

Kinase Domain Receptor is a modulator of Satellite Stem Cell Asymmetric Division

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Abstract

The regulation of muscle stem cell (MuSC) asymmetric division plays an essential role in controlling the growth and repair of skeletal muscle. Perturbations in MuSC function have been demonstrated in disease and aging contexts such as Duchenne's Muscular Dystrophy (DMD) and sarcopenia. We developed and optimized a high content analysis platform combining lineage tracing, myofiber culture, imaging, and bioinformatic analysis to determine modulators of muscle stem cell division. We discover kinase domain receptor (KDR) as a positive modulator of MuSC asymmetric division and confirmed its expression in satellite cells by ddPCR and immunofluorescence. Knockdown of KDR significantly reduces the numbers of asymmetric divisions, whereas ligand stimulation of KDR increases the numbers of asymmetric divisions. KDR signaling is impaired in dystrophin-deficient satellite cells and requires a polarized cell environment established by the dystrophin glycoprotein complex (DGC) to direct asymmetric division. Mice lacking KDR in MuSCs exhibit reduced numbers of satellite cells due to precocious differentiation, and deficits in regeneration consistent with impaired asymmetric division and reduced generation of progenitors. Therefore, our experiments identify KDR signaling as playing an essential role in MuSC function in muscle regeneration. These findings further our understanding of muscle stem cell biology, and in particular, the role of asymmetric division under homeostatic and regenerative conditions.

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List of Abbreviations

4-OHT	4-hydroxy tamoxifen
AurA	Aurora kinase A
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CALCR	Calcitonin receptor
Carm1	Coactivator Associated Arginine Methyltransferase 1
cDNA	Complementary DNA
CEE	Chicken Embryo Extract
Cre	Cre recombinase
CreER	Cre recombinase-estrogen receptor (tamoxifen activated nuclear localization domain) fusion
CTX	Cardiotoxin
Dag1	Dystroglycan
DAPI	4',6-diamidino-2-phenylindole
ddPCR	Digital droplet polymerase chain reaction
DGC	Dystrophin glycoprotein complex
DMD	Duchenne's Muscular Dystrophy
Dmd	Dystrophin
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DsiRNA	Dicer-substrate short interfering RNAs
EC	Endothelial cell
EDL	Extensor digitorum longus
EdU	5-Ethynyl-2'-deoxyuridine
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FAPs	Fibroblast adipogenic progenitors
FBS	Fetal bovine serum
FDB	Flexor digitorum brevis
Fgfr4	Fibroblast growth factor receptor 4
fl or flox	Flanked by LoxP
Flk1	Fetal Liver Kinase 1 (See KDR)
FLT-1	FMS related receptor tyrosine kinase 1 (See VEGFR1)
FLT-4	FMS related receptor tyrosine kinase 4 (See VEGFR3)
FSC	Freshly sorted cells

Fzd7	Frizzled 7
GA	Gastrocnemius
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HRP	Horseradish peroxidase
IGFR-1	Insulin growth factor receptor 1
IgG	Immunoglobulin G
KDR	Kinase domain receptor
KO	Knock out
loxP	Locus of X-over P1
Itga7	Alpha-7 integrin
LWR	Ratio of length to width of an object
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinase
mdx	X chromosome-linked mouse mutant
MHV	Murine coronavirus
MNV	Murine norovirus
MRF	Myogenic regulatory factor
mTOR	mammalian target of rapamycin
MuSC	Muscle stem cell
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein 1
MyoG	Myogenin factor 4
Nrp1	Neuropilin-1
Nrp2	Neuropilin-2
ns	Not significant
OICR	Ontario Institute for Cancer Research Kinase Inhibitor
P2A	perimeter squared to 4PI*area of object
Pax7	Paired box protein Pax-7
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PLA	Proximity ligation assay
PMC	proliferating myogenic precursor
PPIA	Peptidylprolyl isomerase A
R26R	ROSA ^{geo} 26 β locus
ROCK	Rho-associated coiled-coil-containing protein kinase 1
RPS20	Ribosomal protein 20
RSP18	Ribosomal protein 18
RT	Reverse Transcriptase

S6K	Ribosomal protein S6 kinase beta-1
Sdc4	Syndecan-4
TA	Tibialis anterior
Tbp	Tata-binding protein
VCAM1	Vascular cell adhesion molecule 1
VEGFR1	Vascular endothelial growth factor receptor 1
VEGFR2	Vascular endothelial growth factor receptor 2 (See KDR)
VEGFR3	Vascular endothelial growth factor receptor 3
Veh	Vehicle
Wnt7a	Wingless-Type MMTV Integration Site Family, member 7
YFP	Yellow fluorescent protein

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Chapter 1. Introduction

1.1 Skeletal Muscle

Skeletal muscle is highly organized, composed of muscle fascicles consisting of multinucleated myofibers grouped together surrounded by connective tissue (Gillies and Lieber, 2011). Within each myofiber contain myofibrils composed of repeating subunits of actin and myosin protein arrays called sarcomeres (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954). Satellite cells, responsible for the regenerative capacity of muscle, reside on the periphery of myofibers (**Figure 1**) (Kuang et al., 2007; Mauro, 1961).

Skeletal muscle accounts for approximately 40% of total body weight, and is required for numerous critical bodily functions, including voluntary movement, stability, posture, respiration, thermogenesis, circulation, and storage for amino acids and carbohydrates (Aliverti et al., 1997; Huxley and Hanson, 1954; Huxley and Niedergerke, 1954; Ivanenko and Gurfinkel, 2018; Morrison, 2016; Negrini and Moriondo, 2011; Rolfe and Brown, 1997; Sangwan et al., 2014; Wolfe, 2006). Consequently, skeletal muscle loss is an indicator of mortality in age and disease and is associated with numerous comorbidities such as osteoporosis, renal disease, cardiovascular disease, COPD, cancer, obesity and age-related decline (Kalyani et al., 2014; Wolfe, 2006).

