADA are not sufficient for normal levels of EdU incorporation after 6 h of disc culture. Average EdU incorporation in discs cultured with stirring for 6 h in Schneider's medium containing 10 µg/ml insulin, 2.5 nM 20E, 4× pen/strep and 8.3 ng/ml ADA is shown. $n \geq 5$. Scale bar: 75 µm. Error bars indicate s.d. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ by Student's *t*-test.

become EdU negative if the medium is stirred, reaching less than 10% of fresh disc levels after 4 h of culture (Fig. 3C). Excitingly, addition of ADA fully restores EdU incorporation in discs cultured for 3 h to the same level as fresh discs (Fig. 3D,D′).

Adenosine deaminase is an enzyme that converts adenosine to inosine (Zurovec et al., 2002). Adenosine, besides being a structural component of DNA, is released by cells upon stress and is toxic at high concentrations (Hasko et al., 2002; Marunouchi and Miyake, 1978; Zurovec et al., 2002). Inosine is an intermediate in nucleotide biosynthesis pathways, and can be taken up from the medium to generate nucleic acids (Johnson et al., 1980). ADA could thus be beneficial for EdU incorporation either because it detoxifies adenosine, or because it generates inosine, or both. To distinguish between these options, we assayed EdU incorporation of wing discs cultured in medium containing different concentrations of inosine. Supplementation of the medium with 0.1 mM inosine, corresponding to the amount of inosine generated by fully converting all the adenosine present in Schneider's medium, did not promote S-phase progression to the same extent as ADA (Fig. 3E). However, inosine did promote S-phase progression in a

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dose-dependent manner, and 100 times that amount, 10 mM, was able to fully rescue S-phase progression in the absence of ADA (Fig. 3E; Fig. S2B). This suggests that the beneficial effect of ADA on EdU incorporation may be the inosine production, although we cannot exclude that adenosine detoxification is also important.

Stirring of the medium not only oxygenates the discs (Fig. 3B,B′) but also leads to mixing of the nutrients, which might otherwise become locally depleted in the vicinity of the tissues. To test specifically whether oxygenation is required for EdU incorporation, we cultured discs using the stirring condition but placed the entire setup in a hypoxia incubator, thereby allowing us to regulate the oxygen levels in the air (Fig. 3F-F″). Progressive reductions in oxygen levels down to 2% lead to progressive induction of hypoxia, assayed via expression of *LDH* and *CG11652* (Fig. 3F,F′), and a concomitant reduction in EdU incorporation (Fig. 3F″), showing that oxygen is indeed required by wing discs to maintain a proper S-phase progression.

Having achieved good levels of EdU and OPP incorporation for 3 h, we next tested whether we could extend the time of *ex vivo* culturing. Despite oxygenation and ADA, at 6 h of culturing, EdU incorporation was 45% the level of fresh discs (Fig. 3G), indicating that additional parameters need optimization.

Optimized Insulin, 20E and JH levels ensure growth and proliferation in discs cultured for 9 h

In vivo, tightly regulated hormonal signals coordinate growth and proliferation of wing discs. We reasoned that optimized hormone levels are also likely required for discs to grow and proliferate in culture. One important anabolic hormone is insulin. To assay insulin signaling activity in cultured discs, we dissected and lysed wing discs after culturing, and assayed phosphorylation of the downstream targets S6K and Akt by immunoblotting. We found that wing discs cultured in medium lacking insulin quickly shut off insulin signaling. Phosphorylation of S6K and Akt decreased already after 1 h of culture, and they were below the detection limit after 16 h of culture (Fig. 4A). Addition of insulin to the culture medium prevented this drop, with 10 µg/ml insulin sustaining pS6K and pAkt for 16 h at levels similar to those of fresh discs (Fig. 4A). As expected, insulin also promoted protein translation (OPP incorporation) in cultured discs (Fig. 4B,B′). Hence, we use 10 µg/ml insulin in our culture medium in all subsequent experiments.

