

Multicolor fluorescent lineage analysis:
A retroviral-based mouse system for studying muscle stem cells

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Abstract

Adult tissues require stem cells for function and maintenance. Adult stem cell populations are often comprised of cells with varied potential to differentiate or self-renew. Within these heterogeneous populations a subset of stem cells undergo limited rounds of division with the purpose of replenishing the stem cell pool, while the majority rapidly proliferate and differentiate to repair the tissue. In contrast, some stem cells are homogeneous and possess equipotent capacities for differentiation or self-renewal. I investigated satellite cell heterogeneity using an RCAS avian retroviral approach for multi-color fluorescent lineage tracing. I successfully generated retroviral vectors and high-titer retrovirus for lineage tracing. I was able to infect the target myofiber-associated satellite cells, supporting the notion that RCAS can be a powerful tool to label and track satellite cells. I performed two-color lineage tracing of satellite cells, however I was unable to examine satellite cell heterogeneity. My work demonstrates proof of concept that RCAS can be leveraged to interrogate satellite cell behavior and function through *in vivo* cell type-specific retroviral-mediated gene expression.

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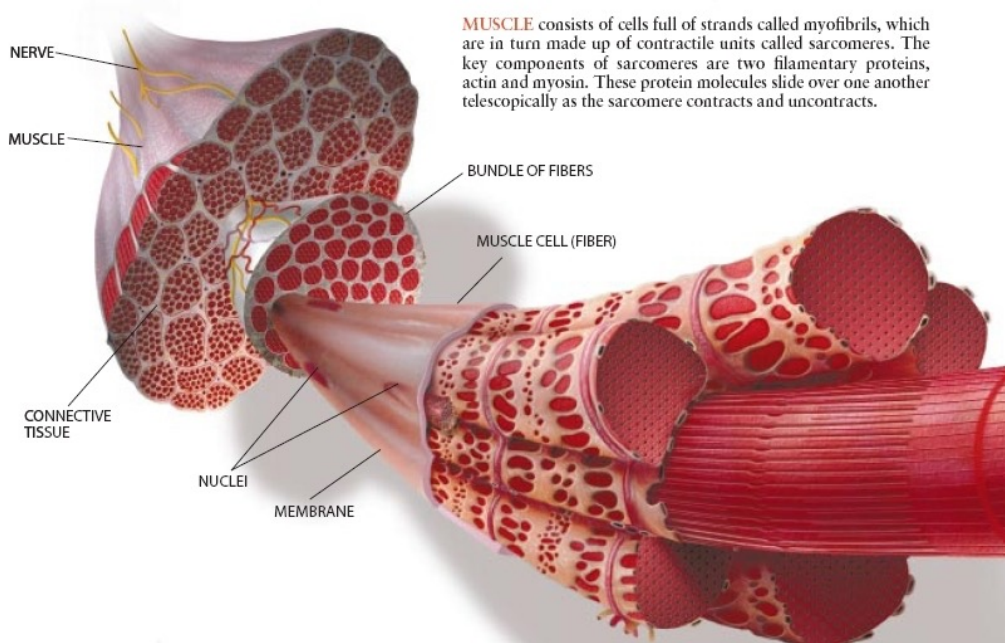
ARAC	arabinosylcytosine or Cytosine Beta-D-arabinofuranoside
BFP	blue fluorescent protein
CFP	cyan fluorescent protein
PFA	paraformaldehyde
DAPI	4',6-diamidino-2-phenylindole
DF1	immortalized chicken cell line
DNA	deoxyribonucleic acid
eGFP	enhanced green fluorescence protein
FGF	fibroblast growth factor
IFU	infectious units of retrovirus
mCherry	red fluorescent protein
mKate	far-red fluorescent protein
MOI	multiplicity of infection
Mowiol	polyvinyl alcohol cell mounting medium
PBS	phosphate buffered saline
RCAS	replication-Competent ASLV long terminal repeat (LTR) with a Splice acceptor
SYTO	red fluorescent nucleic acid stain
TA	tibialis anterior muscle of the hindlimb

Chapter 1: Introduction

Adult organs are homeostatic, with cell loss replaced by new cells, provided by tissue-specific stem cells. Adult stem cells are typically quiescent or cycle slowly and when required for tissue maintenance or repair these cells activate, proliferate, and then differentiate, maintaining homeostasis (Blanpain and Simons, 2013). Adult stem cell populations are often comprised of cells with varied potential to differentiate or self-renew. Within these heterogeneous populations a subset of stem cells undergo limited rounds of division with the purpose of replenishing the stem cell pool, while the majority rapidly proliferate and differentiate to repair the tissue. In contrast, some stem cells are homogeneous and possess equipotent capacities for differentiation or self-renewal (Clayton et al., 2007; Snippert et al., 2010; Graf and Stadtfeld, 2008).

Adult skeletal muscle is one example where adult muscle stem cells, or satellite cells, maintain skeletal muscle homeostasis. Skeletal muscle is dynamic, however, increasing or decreasing mass, depending on physiological demands. The relationships between dynamic changes in skeletal muscle mass and the resident satellite cell population are not understood. Skeletal muscle myofibers comprise the functional, contractile apparatus and are multinucleated syncytia containing hundreds or thousands of myonuclei derived from myoblast fusion during development (Introduction Figure 1) (Mauro, 1961). Myofiber myonuclei are permanently post-mitotic and the addition of new nuclei for growth or repair are derived from satellite cells (Chargé, 2004). Although satellite cells comprise 2-4% of muscle nuclei, they possess a remarkable ability to repair whole muscle tissue (Biressi and Rando, 2010). These stem cells are located underneath the basal lamina and

adjacent to the myofiber plasma membrane (Mauro, 1961). In addition, satellite cells self-renew, replenishing a population that returns to quiescence, though the mechanism is poorly understood (Introduction Figure 2). Satellite cells are difficult to study due to their rarity, and behavior of the population is poorly understood. Satellite cells appear to be a heterogeneous stem cell population, although heterogeneity of satellite cell lineage progression is unclear (Biressi and Rando, 2010). My project is particularly interested in the heterogeneity of adult satellite cell lineage progression and the development of novel techniques to study this complex biological problem.



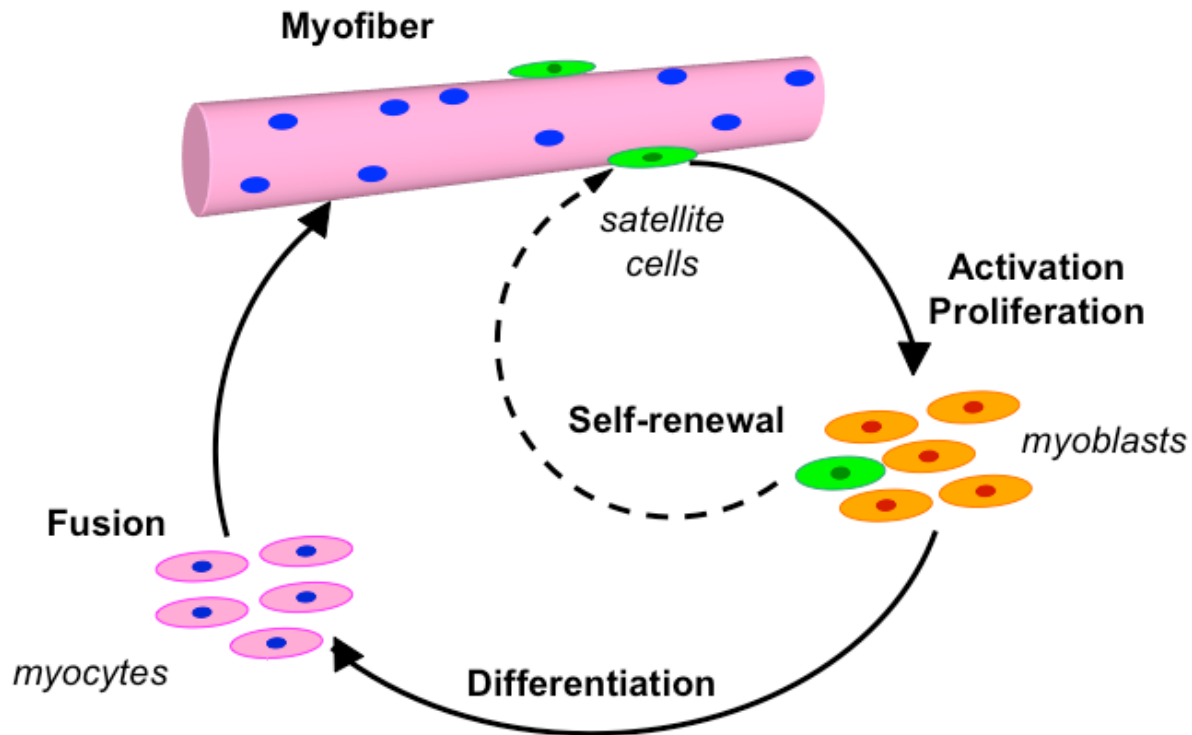
Introduction Figure 1: Skeletal muscle structure

Skeletal muscle is comprised of many bundles of myofibers, which are the multinucleated cells of muscle. Myofibers are the contractile units of skeletal muscle. Image source: (Andersen et al., 2000).

Satellite cells are heterogeneous with respect to gene expression, fiber association, adhesion to the extracellular matrix, and is furthermore demonstrated by the longevity and

proliferative capability of a small subset of satellite cells present in aged muscle.

Heterogeneity in gene expression is observed in a small population of satellite cells that



Introduction Figure 2: Skeletal Muscle Regeneration

Quiescent satellite cells activate, proliferate or undergo self-renewal. Myoblasts, the progeny of satellite cells, are capable of either proliferating or differentiating into myocytes that migrate and fuse with each other or existing myofibers to generate mature myofibers.