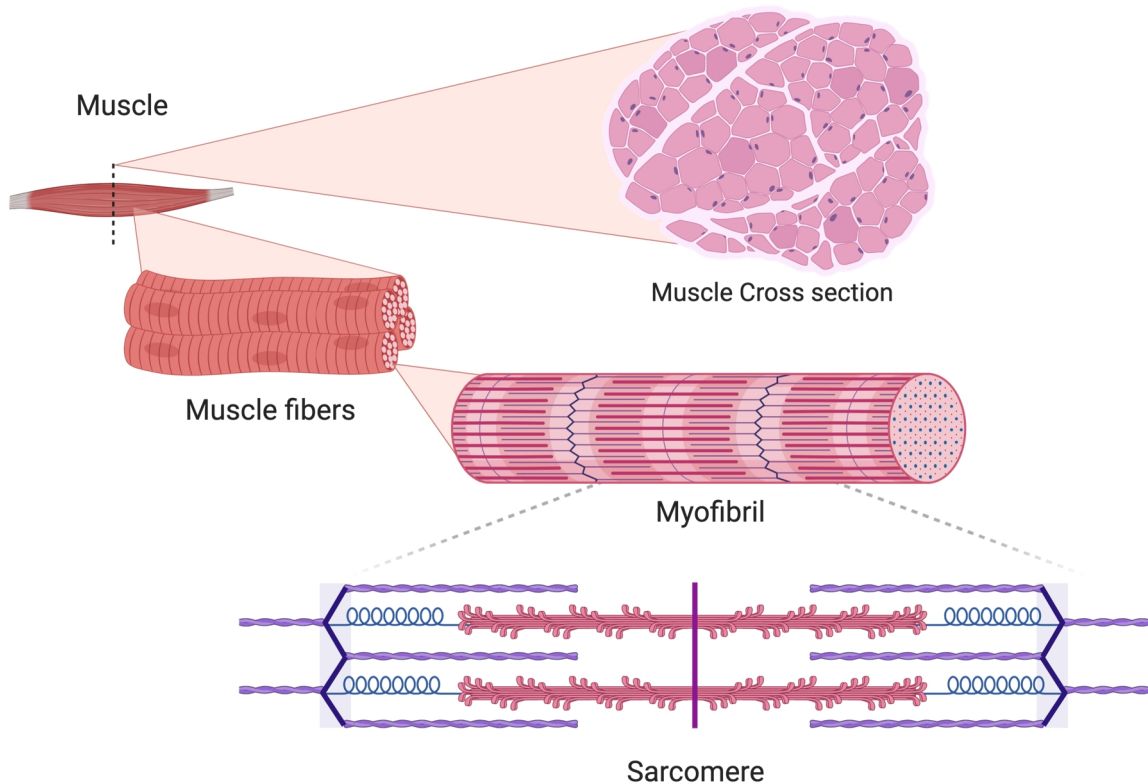


Figure 1. Skeletal Muscle Structure Hierarchy

The basic unit of contraction is the sarcomere, a repeating array of overlapping myosin thick (pink) and actin thin (purple) filaments that create a dark and light banding pattern within myofibrils. Muscle is composed of fascicles, groups of myofibers, which are in turn composed of groups of myofibrils. Muscle cross section depicts the organization of fascicles: groups of myofibers (pink), and satellite cells (purple) that reside on the periphery of myofibers.

1.2 Muscle Dystrophy and Therapeutic Challenges

Muscle dystrophies are a group of diseases that result in the chronic wasting of muscle over time. A multitude of muscular dystrophies and gene mutations have been

identified, including Congenital Muscular Dystrophy (CMD), Limb-Girdle Muscular Dystrophy (LBMD), Emery Dreifuss Muscular Dystrophy (EDMD), Myotonic Dystrophy Type I and Type II (DM1, DM2), Fascioscapulohumeral Muscular Dystrophy (FSHD), Duchenne's Muscular Dystrophy (DMD), and Becker's Muscular Dystrophy (BMD) (Emery, 2002; McNally and Pytel, 2007).

Duchenne's Muscular Dystrophy (DMD) remains the most common muscular dystrophy, affecting approximately 1 in 3500 males (Emery, 2002). The causes of DMD are attributed to frame shift point mutations within the dystrophin gene leading to a truncated dystrophin protein (Brockdorff et al., 1987; Hoffman et al., 1987). The loss of dystrophin results in the inability to assemble the Dystrophin Glycoprotein Complex (DGC) required for maintaining sarcolemma integrity and prevention of contraction-induced damage leading to cycles of degeneration/regeneration and infiltration of fibrotic and adipotic tissue (Eisner et al., 2020; Joe et al., 2010; Kharraz et al., 2014; Petrof et al., 1993). Thus the standard of care for DMD patients has been limited to corticoid steroids aimed at reducing inflammation, non-invasive ventilation, and physiotherapy to maintain muscle function (Birnkrant et al., 2018; Sejerson et al., 2009). Clinical advances in managing DMD have expanded the life expectancy of patients into the 5th decade of life (Saito et al., 2017).

The development of murine, canine and porcine models of DMD have enabled a better understanding of muscle regeneration and disease pathology, yet a number of challenges remain when designing new therapeutics for treating DMD. Disease

modelling has been one challenge, as animal DMD models do not completely replicate human DMD pathology. For example, the *mdx* mouse model demonstrates a much more mild phenotype compared to human dystrophic patients and therapies designed in mouse models has found little success when translating to humans (Selsby et al., 2015). Scale-up of pharmacological dosage, regiment and viral titers is also a challenge in smaller animal models including murine, feline and fish animal models (McGreevy et al., 2015; Selsby et al., 2015). Unfortunately, pharmacological therapeutics are unable to restore muscle function long-term required for full patient autonomy, and an effective cure for genetic diseases such as DMD requires gene therapy. Porcine and canine animal models are emerging as important translational animal models for the design and development of novel gene replacement therapies (Bulfield et al., 1984; López et al., 2018; McGreevy et al., 2015; Selsby et al., 2015).