Drosophila has two major lipophilic hormones that interact to regulate growth and development: the steroid hormone 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH). The molecular function of 20E has been extensively studied. 20E regulates the timing of development, with temporal peaks in 20E concentration triggering larval transitions and metamorphosis (Hodgetts et al., 1977). In contrast, less is known about the function of JH. JH has been proposed to interact with, and fine-tune the effects of 20E (Beckstead et al., 2007). We started optimizing 20E and JH levels by measuring EdU incorporation of discs cultured for 6 h in the presence of 2.5 nM 20E, a concentration similar to that found in early 3rd instar larvae (Fig. 4C-C′) (Hodgetts et al., 1977). We compared this with EdU incorporation of discs cultured at high levels of 20E (25 nM) in the presence or absence of high JH levels (5 µM). Intriguingly, a combination of high 20E and high JH promotes S phase best at 6 h of culturing, yielding levels of EdU incorporation that are similar to those of fresh discs (Fig. 4C,C′). This culture medium also sustains high levels of OPP incorporation in discs cultured for 6 h (Fig. 4D,D′). Unfortunately, however, high 20E and JH levels do not promote S-phase progression in discs cultured for longer time periods, with EdU incorporation dropping dramatically to 20% of fresh disc levels at 9 h of culturing (Fig. 4E). Furthermore, we observed that high concentrations of 20E and JH occasionally lead to premature eversion of the discs, suggesting that these hormone levels are too high for the proliferative phase of disc development. We therefore tested whether we could find a combination of 20E and JH concentrations that was closer to physiological levels and better suited to maintaining S-phase progression in 9 h cultures. We performed a laborious two-dimensional screen, titrating the two hormones against each other, and found that a combination of 5 nM 20E with 1.6 nM JH yields highest EdU incorporation in discs cultured for 9 h (Fig. 4F). Although these lower hormone concentrations are somewhat less effective compared with the higher ones at promoting EdU incorporation at 6 h (Fig. S3A), they are more effective at later time points (9 h, Fig. 4F), suggesting a possible time-dependent trade-off whereby higher hormone levels boost proliferation rates early in culture, but are unable to maintain proliferation in the longer term. This condition also maintains good OPP incorporation rates in wing discs (‘5 nM 20E+1.6 nM JH’; Fig. 4D′; Fig. S3B). Correspondingly, wing discs increase in mass during *ex vivo* culturing in these conditions, quantified by comparing total protein amounts of the left and right wing discs prior to and after culture, respectively (Fig. S3C). Wing discs in these culture conditions also retain phospho-histone H3 staining, but not at the level of fresh discs (Fig. S3D,D′). In summary, our optimized culture setup (stirring in M3 medium+8.3 ng/ml ADA+10 µg/ml insulin+5 nM 20E+1.6 nM JH+1× pen/strep, Table 1) does a reasonable job in maintaining both EdU incorporation and OPP incorporation in explanted wing discs for 9 h (Fig. 4G; Fig. S3B).

As some of the parameters (e.g. hormones and oxygenation) were optimized for promoting EdU incorporation, we tested whether they are also required for OPP incorporation by singly removing each component from our optimized setup. This revealed that oxygen, but not JH, 20E or ADA, is required for OPP incorporation (Fig. S3E,F). We noticed our optimized setup is not capable of sustaining the full proliferative capacity of wing discs, with EdU incorporation rates at 9 h of culture dropping to 44% of those of freshly dissected discs (Fig. 4G). At longer timepoints, EdU incorporation drops even further to 9% of fresh discs at 18 h of culture (Fig. S3G). We do not believe this is due to accumulation of a toxic metabolite, nor depletion of a limiting factor in the medium, because replacing the medium every 3 h does not improve EdU incorporation at 9 h. This suggests one or more growth factors and/or nutrients is still missing in our medium. We extensively searched for factors that need to be added to the medium, but have not yet found any that reproducibly improve EdU incorporation at 9 h of culturing. A summary of all components tested so far, with the concentrations used, is provided in Table S1.