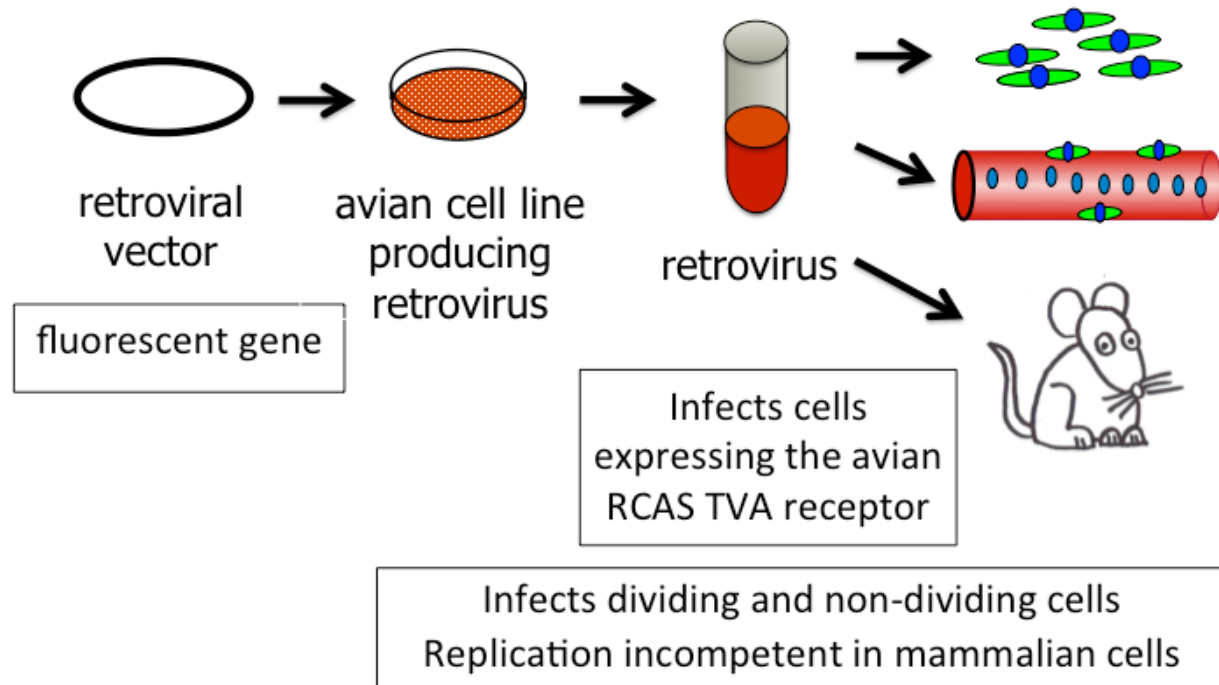
express the cell cycle inhibitors $P27^{kip1}/P21^{cip1}$ and exhibit long term potential for self-renewal (Chakkalakal et al, 2014). Heterogeneity in satellite cells of fast versus slow fibers, classified by their speed of contraction, includes variances in differentiation and proliferation rate in culture (Barjot et al., 1995; Feldman and Stockdale, 1991; Lagord et al., 1998; Biressi and Rando, 2010). Heterogeneity of satellite cell fate is governed by spatial distribution of adhesion to the extracellular matrix and manipulation of adhesive characteristics alters the frequency of asymmetric division events (Yennek et al., 2014). In aged muscle a small population of satellite cells exhibit the same proliferative capacity as

young satellite cells when transplanted along with the associated myofiber into young immunodeficient recipients (Collins et al, 2007). However this proliferative capability appears to be restricted to *in vivo* transplantation assays, as heterochronic culture experiments involving the reseeded of aged satellite cells on young muscle fibers fail to replicate this proliferative capability, indicating the presence of a cell-autonomous defect associated with aging (Bernet et al, 2014).

The aforementioned publications all involved a similar theme: stem cell fate can be elucidated by identifying cells and following their progeny, a process known as lineage tracing. Lineage tracing is traditionally accomplished through the use of fluorescent labels applied to the stem cell and followed into limited progeny (Blanpain and Simons, 2013; Kardon et al., 2002; Wagner et al., 2008). These current fluorescent protein-based methods are useful for studying tissue structure; but they are not useful for studying the heterogeneity of satellite cell lineage progression—the focus of my project. Conventional fluorescent protein based techniques cannot be used in the study of satellite cells in muscle due to the fusion of myoblasts to the syncytial myofiber and subsequent dilution of the fluorescent signal. In satellite cells, stem cell fate must be uncovered by other means of lineage tracing, in this case utilizing a permanent genetic marker to identify satellite cells and their progeny, regardless of final disposition.

To study heterogeneity in adult satellite cells, I devised a novel technique that improved upon existing models of fluorescent protein based lineage tracing. I utilized a vector derived from a member of the avian sarcoma leukosis virus (ASLV) family, the system for which is known as Replication-Competent ASLV long terminal repeat with Splice acceptor (RCAS). Since RCAS retrovirus only infects cells expressing the TVA receptor,

which is endogenous to avian species, mammalian cells require ectopic expression of the TVA receptor for infection. I utilized transgenic murine systems which permitted either ubiquitous or population specific infection of primary cells (Introductory Figure 3).



Introduction Figure 3-RCAS Retroviral Approach

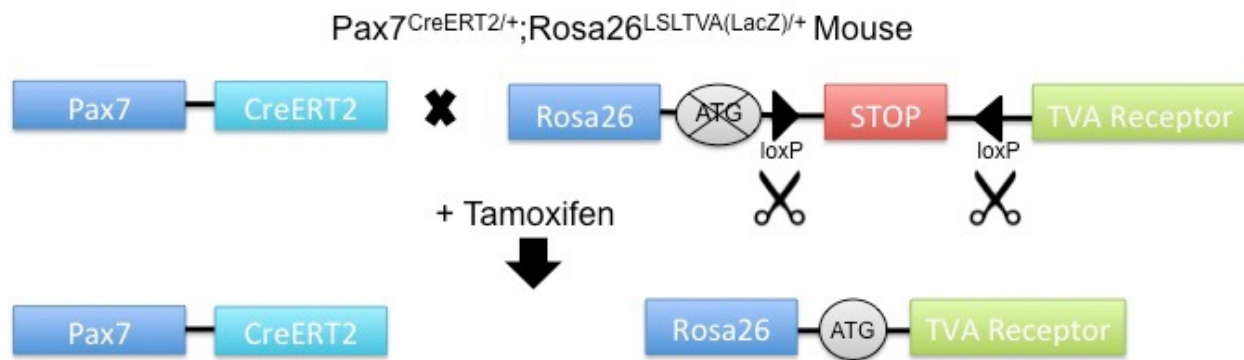
The RCAS vector contains 2.5 kb of available space, into which I inserted genes encoding fluorescent proteins: mKate, (far red), tdTomato (red), BFP (blue), and eGFP (green). RCAS retroviruses are replication competent in avian cells, permitting avian cell line production of retroviruses. RCAS retroviruses are replication incompetent in mammalian cells; individually infected mammalian cells are unable to produce additional virus, preventing infection of other cells. RCAS retrovirus only infects cells expressing the TVA receptor, which is endogenous to avian species. Mammalian cells require ectopic expression of the TVA receptor for infection. These characteristics of the RCAS retroviral system in mammalian cells provide an ideal system in which to study the lineage progression in satellite cells.

RCAS retroviruses possess characteristics that make them an ideal tool for studying heterogeneity in the satellite cell population: a vector with available space in which to insert genes of interest, permanent integration of the retroviral genome into the host DNA, lack of endogenous expression in mammalian cells, and replication incompetence in mammalian cells. The RCAS vector contains 2.5 kb of available space, into which I inserted

genes encoding fluorescent proteins. I generated 4 RCAS retroviruses with 4 different genes that encode fluorescent proteins: mKate (far red), tdTomato (red), BFP (blue), and eGFP (green). Upon infection, RCAS retroviral DNA integrates into the host DNA, providing a permanent genetic marker of infection, in this case expression of a fluorescent protein. RCAS retroviruses can only infect cells expressing the avian TVA receptor, thus the receptor must be expressed exogenously on mammalian cells, effectively controlling the infected population. While RCAS retroviruses are replication competent in avian cells, in mammalian cells RCAS retroviruses are replication incompetent, which is ideal as individually infected cells are unable to produce additional virus to infect other cells. These characteristics of the RCAS retroviral system in mammalian cells provide an ideal system in which to study the lineage progression in satellite cells.

I utilized two genetically modified mice for my RCAS retroviral experiments: a transgenic mouse with the TVA receptor driven by the ubiquitous B-Actin promoter and knock-in mice where the TVA receptor is present but silent in the ROSA locus until recombination occurs, which is inducible and expressed from the satellite cell-specific Pax7 locus and (Pax7CreERT2;LSLTVA) mice. In the B-Actin TVA transgenic mouse all cells express the TVA receptor from a randomly inserted transgene driven by the B-Actin promoter. The Pax7CreERT2;LSLTVA mice are advantageous as expression of the TVA receptor is restricted to cells expressing the satellite cell marker Pax7. In Pax7CreERT2;LSLTVA mice cells expressing Pax7 also express a modified estrogen receptor bound to an intracellular Cre recombinase (CreERT2). Cre-mediated recombination is induced by injection of tamoxifen, which is converted by the liver to 4-hydroxytamoxifen and selectively binds the CreERT2 protein. Following 4-

hydroxytamoxifen binding, CreERT2 translocates to the satellite cell nucleus, where the loxP sites are recombined, removing the stop codon and correcting the defective start codon, inducing robust TVA receptor expression (Introductory Figure 4) (Bockcamp et al., 2008). Satellite cells expressing TVA are then susceptible to RCAS retroviral infection, resulting in the subsequent insertion of retroviral DNA into the cell's genome and retroviral-mediated expression fluorescent proteins (Seidler et al., 2008; Orsulic et al., 2002).



