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Live imaging of muscles in *Drosophila* metamorphosis: Towards high-throughput gene identification and function analysis

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ABSTRACT

Time-lapse microscopy in developmental biology is an emerging tool for functional genomics. Phenotypic effects of gene perturbations can be studied non-invasively at multiple time points in chronological order. During metamorphosis of *Drosophila melanogaster*, time-lapse microscopy using fluorescent reporters allows visualization of alternative fates of larval muscles, which are a model for the study of genes related to muscle wasting. While doomed muscles enter hormone-induced programmed cell death, a smaller population of persistent muscles survives to adulthood and undergoes morphological remodeling that involves atrophy in early, and hypertrophy in late pupation. We developed a method that combines *in vivo* imaging, targeted gene perturbation and image analysis to identify and characterize genes involved in muscle development. Macrozoom microscopy helps to screen for interesting muscle phenotypes, while confocal microscopy in multiple locations over 4–5 days produces time-lapse images that are used to quantify changes in cell morphology. Performing a similar investigation using fixed pupal tissues would be too time-consuming and therefore impractical. We describe three applications of our pipeline. First, we show how quantitative microscopy can track and measure morphological changes of muscle throughout metamorphosis and analyze genes involved in atrophy. Second, our assay can help to identify genes that either promote or prevent histolysis of abdominal muscles. Third, we apply our approach to test new fluorescent proteins as live markers for muscle development. We describe mKO2 tagged Cysteine proteinase 1 (Cp1) and Troponin-I (TnI) as examples of proteins showing developmental changes in subcellular localization. Finally, we discuss strategies to improve throughput of our pipeline to permit genome-wide screens in the future.

1. Introduction

The immense arsenal of genetic tools available for the fruit fly *Drosophila melanogaster* has for over a century advanced our understanding of heredity and fundamental biological processes [1]. Ever since the whole genome was published over a decade ago [2], *Drosophila* has emerged as a model for human diseases due to the evolutionary conservation of genes and pathways between flies and humans [3]. However a recent survey (April 2015) of a list of 15,030 annotated protein coding genes from FlyBase [4] revealed gaps in our knowledge. Only 32.6% (4903) of genes are associated with phenotypic data, while even a smaller proportion of 7.1% (1062) contains information about immunolocalization, highlighting an untapped potential for discoveries. It is difficult to predict biological functions and possible roles in disease situations without taking into account phenotypic effects resulting from gain or loss of gene function. Time-lapse imaging in combination with targeted gene perturbation and reporter gene expression can fill some of the knowledge gaps. *Drosophila* is particular amenable to live imaging because during embryogenesis and metamorphosis, animals are immobile and transparent, allowing the non-invasive study of biological processes. A subset of larval abdominal body wall muscles are located near the cuticle and can be followed continuously throughout metamorphosis. Due to their large size and accessibility, these muscles are a versatile system for the study of fundamental biological problems such as cell size control and programmed cell death, and to gain insights into the subcellular localization of proteins.

1.1. Overview of muscle development in metamorphosis

The first skeletal muscles are formed in embryogenesis when mesodermal precursors called muscle founder cells fuse with

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fusion-competent myoblasts [5]. During the three larval stages which last 4 days, body wall muscle fibers grow about 50-fold. Growth involves endoreplication of myonuclei and depends on the conserved Insulin-Akt-TOR pathway [6]. During metamorphosis, which converts the larval into the adult body plan, most skeletal muscles become obsolete and are removed by cell death, also referred to as histolysis, which is induced by the molting hormone ecdysone (Fig. 1). Muscle histolysis starts at the end of the prepupal stage which lasts around 12 h and gives rise to muscle fragments called sarcolytes. A subset of body wall muscles resists histolysis and survives into adulthood. Some of these persistent muscles are located on the dorsal side of the abdomen and are named DIOMs for dorsal internal oblique muscles (Fig. 1A). The first and second abdominal segments contain three pairs of DIOMs (DIOM1, 2, 3), the third and fourth segment 2 pairs (DIOM 1, 3) and the fifth segment 1 pair (DIOM1) [7]. DIOM1 refers to the most dorsally located muscle. We extend the nomenclature to indicate the abdominal hemi-segment; e.g. DIOM1-2L refers to the most dorsal persistent muscle in the 2nd left abdominal hemi-segment. DIOMs are located basally beneath the doomed DEOMs (dorsal external oblique muscles) which start to degenerate prior to head eversion (HE). For convenience, we use HE as a temporal reference point, where time points are given in hours (h) after head eversion (aHE). Both muscles are amenable to live imaging since they are large and located beneath the transparent cuticle of prepupae or pupae [8,9]. The larval DIOMs (Fig. 1, arrows) undergo morphological remodeling into temporary adult muscles. Remodeling comprises phases of muscle atrophy (loss of cell mass) in early (0–50 h aHE) and hypertrophy (increase of fiber size) in the later stages of pupation between 50 and 100 h aHE. The adult DIOMs degenerate in first 24 h after eclosion. A second group of larval muscles that are protected from histolysis are the 6 longitudinal oblique muscles (Fig. 1B) in the thorax (3 per hemisegment) that serve as templates for a subset of the indirect flight muscles (IFMs), termed the dorsal longitudinal muscles [10]. The templates fuse with proliferating myoblasts to give rise to IFMs which progressively grow in size and express increasing amounts of muscle specific reporter genes. IFMs are also accessible to live cell imaging. Spinning disk confocal microscopy has been used to study myofibrillogenesis and muscle–tendon attachment *in vivo* [11]. The differentiation of IFMs helps in the staging of pupae. Previously, a genome-wide RNAi screen was carried out to find genes involved in the development and function of Drosophila muscles [12]. Assaying embryonic, larval and adult muscles, the screen identified 2785 muscle-specific genes, many of which are phylogenetically conserved and play roles in human muscle diseases.