DISCUSSION

We describe culture conditions that support both growth and proliferation of wing imaginal discs *ex vivo* for 9 h. Although previous reports described conditions that support proliferation for a longer period of time (Tsao et al., 2016), to our knowledge this is the first time both growth and proliferation can be achieved simultaneously for wing discs *ex vivo* for 9 h. One key finding in our opinion is that not only the medium composition matters, but also the physical setup of the culture plays an important role. For example, we find that mixing of the medium to achieve oxygenation of the tissues is important (Fig. 3). Likewise, the presence of

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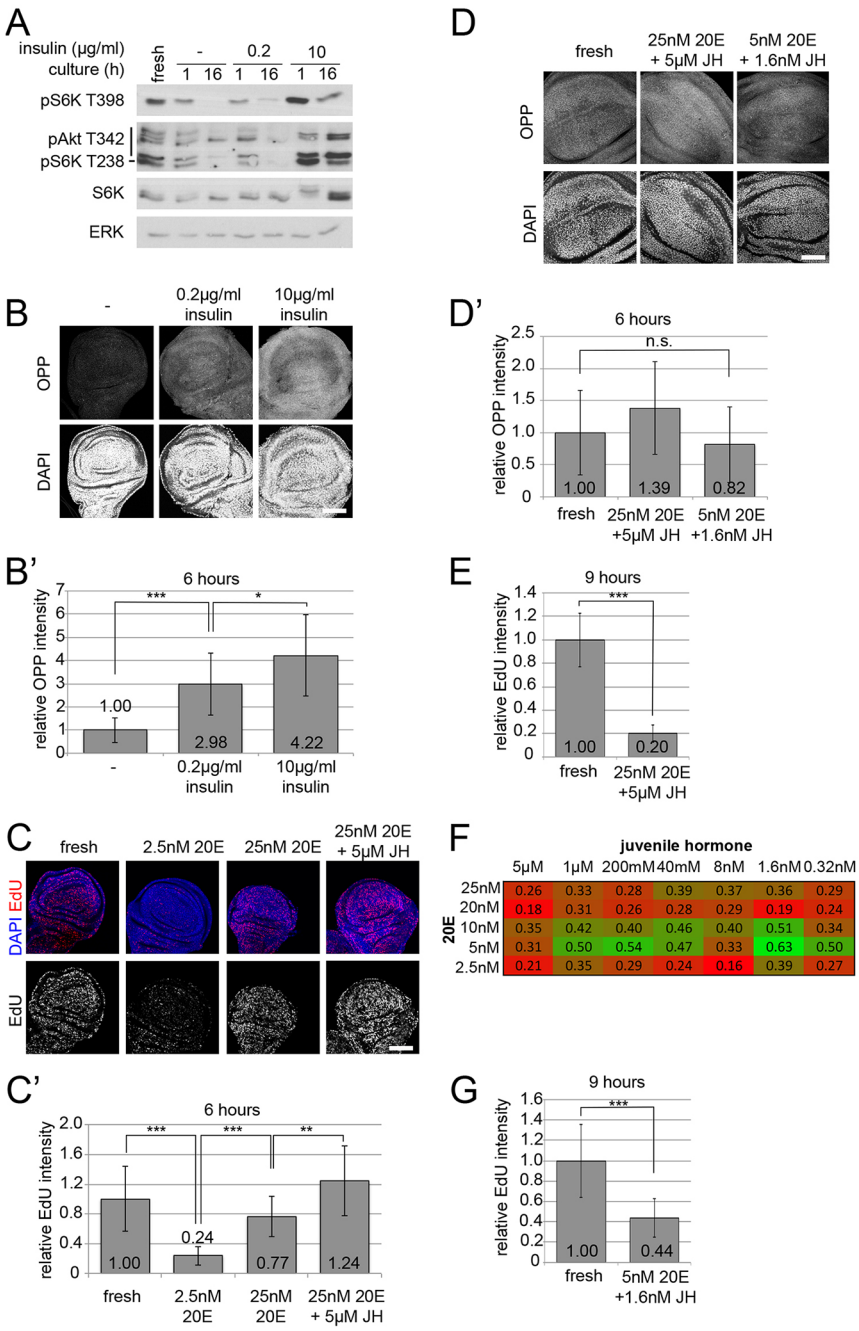