AAV-delivery of mini- or micro- dystrophins have demonstrated great promise in these animal models, achieving high rates of transduction leading to improved cardiac morphology and function, and reduced inflammation and fibrosis (Kupatt et al., 2021). Several human clinical trials utilizing AAV-delivery of micro-dystrophin (NCT03375164, NCT03368742) and mini-dystrophin (NCT03362502) are currently undergoing investigation, with promising findings, showing significant delivery and robust expression of micro-dystrophin in skeletal, diaphragm and heart muscles coupled with a reduction in creatine kinase levels indicating reduced muscle damage. In addition to restoring dystrophin and its structural function within fully differentiated skeletal

muscle, the success of these novel gene therapies may be partially explained by the restoration of dystrophin expression and regenerative capacity in the muscle satellite stem cell population. Duchenne's Muscular Dystrophy is also a muscle stem cell disease, and dystrophin is essential for establishing stem cell polarity required for asymmetric divisions, allowing for the maintenance of the stem cell population and generation of myogenic precursors required for efficient muscle repair (Dumont et al., 2015a).

1.3 Muscle Satellite Cells and Muscle Regeneration

Muscle has a remarkable regenerative capacity, due to the satellite cells positioned on the periphery of myofibers, situated between the myofiber sarcolemma and basal lamina (Mauro, 1961). Satellite cells are required for the growth and repair of skeletal muscle. In response to injury, satellite cells transition from G₀ quiescence or G_{ALERT} to activation, undergoing multiple rounds of division to generate progenitors required for muscle repair (Dumont et al., 2015b; Rodgers et al., 2014; Schultz et al., 1986). Transplantation and serial injury experiments have demonstrated that a small subset of satellite cells are bona fide muscle stem cells, capable of re-establishing the muscle stem cell pool (Collins et al., 2005; Kuang et al., 2007; Sacco et al., 2008).

Satellite cells are identified by the expression of transcription factor *Pax7* (Seale et al., 2000). Satellite cells express a number of membrane markers including syndecan-4 (Sdc4), vascular cell adhesion molecule 1 (VCAM1), alpha-7 integrin (Itga7), notch receptors, calcitonin receptor (CalcR), frizzled 7 (Fzd7), and epidermal growth factor

receptor (EGFR) (Baghdadi et al., 2018a; Cornelison et al., 2004; Fukada et al., 2007; Gnocchi et al., 2009; Grand et al., 2009; Kann and Krauss, 2019; Seale et al., 2000; Wang et al., 2019; Yin et al., 2013; Zhang et al., 2019). The satellite cell population is heterogeneous consisting of a majority short-term repopulating cells, and a minority subset of long-term self-renewing stem cells that give rise to committed progenitors through asymmetric cell divisions (Gurevich et al., 2016; Kuang et al., 2007; Rocheteau et al., 2012). Notably, the long-term self-renewing stem cells do not express the myogenic transcription factor Myf5, which distinguishes stem cells from short-term self-renewing cells and committed progenitors (Kuang et al., 2007).

Muscle regeneration can be divided into three phases: proinflammatory phase, anti-inflammatory phase, and the remodelling phase. Satellite cells may be activated through a number of cues, including changes to the extra cellular matrix (ECM), heat release, mechanical transduction of myofibers, Ca^{2+} release from sheared t-tubules, and pro-inflammatory cytokines released by damaged myofibers into the muscle stem cell niche (Baghdadi et al., 2018a; Moyle et al., 2020; Oishi and Ogata, 2012; Urciuolo et al., 2013; Yamada et al., 2006; Yamaguchi et al., 2015; Yin et al., 2013; Zhang and Anderson, 2014). Within the first 24 hours of satellite cell activation, fast-dividing myogenic precursors and fibro/adipogenic progenitors (FAPs) increase in number, while neutrophils and M1 macrophages migrate to areas of damage. Together, these cell populations release a number of proinflammatory cytokines, mitogenic cell-signaling molecules and ECM deposits to further enhance activation and proliferation (Chazaud et al., 2009; Grand et al., 2009; Joe

et al., 2010; Lazure et al., 2020; Merly et al., 1999; Olguin and Olwin, 2004; Seale et al., 2004; Villalta et al., 2009; Wang et al., 2019; Yin et al., 2013). Approximately 2-4 days post injury, M2 macrophages secrete anti-inflammatory cytokines important for promoting myogenic differentiation and diminishing the FAP population, which is required for efficient muscle regeneration and prevention of fibrotic and adipocytic tissue infiltration (Cantini and Carraro, 1995; Cantini et al., 2002; Joe et al., 2010; Natarajan et al., 2010; Villalta et al., 2009; Yin et al., 2013). During the final remodelling stage of muscle regeneration, vasculature is pruned, FAP numbers return to basal steady state, myofibers mature in size, and satellite cells migrate back to the satellite cell position and withdraw from the cell cycle to return to quiescence (Baghdadi et al., 2018a; Chen et al., 2020; Natarajan et al., 2010; Verma et al., 2018; Woszczyna and Rando, 2018; Yin et al., 2013).

1.4 Asymmetric Division and Muscle Stem Cell Polarity

Asymmetric division is an essential biological process in stem cell biology, required for maintaining stemness, maintenance of the stem cell pool, and the generation of progenitors during regeneration and repair (Bernet et al., 2014; Kuang et al., 2007; Lechler and Fuchs, 2005; Sacco et al., 2008; Wang et al., 2019; Williams et al., 2011; Zismanov et al., 2016). The importance of asymmetric division has been demonstrated in disease and aging, when changes in asymmetric divisions are usually coupled with changes in symmetric stem cell divisions, and prolonged periods of quiescence (Dumont et al., 2015a; Florian et al., 2018; Price et al., 2014).