Introduction Figure 4- Pax7CreERT2;LSLTVA mouse genetics

To induce Cre-mediated recombination, Pax7CreERT2;LSLTVA are treated with the drug tamoxifen which selectively binds the CreERT2 protein. Following tamoxifen binding, Cre-recombinase translocates to the satellite cell nucleus and removes the stop codon located between two loxP sites upstream of the TVA receptor transgene, effectively repositioning the start codon and allowing TVA receptor expression (Bockcamp et al., 2008). RCAS retroviruses are then able to infect TVA-expressing cells resulting in the subsequent insertion of retroviral DNA into the cell's genome and retroviral-mediated expression of fluorescent proteins (Seidler et al., 2008; Orsulic et al., 2002).

Two well-established *in vitro* myogenesis methods aid the study of muscle regeneration, as studying muscle regeneration *in vivo* is difficult. The first includes culturing primary myoblasts (satellite cell progeny) and manipulating culture media to cause them to differentiate into multinucleated myotubes (Rando and Blau, 1994). By culturing myoblasts using media lacking serum, myoblasts terminally differentiate, fuse together, and form myotubes, analogous to myogenesis *in vivo*. The second method

involves isolation and culture of fresh myofibers and associated satellite cells (Bischoff, 1975; Rosenblatt et al., 1995). This method allows limited simulation of the *in vivo* satellite cell niche. Both *in vitro* cultures were combined with RCAS retroviral infection to assay heterogeneity in satellite cell lineage progression.

While conclusions based on *in vitro* studies are limited due to inherent selection via culture conditions and lack of environmental signals, *in vitro* mouse model studies are valuable for studying systems and processes not easily observed *in vivo*. *In vitro* studies can yield information regarding subtleties that may assist in the development of *in vivo* experiments, which are inherently complex. Furthermore *in vitro* assays can be utilized to provide proof of concept and demonstrate functionality of experimental techniques prior to *in vivo* experimentation. For this project, *in vitro* assays allow us to study satellite cell heterogeneity prior to *in vivo* studies. Establishing satellite cell heterogeneity *in vitro* provides a preview of potential *in vivo* satellite cell heterogeneity and potentially alters technical components of future *in vivo* experimentation.

Chapter 2: Materials and Methods

Mice

Mice were housed in a pathogen-free facility. The Institutional Animal Care and Use Committee at the University of Colorado approved all procedures and protocols. Mice were between 3 and 5 months of age. Beta-actin TVA mice were acquired from Dr. Hughes (Federspiel et al., 1996). Pax7CreERT2/+;R26LSLTVA(LacZ)/+ mice were generated by crossing LSL-R26TVA-lacZ mice obtained from Drs. Saur and Songhai (Seidler et al., 2008) and Pax7Cre^{ERT2} obtained from Dr. Fan (Lepper et al., 2009). To induce Cre-recombination in Pax7CreERT2/+;R26LSLTVA(LacZ)/+ mice, Tamoxifen (Sigma Aldrich) was administered to mice at 0.075 mg/gram body weight for five days via intraperitoneal injection (Jackson Laboratory).

RCAS Retroviral Vectors

RCASBPA (Harpavat and Cepko, 2006) retroviral plasmid containing Gateway Cloning recombination sites were used to generate vectors for RCAS-mediated gene expression for eGFP, mCherry, mKate, and BFP. Fluorescent genes were amplified from expression vectors via PCR to introduce flanked DNA sequences containing recombination sites that permit the amplicon containing the gene to be recombined into the RCAS vector via Gateway Cloning (Life Technologies). Plasmid stocks were generated in bacterial stocks and plasmid isolate via Maxi kit (Qiagen). Restriction enzyme analysis and gel electrophoresis was used to confirm gene insertion into the RCAS retroviral vector and to assess plasmid quality before DF1 cell transfection.

RCAS Retrovirus Generation

DF1 chicken cells (American Type Culture Collection) were grown on uncoated tissue culture plates from Cell-Treat (Cell-Treat Scientific Products) in DMEM containing 10% Fetal Bovine Serum and 100 U/ml penicillin and 100 µg/ml streptomycin. DF1 cells were transfected with 5ug RCAS retroviral vectors with Lipofectamine 2000 (Qiagen) according to the manufacturer's instructions for 2 hours. DF1 cells were fixed with 4% Paraformaldehyde for 10min at room temperature and counterstained with 1 ug/ml DAPI (4',6-diamidino-2-phenylindole) or Syto Dye Red 59, SYTO Red Fluorescent Nucleic Acid Stain (Life technologies) at 1:1000 dilution. Visualization of fluorescent proteins to confirm expression and infection was performed using DF1 cells and immunofluorescence microscopy. Fluorescent proteins were excited at the following wavelengths: eGFP (488 nm), mCherry (589 nm) DAPI (350 nm) and far-red (628 nm).

RCAS Retroviral Collection and Titering

Following transfection with an RCAS retroviral vector, DF1's were expanded onto approximately sixteen p150 plates. As DF1s approached confluency, cell culture media was switched to 10 ml high-glucose DMEM, 2% FBS growth media containing 100 U/ml penicillin and 100 µg/ml streptomycin, to further concentrate virus and reduce serum presence. Retroviral supernatants were kept on ice at all times. Supernatant was collected at 48 and 96 hours following low serum media change and centrifuged at 3,000g x 30 minutes at 4°C to pellet floater cells, debris and flocculent serum components. Supernatant was then strained through a 40.0 µm cell strainer followed by filtration through a 0.45 µm acetate-cellulose surfactant free filter. The retroviral supernatant was concentrated via ultracentrifugation at 50,000g for 90min at 4°C using a Beckman L8-70 Ultracentrifuge.

Retroviral pellets were suspended in approximately 200-300ul of 1XPBS, aliquoted into small tubes and stored at -80°C. RCAS retroviral titers were determined using serial dilution method using DF1 cells as previously described (von Werder et al., 2012; Pao et al., 2003; Logan and Tabin, 1998; Fekete and Cepko, 1993).

Myofiber Isolation and Culturing

To generate mouse floating muscle myofibers, the hind limb gastrocnemius muscle was dissected away from the bone with fat and connective tissue being removed. The muscle was digested for 1.5 h at 37°C in 400U/ml Type I Collagenase (Worthington) with gentle agitation every ten minutes. After digestion, myofibers were washed with F12C (Life Sciences Products Inc.) containing 15% horse serum 3 times for five minutes to remove tissue debris. Following washing, myofibers were collected with using fire-polished glass pipets and placed into 6-well tissue culture plates containing growth media, 15% horse serum in F12C media containing 2nM FGF-2 and 100 U/ml penicillin and 100 µg/ml streptomycin. The isolation of individual myofibers by pipette was repeated twice to ensure removal of dead myofibers and cellular debris. Myofiber cultures were incubated in a standard tissue culture incubator at 37°C until fixation.

Myofiber Culture Treatments

For satellite cell ablation experiments, myofiber were cultured in the presence of AraC, Cytosine Beta-D-arabinofuranoside (Sigma Aldrich), at either 10uM, 50uM, or 100uM µM. RCAS retroviral infection of myofibers was performed immediately following clean up of myofibers. For each infection condition, approximately 100 myofibers were infected for 1 hour at 37°C with RCAS retrovirus in a final volume of 200ul. Following infection, myofibers were washed with growth media to remove residual RCAS retrovirus and plated

onto 6-well uncoated tissue culture plates.

Myofiber Immunocytochemistry

Myofibers were washed with 1XPBS and fixed with 4% paraformaldehyde for 10 min at room temperature before immunostaining. For Pax7, MyoD costaining, mouse monoclonal PAX7 (Developmental Hybridoma Bank at Iowa University) was used at 1:1000 dilution, rabbit polyclonal MYOD (C-20, Santa Cruz Biotechnology) was used at 1:1000 dilution. Myofiber cultures were incubated with blocking buffer containing 3% BSA in PBS –T (0.2% Triton X-100) for 60 minutes at room temperature, then incubated overnight at 4°C with primary antibodies. Myofibers were washed 3 times with 1XPBS and incubated for 1 hour at room temperature with 1:1000 Alexa Fluor 555-conjugated goat anti-mouse IgG1 (Life Technologies) and 1:1000 Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life Technologies) secondary antibodies. DNA was stained with DAPI or Syto Dye Red 59, SYTO Red Fluorescent Nucleic Acid Stain (Life technologies) at 1:1000 dilution, and sections were mounted with Moiwol or Vectashield (Vector Labs). Myofiber-associated cells were scored by counting the numbers of DAPI positive cells and fluorescent proteins expressing cells per fiber with three to seven myofibers counted per condition.

Immunofluorescence Analysis

Fluorescence microscopy was performed using a confocal microscope, Zeiss 510 or widefield, Olympus IX81 Inverted Microscope. Fluorescent proteins were excited at the following wavelengths: eGFP (488 nm), mCherry (589 nm) DAPI (350 nm) and far-red (628 nm). Images were captured using Slidebook (Intelligent Imaging Innovations) or Metamorph (Molecular Devices). Multi-channel z-stack fluorescent images of myofibers were taken using Slidebook and summed using Image J software (v1.47). All images were

globally processed for size, brightness and contrast using ImageJ and Adobe Photoshop (11.0.2). Figures were prepared using Adobe Illustrator (14.0) and Prism Graphing software (Scientific Software).

Chapter 3: Results

Generation of Functional RCAS retroviruses containing fluorescent genes

RCAS retroviruses containing genes encoding fluorescent proteins were collected from the supernatant of transfected DF-1 cells and used to infect virgin DF-1 cells. The retroviruses contained genes that code for a single fluorescent protein: either BFP (blue fluorescent protein), eGFP (enhanced green fluorescent protein), mCherry (red), or mKate (far-red). Fluorescent signals corresponding to the emission spectra of all four fluorescent proteins were observed in all conditions compared to the negative control, demonstrating the RCAS retroviruses contain the necessary genetic components to generate functional fluorescent proteins that I can use to infect satellite cells (Figure 1).