Fig. 4. Optimization of insulin, 20E and JH levels allows normal growth and proliferation in cultured discs for at least 6 h. (A) Optimization of insulin levels in the culture medium to maintain active insulin signaling in discs after 16 h of culture. Phosphorylation of S6K (T398) and Akt (T342) in discs cultured for indicated times in the presence or absence of insulin at indicated concentrations were analyzed by immunoblotting. ERK was used as a loading control. Discs were either freshly dissected (fresh) or cultured with rocking in Robb's medium containing 2.5 nM 20E, 8.3 ng/ml ADA and 4× pen/strep. (B,B′) Insulin promotes protein synthesis in cultured wing discs. OPP incorporation in discs was visualized (B) and quantified (B′, $n \geq 5$) after 16 h of rotating the culture in Robb's medium containing 8.3 ng/ml ADA, 4× pen/strep and insulin at indicated concentrations. (C,C′) Superphysiological concentrations of 20E and JH promote robust S-phase progression in 6 h cultured discs. EdU incorporation in discs was visualized (C) and quantified (C′, $n \geq 8$) after 6 h of stirring the culture in M3 medium containing 10 µg/ml insulin, 8.3 ng/ml ADA, 1× pen/strep and indicated concentrations of 20E and JH. (D,D′) Superphysiological concentrations of 20E and JH promote robust protein translation in 6 h cultured discs. OPP incorporation in fresh discs or discs cultured with stirring for 6 h in M3 medium containing 10 µg/ml insulin, 8.3 ng/ml ADA, 1× pen/strep, 25 nM 20E and 5 µM JH and indicated concentrations of 20E and JH was visualized (D) and quantified (D′, $n \geq 8$). (E) Superphysiological concentrations of 20E and JH do not maintain normal levels of S-phase progression in discs cultured for 9 h. Average EdU incorporation in discs cultured with stirring for 9 h in M3 medium containing 10 µg/ml insulin, 25 nM 20E, 5 µM JH, 8.3 ng/ml ADA and 1× pen/strep is shown. $n=12$. (F) Two-dimensional screen, titrating 20E against JH, identifies optimal concentrations for promoting S-phase progression in wing discs cultured for 9 h. 20E and JH were titrated against each other at indicated concentrations in 9 h stirring cultures using M3 medium containing 10 µg/ml insulin, 8.3 ng/ml ADA and 1× pen/strep. Average EdU intensity of cultured discs relative to fresh discs is shown. A combination of 5 nM 20E and 1.6 nM JH yields strongest EdU incorporation. (G) Optimized culture conditions allows S-phase progression of wing discs cultured for up to 9 h. Average EdU incorporation in discs cultured with stirring for 9 h in M3 medium containing 10 µg/ml insulin, 5 nM 20E, 1.6 nM JH, 8.3 ng/ml ADA and 1× pen/strep is shown. $n \geq 9$. Scale bars: 75 µm. Error bars indicate s.d. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ by Student's *t*-test.

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adenosine deaminase in the medium is only required if the medium is stirred, and it is not required in a stationary setup (Fig. 2). In contrast, we tried multiple different basal media, such as Schneider's, M3, Grace's or Robb's, and did not observe major differences between them.