The molecular mechanisms that control and modulate asymmetric division are conserved across both species and stem cell types (Chang et al., 2016; Dewey et al., 2015; Dumont et al., 2015a; Lechler and Fuchs, 2005; Wang et al., 2019). Asymmetric divisions require the polarization of the cellular cortex, and orientation of mitotic spindles in an apical-basal fashion to facilitate the asymmetric segregation of proteins, cell membrane components, and signaling molecules that determine cell fate (Asare et al., 2017; Bernet et al., 2014; Conboy and Rando, 2002; Dewey et al., 2015; Dumont et al., 2015a; Lechler and Fuchs, 2005; Price et al., 2014; Wang et al., 2019). For example, the Par/aPKC complex involving atypical protein kinase C (aPKC), partitioning defect 3 (Par-3), and partitioning defect 6 (Par-6) is essential for polarizing the cell cortex across multiple species (Dewey et al., 2015; Dumont et al., 2015a; Lechler and Fuchs, 2005). Cell polarity is established when aPKC/Par-3/Par-6 are localized opposite to Par-1, modulating the fate-determinant Notch-signaling pathway (Bayraktar et al., 2006; Chang et al., 2016; Conboy et al., 2005; Dewey et al., 2015). The Pins/Mud/Dlg mitotic spindle modulating complex is also well characterized, and conserved across organisms and cell types, required for the asymmetric segregation of fate-determinant signals during mitosis (Dewey et al., 2015; Lechler and Fuchs, 2005; Wang et al., 2019).

Within muscle, the pathways that regulate the choice between MuSC symmetric and asymmetric divisions have a significant impact on the efficiency of muscle regeneration. Wnt7a/Fzd7 stimulation of the planar cell polarity pathway that drives MuSC symmetric expansion increasing satellite cell numbers and enhancing regeneration (Grand

et al., 2009). By contrast, increased JAK2/STAT3 signalling during aging results in reduced numbers of MuSC by inhibiting symmetric divisions (Price et al., 2014; Tierney et al., 2014). Similarly, activation of p38a MAPK signaling in MuSC promotes asymmetric division, commitment to differentiation in ageing, and loss of regenerative potential (Bernet et al., 2014; Cosgrove et al., 2014). Recently, we identified EGFR/Aurka signaling as regulating asymmetric MuSC divisions and that activation of this pathway significantly ameliorated disease progression in *mdx* mice, a mouse model for Duchenne's Muscular Dystrophy (DMD), by rescuing the loss of asymmetric divisions in dystrophin deficient satellite cells (Wang et al., 2019). Therefore, the control of MuSC asymmetric division plays a key role in regulating the efficiency of muscle regeneration in health and in disease.

Polarization of the intrinsic and extrinsic satellite stem cell environment is essential for maintaining stem cell identity and quiescence (Baghdadi et al., 2018b, 2018a; Bernet et al., 2014; Chang et al., 2018; Chen et al., 2020; Cosgrove et al., 2014; Crist et al., 2012; Dumont et al., 2015a; Grand et al., 2009; Haller et al., 2017; Kuang et al., 2007; Morrée et al., 2017; Price et al., 2014; Shea et al., 2010; Verma et al., 2018; Zhang et al., 2019). The immediate satellite cell niche is a polarized environment with the satellite cell situated between the basal lamina and myofiber sarcolemma (Yin et al., 2013). This polarization aids the satellite cell in providing cues to divide asymmetrically in an apical-basal fashion (Kuang et al., 2007; Wang et al., 2019).

The intrinsic polarized satellite cell environment is established in part by the dystrophin-associated glycoprotein complex (DGC) (Dumont et al., 2015a). In muscle

stem cells, dystrophin and Mark2 are colocalized. Mark2 phosphorylation of Pard3 localizes Pard3 expression to the opposite side of the cell, polarizing the cell cortex (Dumont et al., 2015a). Additionally, the EGFR-AurkA pathway orients mitotic centrosomes in an apical-basal orientation, facilitating asymmetric stem cell divisions (Wang et al., 2019). Together, the extrinsic and intrinsic polarized cues and environment are necessary for the asymmetric segregation of transcription factors and proteins required for asymmetric division to occur (Chang et al., 2018; Conboy and Rando, 2002; Dumont and Rudnicki, 2017; Price et al., 2014; Shea et al., 2010; Shinin et al., 2006; Wang et al., 2019). The loss of cell polarity, such as in DMD, results in reduced numbers of asymmetric divisions, reduced generation of progenitors, and a decline in muscle regenerative capacity (Dumont et al., 2015a).

1.5 High Content Analysis and High Throughput Screening

Recent technological advances in microscopy and detection algorithms have enabled the emergence of high content and high throughput screening techniques (Carpenter, 2007). Early use of high content screening started with drug screening and drug discovery, but in recent decades has seen increased use in basic science for the investigations into biological processes (Szymański et al., 2011; Thomsen et al., 2005; Zanella et al., 2010). The advantage of high content and high throughput screening is the ability to identify changes in cell morphologies and biological processes in a relatively short amount of time by testing hundreds or thousands of conditions in parallel (Boutros

and Ahringer, 2008; Kiger et al., 2003; Liu et al., 2009; Szymański et al., 2011; Thomsen et al., 2005).