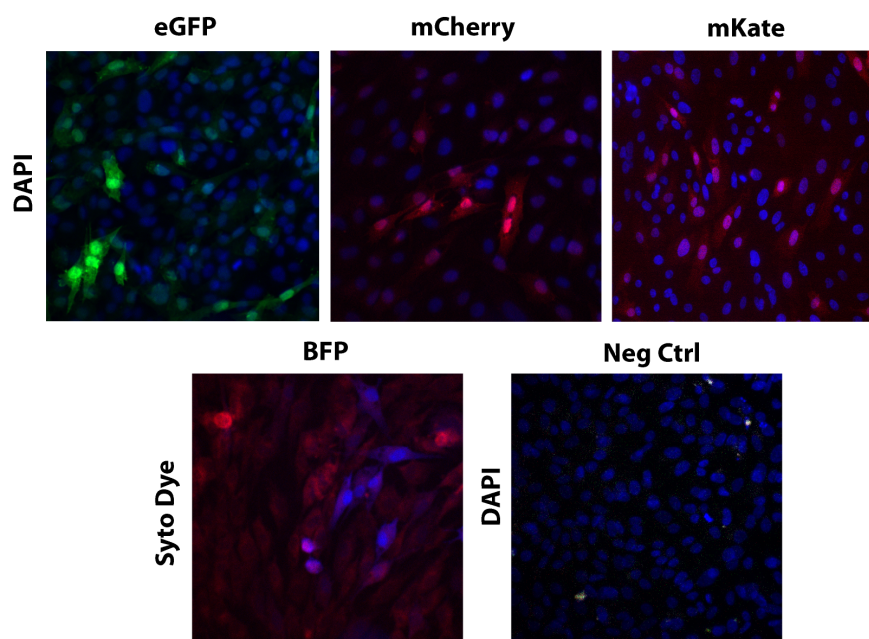


Figure 1 RCAS-mediated gene expression of fluorescent proteins in DF1 cells. Retroviral supernatant was isolated from DF-1 cells transfected with retroviral plasmids that contain a gene encoding a single fluorescent protein, either, BFP (blue), eGFP (green), mCherry (red), or mKate (far red). 72 hours post-infection, infected DF-1 cells were fixed in 4% paraformaldehyde. Following fixation, DF-1 cells were counterstained with DAPI (blue; chromatin) or Syto (red; nucleic acids) to mark nuclei. Images are representative of the stained, infected DF-1 cells. Fluorescent proteins eGFP, mCherry, mKate, and BFP were detected in DF-1 cells.

I utilized a standard serial dilution titering method to calculate RCAS retroviral titers (von Werder et al., 2012; Pao et al., 2003; Logan and Tabin, 1998; Fekete and Cepko, 1993). The fluorescent protein mCherry was detected across the dilution series in wells 1-5 but was absent in well 6, the condition containing no RCAS retrovirus (Figure 2A). After scoring the number of mCherry positive cells in well 5 we calculated viral titer using a retroviral titer equation (Figure 2B). Cells expressing mCherry were not detected in uninfected DF1 cells. This method was used to confirm that all viruses were functional and for determining their titer (Figure 2).

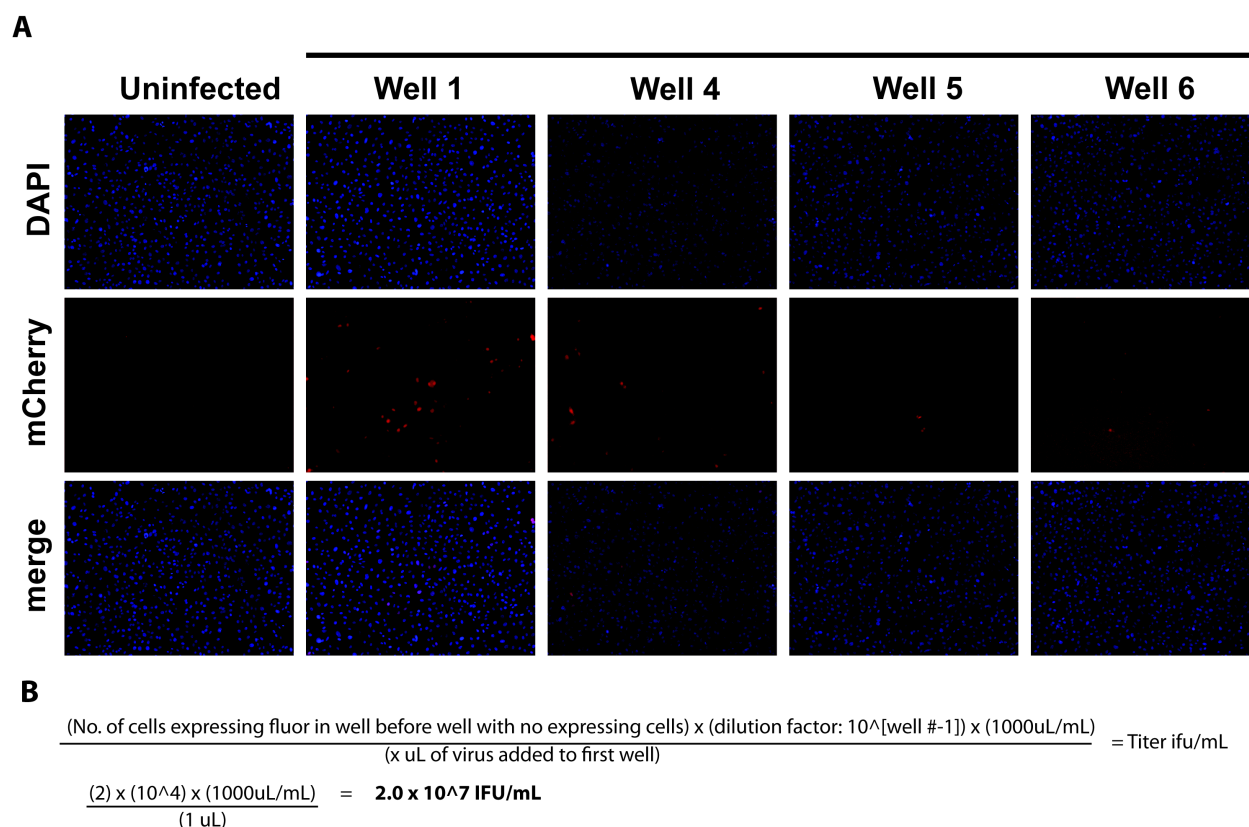


Figure 2 RCAS retroviral titering via serial dilution. To calculate RCAS retroviral titers, DF1 cells were infected with retrovirus at 10-fold serial dilutions as shown in **A**. 72 hours post infection, DF1 cells were fixed with 4% PFA and counterstained with DAPI. Imaging was performed at 10X, with 100 ms exposures for both DAPI (405nm) and mCherry (589 nm). The number of infected cells observed in the images decreased progressively, reflecting that the retrovirus was serially diluted across wells. **B**. To calculate mCherry retroviral titer, I counted mCherry positive cells in the last visible well. For example: in **A**, the well preceding the well with no infected cells is Well 5. Well 5 has two infected cells. Using the equation in **B**, the retroviral titer = 2.0×10^7 ifu/mL.

I examined whether filtering retroviral stocks affected the total yield of infectious units present, as a high titer is necessary for my planned experiments. I compared titers between filtered and unfiltered retroviral supernatant prior to and after ultracentrifugation (Table 1). RCAS retroviral stock for the eGFP construct was concentrated using an ultracentrifuge; in comparison RCAS retroviral stock for the mKate construct was not concentrated to rule out ultracentrifugation as the cause of any differences in titer. Filtration of both mKate and eGFP constructs yielded higher total IFU's compared to unfiltered stock. My data indicate that filtering RCAS retroviral supernatants increases viral yield.

RCAS Retrovirus Fluorophore	Filtering	Concentrated	Total IFUs
eGFP (green)	unfiltered	Ultracentrifuge	0.54×10^8
	filtered	Ultracentrifuge	5.19×10^8
mKate (far-red)	unfiltered	None	1.0×10^5
	filtered	None	5.0×10^5

Table 1: Filtering retroviral supernatant to increase retroviral titer. To determine whether filtering and ultracentrifugation of retroviral stocks affects the total yield of infectious units (IFU), final titers were compared between filtered and unfiltered retroviral supernatant before and after ultracentrifugation. eGFP retroviral stock was concentrated using an ultracentrifuge, while mKate was not concentrated. The highlighted rows in Table 1 correspond to the filtered conditions. For both eGFP and mKate, filtering the retroviral stock resulted in higher total IFUs compared to the unfiltered stock.

Infection of skeletal muscle with RCAS retroviruses

I infected myofiber cultures from B-Actin TVA mice with mCherry and eGFP retroviruses at a multiplicity of infection (MOI) of 6 viral particles per cell with equal representation of each fluorescent protein construct (MOI 12 total per cell). For both eGFP and mCherry infected myofiber cultures, both positive and negative single cells were observed, indicating successful infection of myofiber-associated satellite cells with RCAS

retroviruses (Figure 3). BFP retrovirus was found to be ill-suited for lineage tracing as the emission spectrum overlaps with the most commonly used fluorescent nuclear counter stain and a suitable alternative was not found (data not shown).