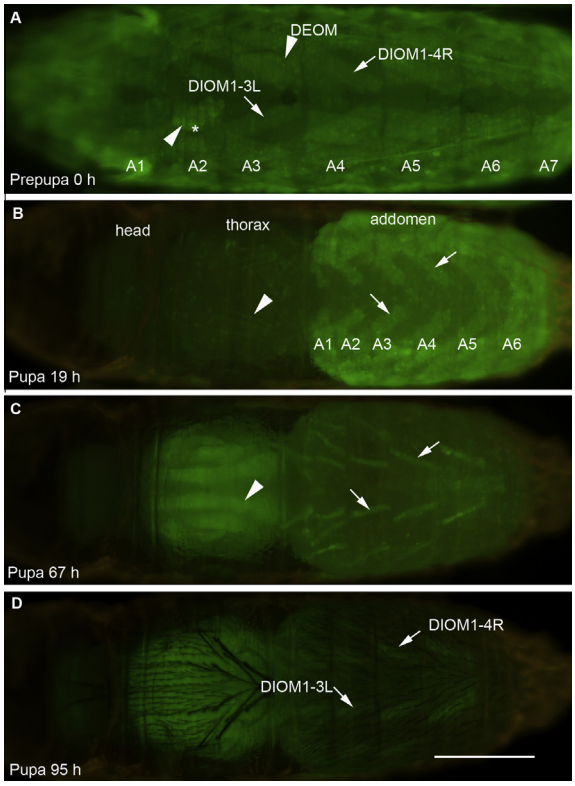

Fig. 1. Development of abdominal muscles in *Drosophila* metamorphosis. Muscle fibers are labeled with Mhc-tau-GFP (green). The specimen was kept at 22 °C and recorded at 4 time points (in hours relative to first time point 0 h) in metamorphosis using a macrozoom microscope. The images show dorsal views, anterior is to the left. The scale bar in (D) corresponds to 500 μm. (A) The transparent cuticle of prepupae allows the visualization of doomed muscles like the DEOMs (arrow head) that start to disintegrate 10 h after pupariation and persistent muscles like the DIOMs (arrow) that survive to adulthood. A1–A7 indicate the position of the abdominal segments. (B) Head eversion establishes the adult body plan and compresses the length of the abdomen, which is shifted posteriorly. The DEOM have histolyzed to give rise to sarcolytes. The larval DLM in the thorax (arrow head) serves as a template for the indirect flight muscle (IFM) seen in (C). (B–D) The DIOMs (arrows) undergo morphological remodeling.

1.2. Drosophila metamorphosis as a model for muscle wasting

Skeletal muscles are essential for mobility and metabolism. They can adjust size and metabolism to varying physiological conditions [13]. Resistance exercise can induce an increase of muscle size and strength (hypertrophy), while immobility, ageing, starvation and disease can lead to loss of muscle mass (atrophy). The two most common muscle wasting pathologies in humans are cachexia, a metabolic syndrome associated with chronic illnesses such as cancer and heart failure, and sarcopenia, the age-related loss of muscle mass and strength [14]. Changes in muscle size are controlled by the ratios between protein synthesis and breakdown. Protein synthesis and cell growth are activated by signaling pathways involving insulin-like growth factor-1 (IGF1), the kinase Akt1 and the mammalian target of rapamycin (mTOR) [15]. Protein degradation and muscle atrophy are induced by a different signaling cascade involving Myostatin, Smad3 and transcription factors of the FoxO family [16]. Two processes mediate protein breakdown: the ubiquitin proteasome system (UPS) [17] and autophagy lysosomal pathway [18].

In many arthropods like the moth Manduca [19], land crabs or lobsters, muscle atrophy takes place as part of normal development [20]. The observation that DIOMs undergo phases of atrophy and hypertrophy during Drosophila metamorphosis gave rise to the speculation that conserved pathways act to control muscle size in human muscle wasting and *Drosophila* development [9]. Recently we were able to demonstrate by that developmental atrophy is indeed regulated by TOR signaling and autophagy as in mammalian cells (manuscript submitted). Studying cell size control is therefore a main application of our method that we illustrate in the Results section.

Loss of muscle mass can either result from reversible shrinking or irreversible degeneration (e.g. by programmed cell death) of muscle fibers. Unlike in muscle wasting pathologies in mammals, *Drosophila* metamorphosis makes it easier to distinguish between these two phenotypes. For instance, our previous study found that overexpression of an N-terminal fragment of the nuclear EAST protein can delay histolysis of doomed DEOMs by several hours [21]. In this paper, we demonstrate how we can identify genetic perturbations that induce premature cell death in persistent muscles.