High content, high throughput screening is extremely versatile technique, and can be adapted for use in any biological system (**Figure 2**). Numerous biological assays have been developed for high content screening using immortal cell lines, primary cells, and stem cells combined with fluorescent reporters and lineage tracing techniques to test small molecule compounds, peptides, RNAi, and CRISPR (Barbuti et al., 2020; Boutros and Ahringer, 2008; Chen et al., 2006; Kiger et al., 2003; Li et al., 2020; Liu et al., 2009; Nazlamova et al., 2020; Valtonen et al., 2020).

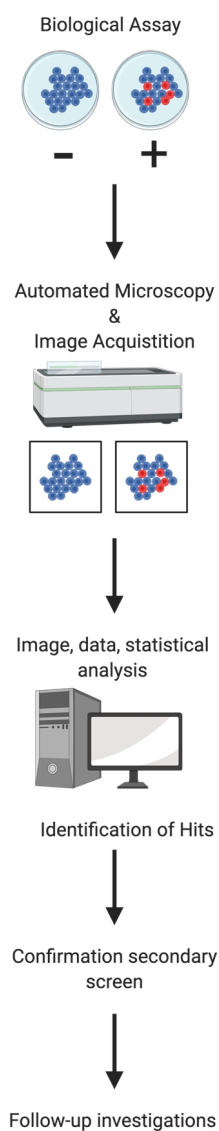


Figure 2. Basic Components of High Content and High Throughput Analysis Screening

A biological assay with positive and negative readouts is imaged. Analysis of test conditions identify prospective hits. Shortlist of hits are confirmed with a secondary screen. Follow-up investigations are performed on confirmed targets.

1.6 The KDR (VEGFR2) Signaling Pathway in muscle satellite stem cells

Multiple reports have provided evidence of an endothelial-like gene expression signature present in satellite cells (Angelis et al., 1999; Cosgrove et al., 2009; Giordani et al., 2019; Goel et al., 2017; Mayeuf-Louchart et al., 2014; Motoike et al., 2003). Interestingly, Pax7-expressing satellite cells are derived from the same mesodermal multipotent Pax3⁺ population that gives rise to CD31⁺ endothelial cells (Mayeuf-Louchart et al., 2014; Motoike et al., 2003). Kinase Domain Receptor (KDR), also known as VEGFR2 and Flk1, is a receptor tyrosine kinase activated by binding of VEGFA, VEGFC and VEGFD, that plays an essential role in angiogenesis, vascular development, vascular permeability, and embryonic hematopoiesis (Apte et al., 2019). Notably, VEGFA ligand is abundantly expressed within the muscle by nearly all components of the satellite cell niche including myofibers, satellite cells, endothelial cells, and fibroadipogenic cells (Blackburn et al., 2019; Christov et al., 2007; Micheli et al., 2020; Rissanen et al., 2002; Verma et al., 2018; Wagatsuma et al., 2006). However, a physiological role for KDR in muscle stem cell biology has never been previously reported.

Here we employed an *ex vivo* high content MuSC screen and identified KDR signaling as a modulator of asymmetric division. siRNA mediated knockdown of KDR significantly inhibits asymmetric divisions. Conversely, ligand stimulation of KDR increases the numbers of muscle stem cell asymmetric divisions. This pathway is impaired

in *mdx* mice and cannot be rescued by ligand treatment. Impairment of KDR-induced muscle stem cell asymmetric divisions result in a cascading effect on muscle regeneration, leading to fewer progenitors in response to injury. Therefore, we conclude the KDR pathway is a critical component of stem cell function, and this process is necessary for both muscle regeneration and maintenance.

1.7 Rationale and Objectives

Our laboratory has demonstrated that DMD is also a stem cell disease. Dystrophic satellite cells demonstrate a number of severe intrinsic defects that affect proper cell division including loss of cell polarity, irregular cell division patterns, and prolonged cell divisions that lead to stem cell hyperplasia (Dumont et al., 2015a). Consequently, satellite stem cells lacking dystrophin are unable to efficiently divide asymmetrically to maintain proper numbers of functional stem cells. We hypothesize that there are additional cellular pathways that are essential for asymmetric cell division. Our objective is to investigate these additional pathways modulating asymmetric satellite stem cell division through three aims:

1. Design and develop a high content screening platform to identify novel targets of asymmetric stem cell division.
2. Confirm the target effect on asymmetric division using *in vitro* and *ex vivo* models.
3. Investigate the regenerative impact *in vivo* of our target in the context of asymmetric division.

Chapter 2. Methods

2.1 Mice and Animal Care

We performed all experiments in accordance with the Canadian Council Animal Care Guidelines and the University of Ottawa Animal Care Committee. We used adult 2-3 month old mice for all experiments. Mice were housed in ventilated cages and ¼” corncob bedding changed every 14 days. Mice were supplied food *ad libitum* with automated acidified RO water or bottles changed every 7 days. Light cycles were 12-12h cycle with simulated sunrise and sunset. Health and immune status of experimental mice were normal where Sentinels are routinely monitored (fecal, fur, and oral swab) for *MNV*, *MHV*, Mouse *Pneumocystis*, pinworm, and *Spironucleus muris*. No animals were subjected to previous experimental procedures.

The following mouse lines were used: C57BL/B10ScSn-*Dmd*^{mdx}/J (mdx, homozygous *Dmd*^{mdx} females and hemizygous *Dmd*^{mdx} males). B6.Cg-*Pax7*^{tm1(cre/ERT2)Gaka/J} (*Pax7-Cre*^{ERT2}), B6.129S4-*Myf5*^{tm3(cre)Sor/J} (*Myf5-Cre*), B6.129X1-*Gt(ROSA)26Sor*^{tm1(EYFP)Cos/J} (*R26R-eYFP*), B6.SJL-*Pax7*^{tm1.2Tajb} (*Pax7-nGFP*), B6.129S1-*KDR*^{tm2Sato/J} (*KDR*^{fl}), *KDR*^{tm2.1Jrt} (*Flik1-GFP*) (Bulfield et al., 1984; Cheers and McKenzie, 1978; Ema et al., 2006; Hooper et al., 2009; Murphy et al., 2011; Sambasivan et al., 2009; Srinivas et al., 2001; Tallquist et al., 2000) .