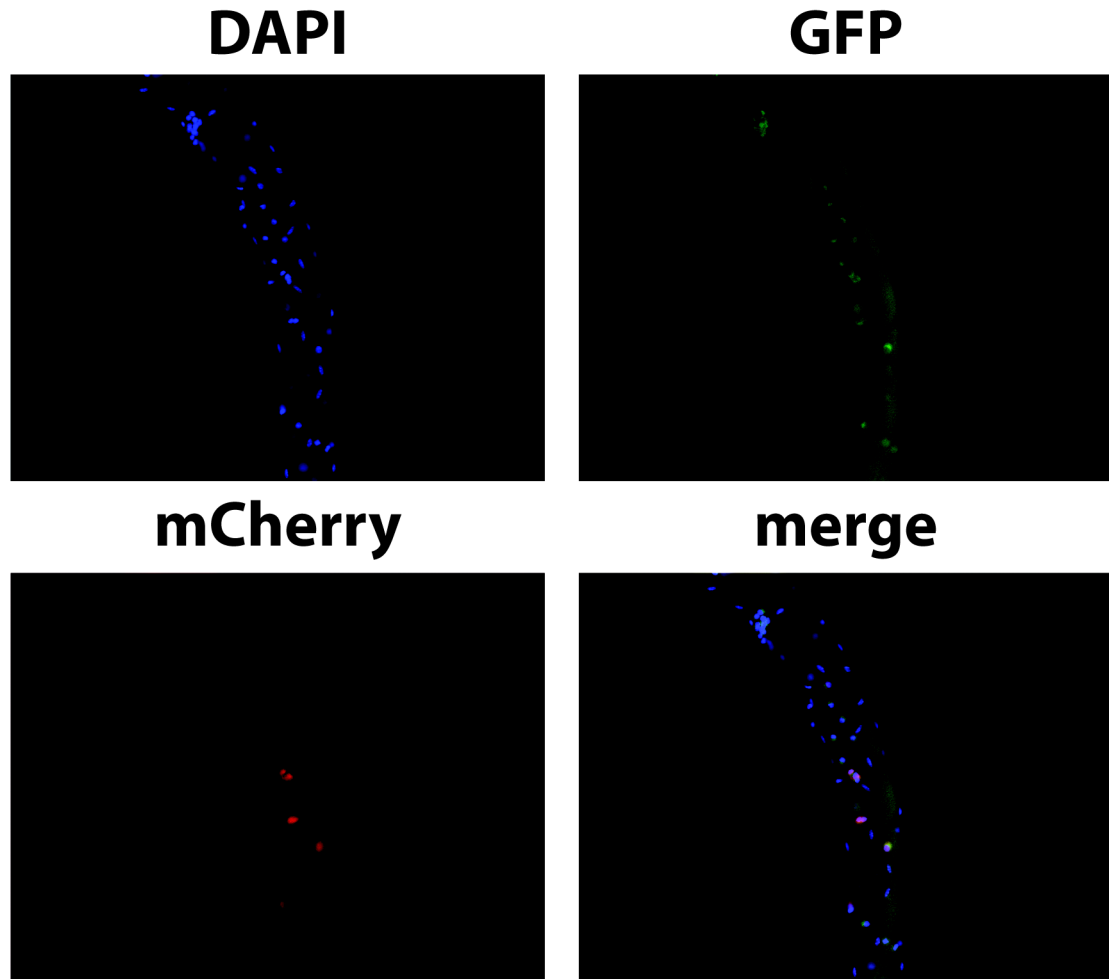


Figure 3: RCAS retroviral infection of myofiber-associated satellite cells.

Myofibers from B-ActinTVA mice were infected with equal amounts of RCAS retroviruses for mCherry and eGFP. Myofibers were fixed with 4% paraformaldehyde 72 hours post infection and counterstained with DAPI. Positive and negative single cells were observed at both eGFP (488 nm) and mCherry (589 nm) showing successful infection of myofiber-associated cells with RCAS retrovirus.

I ablated myofiber-associated satellite cells *in vitro* to determine if myofibers express sufficient TVA driven by the β -actin promoter and can be infected by RCAS retrovirus without the fusion of previously infected satellite cells. I treated myofibers with

varying concentrations of the mitotoxin AraC, a deoxycytidine analog that kills dividing cells but not the post mitotic myofibers. Myofiber-associated satellite cells were present on untreated myofibers (0 uM AraC) at 48 and 72 hours (Figure 4A). The increase in cell numbers between 48 and 72 hour untreated cultures is consistent with expected satellite cell proliferation. Few myofiber-associated cells were observed with either a 48 hour 10 uM AraC treatment or a 48h 50 uM AraC treatment, and no cells were observed with a 48 hour 100 uM AraC treatment (Figure 4A). Quantification of myofiber-associated cells revealed effective ablation of all satellite cells at 48 hours with 100 uM AraC, while cell numbers were reduced with either a 48h treatment of 10 uM AraC or 50 uM AraC compared to untreated controls (Figure 4B). At 72 hours, effective ablation of all satellite cells was observed for 10 uM AraC, 50 uM AraC, and 100 uM AraC, compared with untreated. Effective myofiber associated satellite cell ablation was observed with 10 uM AraC treatment for 72h, which is consistent with prior published findings (Troy et al., 2012).

Myofibers isolated from β -actin TVA mice subjected to satellite cell ablation by AraC treatment were infected with equivalent MOIs for eGFP and mCherry retroviruses. Greyscale images of eGFP (488 nm) and mCherry (586 nm) fluorescence were compared to fluorescence intensities of uninfected myofibers (negative control). Representative Z-stack images for the highest and lowest intensity myofibers are shown for each condition (Figure 5). The variability in background autofluorescence of myofibers observed in all conditions prevented determination of myofiber infection by either eGFP or mCherry RCAS retroviruses.

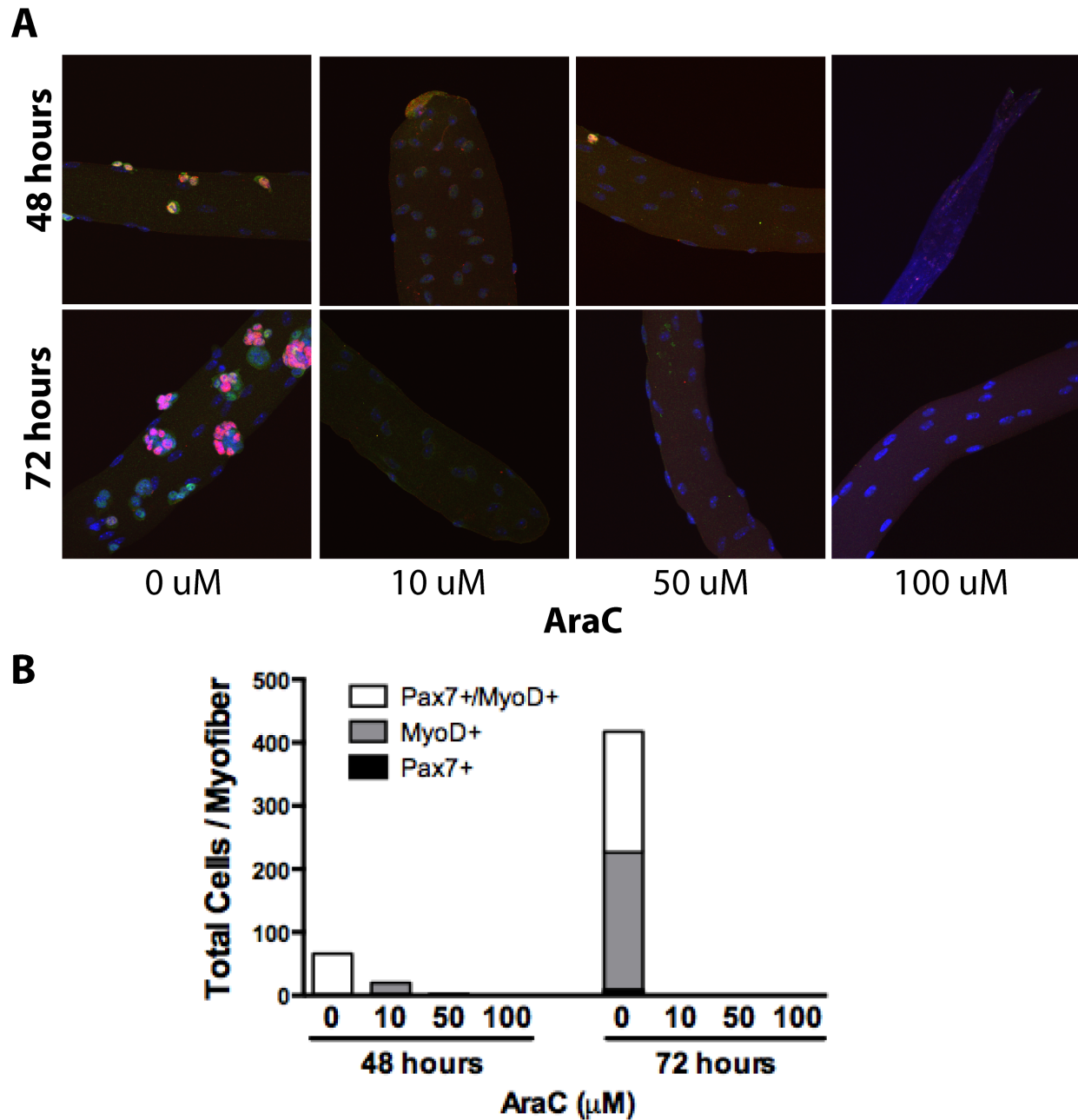


Figure 4: Ablating satellite cells to study RCAS infection of myofibers. To ablate satellite cells, myofibers were treated with AraC, a deoxycytidine analog, which kills dividing cells. **A) AraC concentration and timecourse required for satellite cell ablation.** The AraC treatment survival curve was generated in vitro to determine the concentration of AraC and timecourse needed to ablate myofiber associated satellite cells. Myofibers isolated from the hindlimb of Pax7CreERT2/+;R26LSLTVA(LacZ)/+ mice were treated with 0 uM, 10uM, 50uM, or 100uM AraC and fixed at either 48 or 72 hours. Fibers were immunostained with antibodies targeting MyoD (green) and Pax7 (red) and counterstained with DAPI (blue) to mark nuclei, then scored. One complete representative myofiber was imaged and analyzed per condition. Myofiber-associated satellite cells were observed for untreated conditions (0 uM) at 48 and 72 hours, while few cells

were observed on myofibers for AraC treated conditions **B) Quantitative analysis of satellite cell ablation.** Confocal images from (A) were used to quantify the total number of satellite cells per myofiber by scoring cells as Pax7+/MyoD+, MyoD+, or Pax7+, which mark all mononucleated muscle cells. At 48 hours, effective ablation of all satellite cells was observed for 100 uM AraC, while cell numbers were reduced for 10 uM (20 cells) and 50 uM (2 cells) compared with untreated 0 uM (66 cells). At 72 hours, effective ablation of all satellite cells was observed for 10 uM, 50 uM, and 100 uM, compared with untreated 0 uM (417 cells). The increase in cell numbers between 48 and 72 hour untreated conditions is consistent with satellite cell proliferation. Effective myofiber associated satellite cell ablation was observed for the 10 uM, 72 hour condition.