We identified adenosine deaminase-related growth factors (Adgfs) being actively secreted by fat bodies or S2 cells and necessary for S-phase progression in cultured wing discs. Interestingly, Adgfs were first identified by Michal Zurovec in Peter Bryant's lab as growth factors required to promote survival and cell cycle progression of the wing imaginal disc cell line Cl.8^{+} (Zurovec et al., 2002). Thus, the requirement for Adgfs is logical in retrospect. It is possible that adenosine deaminase is one of the key activities provided by the FBS or FEX used by other groups in their culture setups. *Drosophila* has six Adgfs with predicted deaminase activity, although Adgf-A and Adgf-D appear to be more active than Adgf-E (Zurovec et al., 2002). *In vivo*, Adgfs are required for viability. Adgf-A mutants are lethal, with only 2% of mutants reaching adulthood (Dolezal et al., 2005), and Adgf-C and Adgf-D double mutants are semi-lethal, suggesting a redundant function for this family of enzymes. It will be interesting to study the roles of circulating adenosine and inosine on imaginal disc proliferation *in vivo*.

Although the ability to maintain both growth and proliferation *ex vivo* for 9 h is a step forward compared with previously discovered culturing methods, additional work and further improvements will be required to allow longer term cultures as well as live imaging. With our optimized setup, EdU incorporation rates at 9 h of culture are 44% of fresh discs. We believe this reflects the fact that our culture medium is still missing a nutrient or growth factor that we have not been able to identify thus far. By frequently replacing the medium, we can exclude the possibility that the progressive drop in EdU incorporation rates is due to accumulation of a toxic metabolite or to depletion of a nutrient that is present at limiting levels. Further work in the lab will hopefully identify this missing factor. Furthermore, the current setup is not adequate for live imaging of the discs because of the requirement of a large culture volume that needs mixing. Eventually, one solution we can envisage would be a microfluidic device that allows the disc to be imaged from the bottom while continuously flowing fresh oxygenated medium over the top of the disc. Alternatively, a SPIM or lightsheet microscope could be used if the sample can be sufficiently perfused with fresh medium. In combination with the identification of the missing factor, such a device would allow imaging of long-term cultures. In summary, we show here that a combination of oxygenation and adenosine deaminase activity in the medium allows *ex vivo* cultures of *Drosophila* wing imaginal discs that maintain both growth and proliferation for almost one cell cycle.

Table 1. Medium composition of our final optimized setup

Component	Supplier and catalog code	Final concentration
Shields and Sang M3 insect medium	Sigma S3652	
Penicillin/streptomycin	Gibco 15140-122	1× for 9 h culture 4× for longer cultures
Calf intestine adenosine deaminase	Roche 10102105001	8.3 ng/ml
Bovine pancreas insulin	Sigma I0516	10 µg/ml
20-hydroxy ecdysone (20E)	Sigma H5142	5 nM
Juvenile hormone (JH, methoprene)	Fluka 33375	1.6 nM

MATERIALS AND METHODS

Fly stocks

All experiments were performed using 12-24 h pre-wandering w^{1118} larvae. For Adgf-a knockdown, tubulin-Gal4 was crossed to a Adgf-a RNAi line (VDRC ID 110152).

Media and supplements

Basal media used were: serum-free media (SFM, Gibco 10486-025) supplemented with 1×L-glutamine (Gibco 25030-024), Schneider's *Drosophila* medium (Gibco 21720-024), Shields and Sang M3 insect medium (Sigma S3652) or Robb's medium (described by Robb, 1969). All media were supplemented with penicillin/streptomycin (pen/strep) (Gibco 15140-122).

Our optimized medium consists of M3 containing 10 µg/ml insulin, 5 nM 20-hydroxy ecdysone (20E, Sigma H5142), 1.6 nM juvenile hormone (JH, Methoprene, Fluka 33375), 8.3 ng/ml ADA (Roche 10102105001) and 1× pen/strep. To prevent any nutrients from being exhausted during culturing, the medium was replaced every 3 h during culture.