Name	Allele	Background	Source	Identifier
Wild type	-	C57BL/10 ScSn	JAX	000476 (Cheers and McKenzie, 1978)
<i>DMD^{mdx}</i>	<i>DMD^{mdx}</i>	C57BL/10 ScSn	JAX	001801 (Bulfield et al., 1984)
<i>Pax7^{creER}</i>	<i>Pax7^{tm1(cre/ERT2)Gaka}</i>	B6.Cg	JAX	017763 (Murphy et al., 2011)
<i>Myf5^{Cre}</i>	<i>Myf5^{tm3(cre)Sor}</i>	B6.129S4	JAX	007893 (Tallquist et al., 2000)
<i>R26R-eYFP</i>	<i>Gt(ROSA)26Sor^{tm1(EYFP)Cos}</i>	B6.129X1	JAX	006148 (Srinivas et al., 2001)
<i>Pax7-nGFP</i>	<i>Pax7^{tm1.2Tajb}</i>	B6.SJL	-	(Sambasivan et al., 2009)
<i>KDR^{fl}</i>	<i>KDR^{tm2Sato}</i>	B6.129S1	JAX	018977 (Hooper et al., 2009)
<i>Flk1-GFP</i>	<i>KDR^{tm2.1Jrt}</i>	B6.129S1	JAX	017006 (Ema et al., 2006)

Table 2-1. Primary Mouse Lines

Myf5-Cre:ROSA26-eYFP mice (Kuang et al., 2007) were F1 progeny from *Myf5-Cre* x *R26R-eYFP* breeding pairs (Srinivas et al., 2001; Tallquist et al., 2000). Tamoxifen inducible conditional genetic knockout animals were F2 crosses between offspring of *Pax7-Cre^{ER/+}* mice (Murphy et al., 2011) and *KDR^{fl/fl}* mice (Hooper et al., 2009) generating *Pax7-Cre^{ER/+}:KDR^{+/+}* and *Pax7-Cre^{ER/+}:KDR^{fl/fl}*. Unless otherwise stated, 6-8 week old mice were used for all experiments.

2.2 Lineage Tracing with Pax7-nGFP and Myf5-Cre:R26R-eYFP

Pax7-nGFP transgenic mice possess a knock-in of nuclear localized EGFP (*nGFP*) within the first exon of *Pax7* (Sambasivan et al., 2009). Expression of *nGFP* corresponds with endogenous *Pax7* gene expression. *Pax7-nGFP* mice allow for the visualization of *Pax7*-expression satellite cells.

Myf5-Cre:R26R-eYFP transgenic mice (Kuang et al., 2007) possess a knock-in of Cre recombinase within the 5'-UTR of the *Myf5* gene crossed with a knock-in of Cre-mediated activation of yellow fluorescent protein (eYFP) within the *Gt(ROSA)26Sor* locus (Srinivas et al., 2001; Tallquist et al., 2000). *Myf5-Cre:R26R-eYFP* mice allow for the differentiation between committed satellite cells that have expressed Myf5-Cre from satellite cells that have never expressed Myf5-Cre (Kuang et al., 2007).

2.3 Cardiotoxin Injury and Tamoxifen Treatment

We performed five daily intraperitoneal tamoxifen injections (20 mg tamoxifen/ml corn oil, 100 µl/20g mouse) (Sigma Aldrich) followed by a recovery day before use. Mice were then switched over to a tamoxifen diet (0.5 g tamoxifen/kg feed, 5 g feed/day/mouse, Envigo) until end point. We performed cardiotoxin (Cedarlane) injections (50 µl cardiotoxin at 10 µM in saline) on mice under general anesthesia intramuscularly in the right *tibialis anterior* (TA) muscle. Mice were given the appropriate dosage of buprenorphine (0.3 mg buprenorphine/ml saline, 0.10 mg/kg mouse, McGill University) before going under general anesthesia. Eye lubricant (Aventix) was applied and surgical sites were washed and disinfected (Ecolab, Baxedin) before proceeding with injections. See Table 2-2. Animal Care Reagents.

Product	Source	Identifier	Final Concentration	Amount used
NERL™ Blood Bank Saline	ThermoFisher Scientific	8504	-	-
Cardiotoxin Gamma (Latoxan)	Cedarlane	L8102	10 µM in saline	50 ul
Buprenorphine (Vetergesic)	McGill University	n/a	0.3 mg/ml	0.10 mg/kg mouse
Tamoxifen >99%	Sigma-Aldrich	T5648	20 mg/ml	100ul/20g mouse
Corn Oil	Sigma-Aldrich	C8267	-	-
Optixcare Eye Lubricant	Aventix	13780013	-	-
Endure 400 Scrub-Stat 4	Ecolab	47593-265	-	-
Chlorhexidine Gluconate 2% w/v in 70% v/v Isopropyl Alcohol	Baxedin	L0000017	-	-
Teklad Custom Tamoxifen Diet	Envigo	TD.130857	0.5 g/Kg	5g/day/mouse

Table 2-2. Animal Care Reagents.

2.4 Compound Libraries

The Ontario Institute for Cancer Research Kinase Inhibitor and toolkit compound libraries consists of 400 specific kinase inhibitors and 160 additional small molecule compounds targeting cellular and molecular developmental pathways. Compounds were received as 1 mM solutions dissolved in DMSO. See 6.1 for list of OICR compounds.