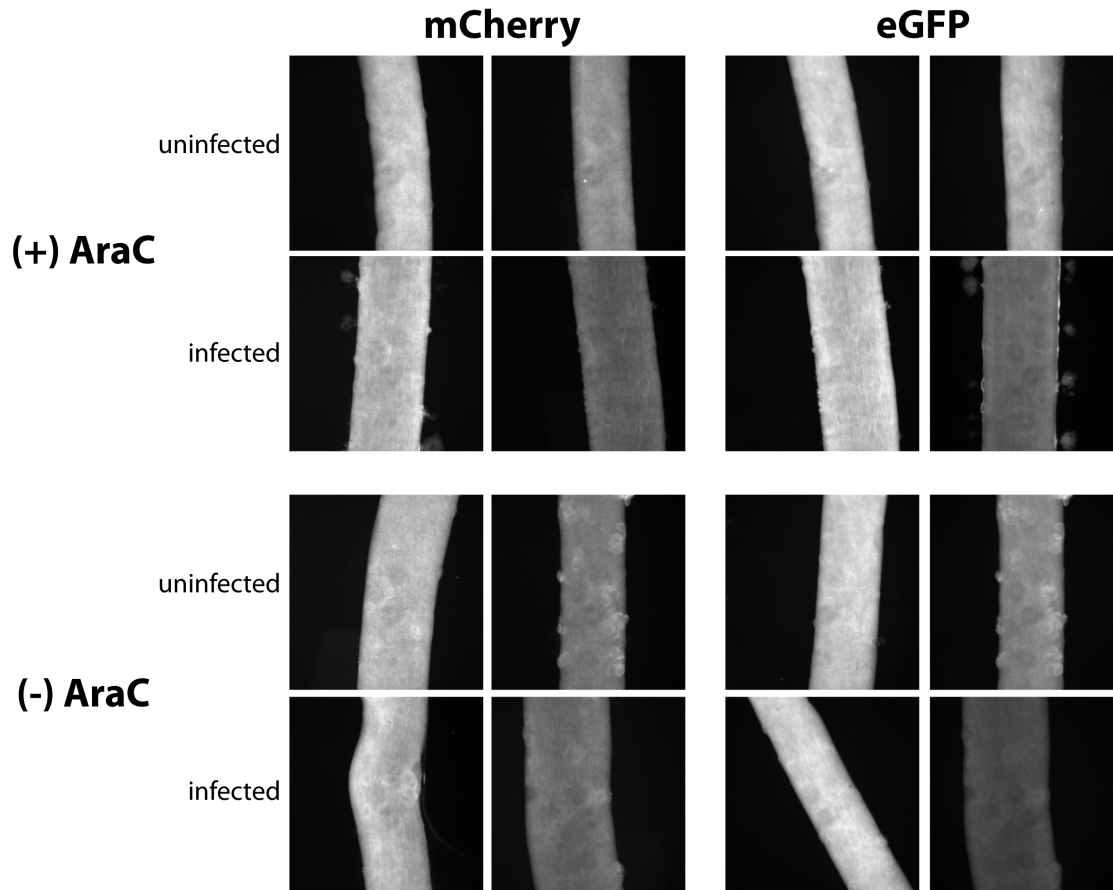


Figure 5: RCAS retroviral infection of myofibers. To determine if myofibers can be infected with RCAS retrovirus in culture, myofibers were isolated from B-actin TVA mice and infected with equal parts eGFP and mCherry RCAS retrovirus. Prior to infection, myofibers were treated with 10 uM AraC for 72 hours to ablate all myofiber associated satellite cells (Figure 4), assuring that any fluorescence detected in the myofibers is due to infection of the myofiber and not infection of the myofiber associated satellite cells that have fused into the myofiber. Images are representative Z-stacks of myofibers fixed at 96 hours. Greyscale images were taken at eGFP (488 nm) and mCherry (586 nm) to compare relative fluorescence intensities between infected and uninfected myofibers (negative control) to detect retrovirus-mediated fluorescent protein expression. Representative images for the highest and lowest intensity myofibers are shown for each condition. The variability in background autofluorescence of myofibers observed in all conditions hinders interpretation of successful detection of eGFP or mCherry infection with RCAS retrovirus.

Lineage tracing myofiber-associated satellite cells using RCAS-mediated fluorescent proteins

As the number of viral infections (MOI) increases, cell number and behavior can be affected as a result of multiple retroviral integrations in the host genome including those which regulate cell division or death. Therefore an MOI of 1, corresponding to a single viral infection within the cell and thus limiting the potential for problematic integration is desirable for single cell lineage tracing. A higher MOI can affect cell numbers either through cell death or over-proliferation of cells. These issues can result in aberrant cell behavior, which would confound lineage tracing analysis. To empirically determine if higher MOI infections affects cell numbers, B-actin TVA myofibers were infected with eGFP and mCherry RCAS retroviruses at a multiplicity of infection (MOI) of 0, 1, or 6 per condition, or 0, 2 and 12 combined conditions. An MOI of 1 correlates to 63% cell infection (which results in one retroviral infection per cell) and an MOI of 6 correlates to 100% cell infection (which results in 6 retroviral infections per cell) (Brown and Faulker, 1975). The average number of cells per myofiber was calculated for 0, 2, and 12 MOI. I found that as MOI increased, the average number of cells per myofiber increased (Figure 6A). These data suggest that a higher MOI may increase cell divisions. Since cell clusters or clones may behave differently than single cells, I also scored cell clusters per myofiber. The average number of cell clusters remained fairly constant among the different MOIs (Figure 6B).

I determined infection efficiency of satellite cells at the individual myofiber level by quantifying the percentage of infected cells per myofiber. The percentage of infected cells per myofiber ranged from 60-90% for MOIs 2 and 12, with the exception of myofiber “c” in MOI 2, which was below 40%. A 100% infection of cells corresponds with an MOI of 6,

however, at twice that, an MOI 12, I did not observe 100% infection of myofiber-associated satellite cells (Figure 6C).

Since multi-color fluorescent protein lineage tracing will require equal delivery of eGFP and mCherry retroviral particles, I concurrently infected untreated avian DF-1 cells with equivalent proportions of eGFP and mCherry corresponding to MOIs of 60, 6, and 1. Equal infection by mCherry and eGFP was observed at MOIs of 60, 6, and 1, with the numbers of infected cells decreasing as MOI decreased, demonstrating functionality of the viral particles (Figure 6D). These data suggest that equal mCherry and eGFP retroviral particles were delivered to myofiber-associated satellite cells in Figure 6. Concurrent delivery of multiple retroviral stocks allows attribution of differences seen in fluorescent expression during lineage tracing to biological mechanisms rather than the unequal delivery of retroviruses to cells.