Medium conditioning

S2 cells in SFM (verified mycoplasma free using the Multiplex Cell Contamination Test, Multiplexion) seeded in 6-well plates at 3×10^{6} cells/well/ml were treated with 12 µg dsRNA for 4 h before 2 ml SFM were added. After 4 days of knockdown, cells were reseeded into 24-well plates at 10^{6} cell/well/ml. Plates were spun for 1 min at 50 ***g*** and medium was replaced with fresh M3 to allow conditioning for 6 h. Conditioned medium was kept frozen at −20°C. For fat body-conditioned medium, fat bodies of 30 wandering larvae were incubated for 3 h in 300 µl culture medium.

Testing for the S-phase-promoting factor

Six larvae were rotated in 300 µl fresh or conditioned M3 containing 100 nM 20E and 1× pen/strep for 5 h with 25 µM EdU added to the culture afterwards for 1 h.

Preparation, fractionation and delipidation of FEX

Fly extract was prepared from female flies as described previously (Currie et al., 1988). FEX was fractionated on a 3 kDa Pierce protein concentration column yielding a 'small molecules' fraction (flow through) and a '>3 kDa' fraction that was re-diluted to the original volume and applied at a final concentration of 5%.

To delipidate FEX, two volumes of premixed butanol:diisopropyl ether (25:75) were added to 1 volume of FEX containing 0.1 mg/ml EDTA. The mixture was rotated end-over-end for 3 h, then centrifuged at 800 ***g*** for 2 min at room temperature and the aqueous layer was saved and degassed.

Proteinase K treatment was carried out at 37°C for 1 h prior to a phenol-chloroform extraction to remove proteinase K.

Disc preparation

Discs were cultured attached to the anterior part of the carcass to prevent loss of discs during the culture and staining procedure. Pre-wandering larvae at 12 h were dissected in M3 medium. The posterior end of the larva was removed prior to inversion of the anterior end, and all inner organs were removed except for trachea and wing discs. Experiments include a 'fresh' control of larvae directly labeled after dissection. Six larvae were dissected per condition.

Stirring culture setup

A cage was built by cutting off the bottom of a 2 ml eppendorf tube and attaching a metal mesh to it. The pores of the mesh were chosen to be large enough to allow medium flow but small enough to prevent discs passing through. The mesh was attached to the tube by melting the plastic at the bottom of the tube and quickly pressing the mesh onto the hot plastic. Chemical glues are not suitable as they prevent EdU incorporation in our hands. This cage is placed into a glass vial (~5 ml, 40×19 mm) containing a

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stir bar and 3 ml culture medium. Six larvae were then placed into the cage and cultured in medium with stirring at 100 rpm.

Quantitative RT-PCR

Oligos used for quantitative RT-PCR were (forward/reverse): LDH, ACCTCCGTTTTGGGCGA/ATGTTGGCGGACTTCTGC; CG11652, CCCGTTGCTAAAGGCTTA/CGTCCAGGCGCAGATTCT; rp49, GCTAAGCTGTCGCACAAA/TCCGGTGGGCAGCATGTG. Total RNA was reverse transcribed using Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific) following the manufacturer's instructions, and cDNA was quantified using a StepOnePlus Real-Time PCR machine (ThermoFisher Scientific).

EdU incorporation assay

EdU incorporation was assayed using the Click-iT EdU Alexa Fluor 555 Imaging Kit (Life Technologies, C10338) following the manufacturer's instructions: After disc culturing, EdU was added to a final concentration of 25 µM for 1 h, followed by 20 min fixation with 4% paraformaldehyde. Discs were rinsed in PBS/0.2% Triton (PBT) and subsequently blocked in PBT/0.1% BSA for 30 min. After another PBT rinse, discs were incubated in staining solution (12 discs/100 µl) for 30 min and subsequently washed again in PBT. After 30 min incubation in PBT+DAPI, discs were equilibration in glycerol mounting medium (160 ml glycerol, 20 ml 10× PBS, 0.8 g n-PG, 12 ml water). Images were quantified as described below.