2.5 FDB Myofiber High Content Analysis

We performed high content analysis on Flexor Digitorum Brevis (FDB) myofibers isolated from *Myf5-Cre:R26R-eYFP* mice (Kuang et al., 2007; Shefer and Yablonka-Reuveni, 2005). Myofibers from 3-4 mice were pooled per 96-well plate (Fisher Scientific). FDB muscles were dissected and incubated in collagenase type I

(0.4% in DMEM, ThermoFisher Scientific) for 3 hours. Myofibers were separated using trituration with a glass pipet. We cultured myofibers in DMEM (Dulbecco's modified Eagle's medium; high glucose, L-glutamine with 110 mg/ml sodium pyruvate) containing 20% FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin, 2.5 ng/ml bFGF and supplemented with either vehicle (1 μ M DMSO in growth medium), EGF ligand (100 ng/ml), Wnt7a ligand (50 ng/ml), or small molecule compound (1 μ M). See Table 2-12.

Single Myofiber Isolation and Culture Reagents.

Compounds were screened in duplicate at 1 μ M concentration. Image acquisition and satellite cell identification was performed on the Cellomics™ ArrayScan platform (ThermoFisher) and a custom Cellomics-based script developed in house. Quantification satellite cell divisions was performed on output from Cellomics using a secondary custom Matlab-based script.

2.6 Real-Time PCR

We isolated RNA according to the NucleoSpin commercial kit (FisherScientific) instructions. We performed reverse-transcriptase according to the iScript kit instructions, which utilizes a modified Moloney murine leukemia virus reverse transcriptase and a proprietary mixture of oligo(dT) and random hexamer primers for cDNA synthesis (iScript, Bio-Rad).

Primers were reconstituted in TE buffer pH 8.0 to create primer stock solutions (100 μ M). Working primer solutions contained forward and reverse primers (5 μ M) in Tris pH 8.0 for a final concentration of 300-500 nM.

We performed real-time PCR analysis (SsoFast™ EvaGreen Supermix, Bio-Rad) using SYBR Green and the CFX384 real time PCR detection system (Bio-Rad) for 40

cycles. See Table 2-3. Real-Time PCR Protocol. We normalized to *PPIA*, *RPS18*, and *RPS20* and analyzed results using the Bio-Rad CFX Manager software.

	cDNA or Plasmid DNA		Genomic DNA		
Cycling Step	Temperature	Time	# Cycles	Temperature	Time
Enzyme activation	95°C	30s	1	98°C	2 min
Denaturation	95°C	1-5s*	30-40	98°C	1-5s
Annealing/extension	55-60°C	1-5s**		55-60°C	1-5s
Melt Curve	65-95°C (in 0.5°C inc.)	2- 5s/step**	1	65-95°C (in 0.5°C inc.)	2- 5s/step

Table 2-3. Real-Time PCR Protocol

Modify step duration *15s, **30s for SYBR

Product	Source	Identifier
Macherey-Nagel Supplier Diversity Partner NUCLEOSPIN RNA II	Fisher Scientific	740955.250
iScript™ cDNA Synthesis Kit	Bio-Rad	170-8891
SsoFast™ EvaGreen Supermix	Bio-Rad	172-5202

Table 2-4. qPCR Reagents

We used the following qPCR primers:

Gene	Forward (5'-3')	Reverse (5'-3')	Tm (°C)	Size (bp)
<i>Pax7</i>	GACGACGAGGAAGG AGACAA	ACATCTGAGCCCTC ATCCAG	60	234
<i>Myf5</i>	TGACGGCATGCCTGA ATGTA	ATCTGCAGCACATG CATTTGATA	60	100
<i>KDR</i>	Qiagen (proprietary sequence)		60	133
<i>PPIA</i>	CACTGCCAAGACTG AATG	GTCGGAAATGGTGA TCTTC	60	136
<i>RPS18</i>	AACGGTCTAGACAA CAAGCTG	AGTGGTCTTGGTGT GCTGAC	60	21
<i>Gapdh</i>	CCCAGAAGACTGTG GATGG	ACACATTGGGGGTA GGAACA	60	171
<i>Tbp</i>	AGAACAATCCAGAC TAGCAGCA	GGGAACTTCACATC ACAGCTC	60	120
<i>Dmd</i>	CAACAACCTCCTTCCC TAGTT	GCTCTGCCCAAATC ATCT	60	107
<i>Dag1</i>	CTCATTTTCGAGTGAG CATTCC	ACTGTGTGGGTCCC AGTGTA	60	115

Table 2-5. qPCR Primer Sequences

2.7 Digital Droplet PCR

We isolated RNA according to the PicoPure RNA Isolation commercial kit instructions (Life Technologies). We performed reverse-transcriptase according to the iScript kit instructions, which utilizes a modified Moloney murine leukemia virus reverse transcriptase and a proprietary mixture of oligo(dT) and random hexamer primers for cDNA synthesis (iScript, Bio-Rad).

We generated ddPCR reactions using predesigned and pre-validated primer probes (final concentration 500 nM primers, 250 nM probes, IDT) diluted in a proprietary mixture of components designed for hydrolysis probe-based ddPCR (Supermix, Bio-Rad), water, and template cDNA for a final volume of 20 µl per reaction. See Table 2-8 for ddPCR reaction mixture and concentrations. Oil droplet generation was performed according to kit instructions using droplet generation apparatus (DG8 Cartridges and

Gaskets, Bio-Rad), droplet generation oil (70 µl), and droplet generator (Bio-Rad) for a final volume of 40 µl per reaction. We performed end-point PCR (C1000 Thermotouch Cyclcr, Bio-Rad) and quantified transcript amplification on the QX200 Droplet Reader (Bio-Rad).