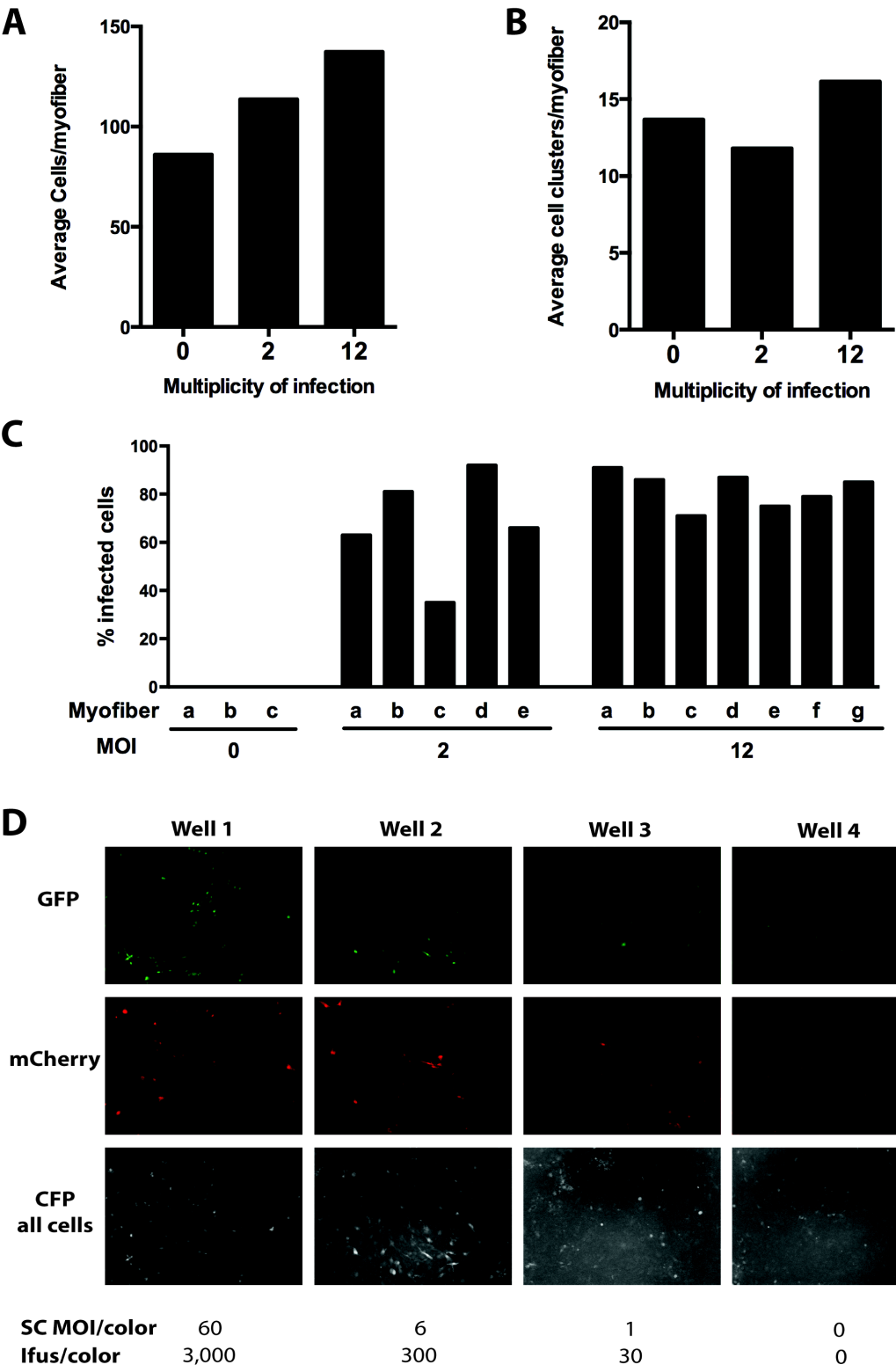


Figure 6: RCAS infection of myofiber-associated satellite cells and DF1 cells at different multiplicities of infection. To assess whether higher MOI infections affects cell numbers and behavior, myofibers were isolated from B-actin TVA mice and infected with eGFP and mCherry RCAS retroviruses at a multiplicity of infection (MOI) of 0, 1, or 6 per condition, or 0, 2 and 12 combined conditions. 72 hours post-infection, myofibers were fixed with 4% PFA, counterstained with DAPI to mark nuclei. **A) Average cells per myofiber.** Total cells per myofiber was calculated for 0, 2 and 12 MOI by counting the number of myonuclei at DAPI (405 nm) and is shown in A. As MOI increased, total cells per myofiber increased suggesting that a higher MOI may increase cell divisions. **B) Average cell clusters per myofiber.** Cell clusters observed at DAPI (405 nm) were scored for each myofiber, excluding single cells, with the average number of cell clusters per myofiber determined for 0, 2 and 12 MOI. Total cell clusters per myofiber remained fairly constant among the different MOIs suggesting that a higher MOI does not alter this aspect of cell behavior. **C) Percentage of cells infected per myofiber.** The percentage of infected cells per myofiber ranged from 60-90% for both MOIs 2 and 12, with the exception of myofiber “c” in MOI 2 which was below 40%. A 100% infection of cells corresponds with an MOI of 6, however, at MOI 12, we did not observe 100% infection of myofiber-associated cells suggesting that some myofiber-associated satellite cells are not accessible to virus during infection. **D) Equal infection of DF-1 cells with eGFP and mCherry RCAS retrovirus.** To confirm equal delivery of eGFP and mCherry RCAS viral particles to myofiber-associated satellite cells, untreated avian DF-1 cells were concurrently infected with the same eGFP and mCherry RCAS retroviral preparations as myofiber cultures in A), B), and C) at an MOI of 60, 6, 1, or 0 per fluorescent protein. Equal infection of DF1 cells with mCherry and eGFP was observed at MOIs of 60, 6, and 1, with the numbers of infected cells decreasing as MOI decreased.

RCAS-mediated fluorescent protein expression was used to lineage trace satellite cells. Myofibers isolated from B-actin TVA mice were infected with equal IFUs of eGFP and mCherry retrovirus (Figure 6D) at a MOI of 0, 1, or 6. Positive and negative clusters of myofiber-associated satellite cells were observed (Figure 7A) along with single cell clones (Figure 3). At an MOI of 0, only negative cells were observed. At MOIs 1 and 6, negative cells, eGFP positive cells, and double positive cells were observed. Few single mCherry cells were detected at MOIs 1 and 6 despite equal delivery of virus as shown in Figure 6D, while mCherry was detected in the double positive condition. The fluorescent protein expression profile of myofiber-associated cells varied between MOIs 1 and 6 for double negative cells, but was similar for negative and eGFP positive cells.

I generated individual plots for each myofiber to investigate whether cell satellite cell fluorescent protein expression varied for individual myofibers. At an MOI of 0, only

negative cells were observed (Figure 7C). At MOIs 1 and 6, negative cells, eGFP positive cells, and double positive cells were observed, however the trend observed in Figure 7B did not represent the variability in trends observed for Figure 7C. Few single mCherry cells were detected at MOIs 1 and 6, while mCherry was detected in double positive cells, suggesting that mCherry is not suitable for lineage design. While the eGFP expression profile of satellite cells varied between individual fibers between and within MOIs 1 and 6, suggesting heterogeneity in eGFP infected satellite cells, I cannot empirically know that I infected the same number of eGFP cells for individual myofibers.

To determine whether single cells or clusters of cells on myofibers behaved differently during lineage tracing, I examined the fluorescent protein expression profile (Figure 7D and E). At an MOI of 0, only negative individual cells and cell clusters were observed. Similar to my results in Figure 7C, the fluorescent protein expression profile for single cells and cell clusters varied dramatically both between and within MOIs 1 and 6 with variability in detection of eGFP positive and double positive cells with little to no detection of single mCherry expressing cells.

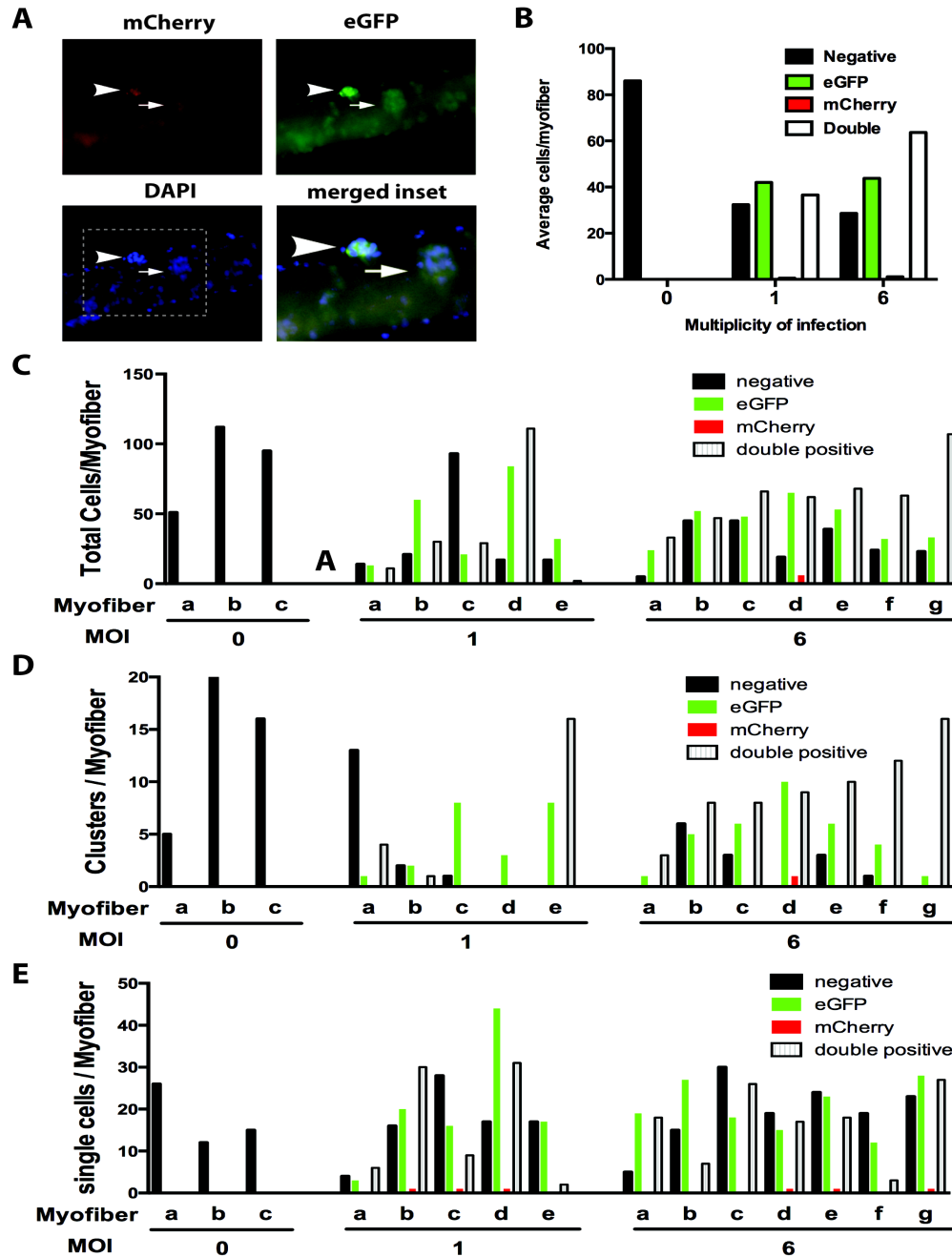


Figure 7: Lineage tracing of myofiber-associated satellite cells. To lineage trace myofiber-associated satellite cells, myofibers were isolated from B-actin TVA mice and infected with equal parts of eGFP and mCherry RCAS retrovirus as performed in Figure 6D at a multiplicity of infection (MOI) of 0, 1, or 6 for each fluorescent protein. 72 hours post-infection, myofibers were fixed and counterstained with DAPI. Myofibers were imaged and analyzed at DAPI (405 nm), eGFP (green; 488 nm) and mCherry (red; 589 nm). **A) Fluorescent protein expression of myofiber-associated satellite cells for lineage tracing.** Clusters of myofiber-associated satellite cells were observed on myofibers along with single cell clones. The white arrowhead denotes a positive cell cluster for eGFP and mCherry; the white arrow denotes a negative cell cluster for both fluorescent proteins. Examples of single positive and negative cells for eGFP and mCherry are shown in Figure