OPP incorporation assay

OPP incorporation was assayed using the Click-iT Plus OPP protein Synthesis Assay Kit (Life Technologies, C10456) following the manufacturer's instructions. After disc culturing, OPP was added to a final concentration of 20 µM for 30 min, followed by a PBS wash and 20 min fixation with 4% paraformaldehyde. Discs were rinsed in PBS/0.2% Triton (PBT) and permeabilized in PBT for 15 min. After two PBS rinses, discs were incubated in staining solution (12 discs/100 µl) for 30 min while protected from light, followed by a rinse in component F, a 30 min incubation in nuclear stain/PBS, a PBS wash and equilibration in glycerol mounting medium (160 ml glycerol, 20 ml 10× PBS, 0.8 g n-PG, 12 ml water). Images were quantified as described below.

Image acquisition and quantification

Images were taken with a 40× objective using a Leica TCS SP8 confocal microscope. For all quantifications, a single focal plane image was taken using a large aperture (300 µm) so that the wing pouch was in focus. Using ImageJ software, EdU or OPP intensity was measured in this image as the integrated density of the red/green channel (EdU/OPP), which was then normalized to wing disc area, defined by presence of DAPI/nuclear stain. Phospho-histone 3 staining was quantified by counting pH3-positive particles per disc in this image.

Total protein quantification of discs

Mid 3rd instar larvae were dissected in Schneider's medium lacking serum. Ten larvae were dissected for each of four biological replicates. From each larva, one wing disc was removed and transferred to a 200 µl PCR tube containing Schneider's medium, yielding 10 wing discs per tube. The wing discs were allowed to settle to the bottom of the tubes, the Schneider's medium was almost completely removed (leaving circa 3 µl) and the wing discs were lysed by adding 12 µl of denaturing lysis buffer (10 M urea, 0.1% SDS in PBS) and pipetting up and down repeatedly. The discs were then transferred to 4°C for storage. The inverted larvae with one remaining wing disc were then transferred into *ex vivo* culture for 6 h in optimized medium with stirring, after which the remaining wing discs were dissected and lysed as described above. Total protein content was then measured by adding 1 ml of Bradford reagent (Biorad) to each sample and measuring OD595.

Immunoblotting and antibodies

Twenty-four discs were dissected into PCR tubes containing ice-cold medium lacking serum, then gently spun down. Medium was removed under the microscope and discs were lysed in 24 µl Laemmli buffer containing protease and phosphatase inhibitors. Samples (10 µl/lane) were subjected to SDS-PAGE and immunoblot analysis. Antibodies used were rabbit anti-phospho-S6 kinase (T398) (PhosphoSolutions, p1705-398) diluted 1:1000; rabbit anti-phospho-Akt (T342) (PhosphoSolutions, p104-342) diluted 1:1000; guinea pig anti-*Drosophila* S6 K (Hahn et al., 2010) diluted 1:2000; rabbit anti-ERK (Cell Signaling, 9102) diluted 1:2000; and rabbit phospho-histone H3 (Ser10) (Cell Signaling, 9701S) diluted 1:100.

FEX fractionation and mass spectrometry

Conditioned SFM and fly extract were fractionated first by size exclusion chromatography and then ion-exchange chromatography, and proteins were identified by electrospray ionization mass spectrometry. For additional details, please see the supplementary Materials and Methods, Table S2 and Table S3.

Acknowledgements

We thank Martina Schnölzer and the DKFZ Proteomics Core Facility for mass spectrometry analysis of fractions containing S-phase-promoting activity.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.S., F.K.L., A.A.T.; Methodology: K.S., F.K.L., M.L., F.G., A.A.T.; Formal analysis: K.S., F.K.L., A.A.T.; Investigation: K.S., F.K.L., M.L., F.G., A.A.T.; Resources: M.B.; Writing - original draft: K.S., F.K.L., F.G., A.A.T.; Writing - review & editing: K.S., F.K.L., A.A.T.; Visualization: K.S., F.K.L., A.A.T.; Supervision: M.B., A.A.T.; Project administration: M.B., A.A.T.; Funding acquisition: M.B., A.A.T.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.147538.supplemental

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