3. B) Fluorescent protein expression in myofiber-associated cells during lineage tracing. At MOI of 0, only negative cells were observed. Negative cells, eGFP positive cells, and double positive cells were observed at both MOI 1 and 6. Few single mCherry cells were detected at MOIs 1 of 6 despite equal delivery of virus as shown in Figure 6D, suggesting that fluorescent protein detection between eGFP and mCherry in satellite cells is not equivalent. **C) Fluorescent protein expression expression per myofiber during lineage tracing.** At MOI of 0, only negative cells were observed. Negative cells, eGFP positive cells, and double positive cells were observed at both MOI 1 and 6. Few single mCherry cells were detected at MOIs 1 of 6. The fluorescent protein expression profile of myofiber-associated cells on individual myofibers varied between and within MOIs 1 and 6. **D) Fluorescent protein expression in cell clusters on individual myofibers.** Since cell clusters may behave differently than single cell clones, I examined the fluorescent protein expression in cell clusters for each myofiber. Clusters were scored for presence of eGFP and mCherry, and as double positive or double negative. At MOI of 0, only negative clusters were observed. Negative clusters, eGFP positive clusters, and double positive clusters were observed at both MOI 1 and 6. Few single mCherry clusters were detected at MOIs 1 of 6. The profile of fluorescent protein expression for cell clusters varied between and within MOIs 1 and 6. **E) Fluorescent protein expression in single cells on individual myofibers.** Since single cells may behave differently than cell clusters, I examined the fluorescent protein expression of single cells per myofiber. Single cells on individual myofibers were scored for presence of eGFP and mCherry, and as double positive or double negative. At MOI of 0, only negative cells were observed. Negative cells, eGFP positive cells, and double positive cells were observed at both MOI 1 and 6. Few single mCherry cells were detected at MOIs 1 of 6. The profile of fluorescent protein expression for single cells varied between and within MOIs 1 and 6. In B), C) and D) Non-equivalent detection between eGFP and mCherry in satellite cells undermines lineage tracing analysis.

Chapter 4: Discussion

I developed a method utilizing avian retroviruses expressing distinct fluorescent proteins to lineage trace individual satellite cells to address satellite cell heterogeneity. I successfully generated retroviral vectors and high-titer retroviruses expressing eGFP, mCherry and mKate for lineage tracing. I was able to infect myofiber-associated satellite cells, supporting the notion that RCAS can be a powerful tool to study satellite cell behavior. I performed two-color lineage tracing of satellite cells. In my approach, a bias in the retroviral-mediated expression of fluorescent proteins in satellite cells over time would represent a heterogeneous satellite cell population. I was unable to examine satellite cell heterogeneity, however, this approach could easily be modified to investigate satellite cell lineage progression.

Generation of RCAS retroviruses containing fluorescent genes

I successfully generated four RCAS retroviruses: eGFP, mCherry, mKate, and BFP (Figure 1). I encountered nuclear counter-staining issues with BFP, as the nuclear stain DAPI (405 nm) excites near the same wavelength as BFP (402 nm). I attempted to use the ToPro3 (far red) to mark nuclei when imaging samples containing BFP, but detected ToPro3 emission in the eGFP and mCherry channels. As such, (absent a compatible nuclear stain) BFP is not optimal for lineage tracing. To titer retroviruses, I utilized a serial dilution titrating protocol to calculate RCAS retroviral titers (Figure 2) that is both accurate and similar to a prior published titrating protocol (Werder et al 2012, Pao et al 2003, Logan and Tabin 1998, Fekete and Cepko 1993). I calculated retroviral titers to ensure delivery of equal viral particles when infecting satellite cells for fluorescent protein lineage tracing. I hypothesized that filtering RCAS retroviral stocks would decrease retroviral titer, but in

fact discovered that filtering RCAS retroviral stocks increased retroviral titer (Table 1). It is possible that DF1 membrane fragments and retroviral aggregates present in the unfiltered stocks could bind retroviral particles, decreasing total IFUs available for infection.

Infection of skeletal muscle with RCAS retroviruses

I successfully infected myofiber-associated satellite cells with RCAS retroviruses (Figure 3). I labeled, traced, and interrogated satellite cell behavior over time. In addition to lineage tracing, future research can use the RCAS approach to interrogate satellite cell behavior.

I was unable to confirm RCAS retroviral infection of myofibers in the absence of myofiber-associated satellite cells (Figure 5). I successfully ablated myofiber-associated satellite cells by treating them with 10 μ M AraC (Figure 4) which is consistent with published data (Troy et al 2012). Ablation of satellite cells was essential for the attempted RCAS retroviral infection of myofibers, as satellite cell infection would confound analysis of infection of the myofiber as infected satellite cells could fuse into myofibers. The variability in background autofluorescence of myofibers hindered interpretation of RCAS retroviral infection. Variability in autofluorescence could be due to different muscle fiber types, with each fiber type having different levels of autofluorescence.

I performed PCR to detect potential RCAS retroviral infection as a secondary method for confirming infection of myofibers in the absence of associated satellite cells. Using genomic DNA from infected myofiber cultures, I attempted to amplify regions of the RCAS viral construct that would have integrated, but the DNA yield for approximately 100 myofibers was too low to detect viral DNA (data not shown). To avoid autofluorescence

observed in my experiments, future RCAS retroviral infection should focus on the expression of genetic markers not requiring fluorescence microscopy.

Lineage tracing satellite cells using RCAS-mediated fluorescent protein expression

A single retroviral infection (MOI) per satellite cell is essential for adequate lineage tracing, as a high number of retroviral infection events per cell can lead to multiple integrations, marking the cell with more than one fluorescent protein. Multiple infections per cell could lead to aberrant cell behavior due to integration in regions required for survival, confounding lineage tracing analysis. I determined that increasing MOI of RCAS retrovirus correlated with a subtle increase in cell number (Figure 6A,B). While the data showed a subtle correlation, the experiments were not designed to determine the cause of increased cell number, therefore I cannot directly attribute increased cell numbers to multiple retroviral integrations. However, experimental data does not always replicate theoretical biology. For example, a theoretical MOI of 6 corresponds to 100% infection of cells corresponds to an MOI of 6 (six infections per cell) (Brown and Faulker, 1975). Even at twice the theoretical value yielding 100% infection (MOI = 12), I did not observe 100% infection of satellite cells (Figure 6C). A subset of satellite cells may be protected by the basal lamina, preventing satellite cell infection under culturing conditions by restricting access to viral particles.

Multiple fluorescent protein-based lineage tracing requires equal delivery of all retroviruses to allow the attribution of differences in fluorescent protein expression during lineage tracing to biological mechanisms. I successfully delivered equal mCherry and eGFP retroviral particles to DF1 cells and myofiber-associated satellite cells (Figure 6D). However, I observed variability in numbers of eGFP positive cells between individual

myofibers at the same MOI (Figure 7C,D,E). Perhaps some eGFP positive cells divide more often than other eGFP positive cells, potentially due to either heterogeneity or integration of the viral genome in genes controlling proliferation. In addition, there may have been more cells infected with eGFP on some myofibers compared to others. I was unable to determine whether each fiber was infected with an equal number of viral particles for eGFP and mCherry. The underlying conceptual issue in the lineage tracing design is the fact that each infection is an independent event. If bias in one particular viral construct is observed, the bias likely reflects differences in the actual number of cells initially infected with said construct rather than a biological difference in satellite cell behavior.

I encountered problems with detecting mCherry positive cells during lineage tracing. Optimal excitation for mCherry occurs at a wavelength of 586 nm. Unfortunately, the available microscope laser excites at 546 nm, preventing optimal excitation and visualization of the emission spectra. mCherry was frequently observed in double positive (mCherry+/eGFP+) cells, but rarely was detected in single cells or cell clusters. Originally I considered the possibility of spectral bleedover from the emission of the eGFP channel into the mCherry channel. However, if that had been the case, I would have seen a mirroring of data with single eGFP positives and double positives, which was not observed. In fact, on a single myofiber I often observed many double positives, but few eGFP single positives. There may be some biological phenomena where the mCherry and eGFP synergistically interact to enhance detection of mCherry. This highlights the importance of generating vectors that match the detection capabilities of microscopes available at the time of vector generation. In future lineage tracing studies, viral constructs should be generated with

fluorescent proteins matching available imaging equipment, and consideration should be given to overlap between the emission and excitation spectra of fluorescent proteins.

The fluorescent protein expression profiles for total cells, single cells, and cell clusters per individual myofiber varied between and within MOIs 1 and 6. The trends observed for average cells per myofiber (Figure 7B) were not observed for total cells per individual myofiber, cell clusters per individual myofiber, or single cells per individual myofiber. (Figure 7C,D,E). The variability of fluorescent protein expression for single cells and clusters between myofibers at each MOI suggests the current technique cannot be used to delineate satellite cell heterogeneity. Furthermore, assuming a subset of satellite cells are heterogeneous, I cannot control for infecting the same subset of cells for each myofiber to compare fluorescent protein expression profiles. Overall, I cannot control equal infection of individual satellite cells nor can I preferentially infect a subset of satellite cells if they exist. RCAS retroviruses containing unique variable DNA barcodes (DNA sequence tags) instead of fluorescent proteins could allow lineage tracing of myofiber-associated satellite cells (Lu et al., 2011; Bystrykh et al, 2012). This method of lineage tracing would allow for unique genomic marking for each individual satellite cell both *in vitro* and *in vivo*.

Through the course of this project I utilized a combination of *in vitro* satellite cell analysis and RCAS retroviral infection to demonstrate proof of concept for several techniques, although I was unable to generate functional data indicating heterogeneity. I generated high titer RCAS retrovirus and was able to obtain retroviral-mediated expression of varied fluorescent proteins via the infection of both avian and primary mammalian cells expressing the TVA receptor. While I was unable to lineage trace myofiber-associated satellite cells with a fluorescent protein-based experimental design, my work demonstrates

proof of concept that RCAS can be a powerful tool to interrogate satellite cell behavior and function through *in vivo* cell type-specific retroviral-mediated gene expression.

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