Product	Source	Identifier
PicoPure RNA Isolation	Life Technologies	KIT0204
IDTE buffer	IDT	11-01-02-05
DG8 Cartridges	BioRad	186-4008
DG8 Gaskets	BioRad	186-3009
Foil Plate Sealer	BioRad	181-4040
Droplet Generation Oil	BioRad	186-3005
2x ddPCR Supermix (no dUTP) 200 rxns; 500 rxns	BioRad	186-3023 186-3024
96w plate	Eppendorf	951020303
ddPCR 2x buffer	BioRad	1863052

Table 2-6. ddPCR Reagents

We used the following ddPCR primer probes:

Gene	Source	Identifier
Pax7	Integrated DNA Technologies	Mm.PT.58.9286978
Myf5	Integrated DNA Technologies	Mm.PT.58.5271235
MyoD	Integrated DNA Technologies	Mm.PT.58.8193525
MyoG	Integrated DNA Technologies	Mm.PT.58.6732917
VEGFR1 (FLT-1)	Integrated DNA Technologies	Mm.PT.58.43852013
KDR (FLK-1, VEGFR2)	Integrated DNA Technologies	Mm.PT.58.5869721
VEGFR3 (FLT-4)	Integrated DNA Technologies	Mm.PT.58.13229320
Nrp1	Integrated DNA Technologies	Mm.PT.58.9810806
Nrp2	Integrated DNA Technologies	Mm.PT.58.30988321
VEGFA	Integrated DNA Technologies	Mm.PT.58.14200306
VEGFB	Integrated DNA Technologies	Mm.PT.58.16515314
VEGFC	Integrated DNA Technologies	Mm.PT.58.10862583
VEGFD	Integrated DNA Technologies	Mm.PT.58.43645237
DMD	Integrated DNA Technologies	Mm.PT.58.9899092
TBP	Integrated DNA Technologies	Mm.PT.39a.22214839
TBP	Integrated DNA Technologies	Mm.PT.58.17504874
RSP18	Integrated DNA Technologies	Mm.PT.58.12109666
RSP20	Integrated DNA Technologies	Mm.PT.58.41623895.g
PPIA	Integrated DNA Technologies	Mm.PT.39a.2.gs

Table 2-7. ddPCR Primer Probes

Reagent	Stock Concentration	Volume (μL)	Final Concentration
Supermix	2x	10	1x (Proprietary mixture)
Primer Probe	20x	1(+1)	1x (500 nM primers, 250 nM probe)
H2O	-	4 (3)	-
cDNA	-	5	-
Final Volume	-	20	-

Table 2-8. ddPCR reaction components

Primers may be duplexed when utilizing different fluorophores (i.e. HEX and FAM). Values for duplexed reactions are described in brackets.

2.8 Western Blot

Proteins were isolated using RIPA buffer (ThermoFischer) and a protease inhibitor (cOmplete™, Millipore) and centrifuged at 14.8K RPM for 30 min at 4°C to remove cell debris. Protein concentrations were determined by a 6-point standard curve Bradford Assay. Protein samples were prepared in 25 μg aliquots, diluted in Laemmli's buffer and boiled for 5min at 100°C prior to use. Protein samples were separated on a

10% SDS-PAGE gel (120V stacking gel, 160V resolving gel, 120V gradient gel), and transferred to PVDF membrane (EMD Millipore) (1h, 100V).

Membranes were probed with primary antibodies at 1:1000 in TBST with 5% milk and incubated overnight at 4°C (See Table 2-15 for list of antibodies). Membranes were washed 3 times for 5-10 mins in TBST. Secondary antibody incubation was performed by HRP-conjugated secondary antibodies at 1:5000 (See Table 2-10, BioRad) for 1-2hrs room temperature. Membranes were washed 3 times for 5-10 mins in TBST before proceeding to develop using Immobilon Western HRP substrate (EMD Millipore). Membranes were exposed to BioMax film (Eastman Kodak).

Product	Source	Identifier
RIPA Lysis and Extraction Buffer	ThermoFischer Scientific	89900
cOmplete™	Millipore Sigma	5056489001
Semi-micro Cuvettes, PS	Sarstedt	67.742
Protein Assay Dye	BioRad	5000006
Ultraspec 2100 pro UV/Visible Spectrophotometer	Biochrom	80-2112-21
Immobilon-P PVDF Membrane	EMD Millipore	IPVH 00010
Immobilon Western substrate	EMD Millipore	WBKLS0500
Carestream Kodak BioMax XAR Film	Sigma	F5513-50EA

Table 2-9. Western Blot Reagents

Antibody	Company	Identifier	Concentration
Goat anti-mouse IgG	BioRad	170-6516	1:5000
Goat anti-rabbit IgG (H+L)	BioRad	170-6515	1:5000
Rabbit anti-goat IgG (H+L)	BioRad	1:5000	1:5000

Table 2-10. Western Blot Secondary Antibodies

2.9 Cryosection

To prepare the TA muscle for cryosection, isolated TA samples were lowered into a tinfoil receptacle filled with O.C.T (Sakura Finetek). The receptacles were immediately lowered into an isopentane bath (ThermoScientific) cooled by liquid nitrogen. Samples were then cut by microtome at 10 µm sections and transferred onto microscope slides and stored at -80°C. Cryosections were fixed with 4% PFA (Cedarlane) in PBS for 5-10 mins. Samples were permeabilized in 0.1% Triton, 0.1 M glycine in PBS at for 5 mins prior to immunostaining protocol. See section 2.12 for cryosection processing for immunostaining.

Product	Source	Identifier
2-Methylbutane (Certified ACS)	ThermoScientific	O35514
Tissue-Tek® O.C.T. Compound	Sakura® Finetek	25608-930
Superfrost™ Plus Microscope Slides	Fisher Scientific	12-550-15
Cover Glasses	Fisher Scientific	12-544D

Table 2-11. Cryosection Reagents

2.10 Single Myofiber Isolation and Culture

We performed extensor digitorum longus (EDL) dissection and isolation of single myofibers as previously described (Kuang et al., 2007; Shefer and Yablonka-Reuveni, 2005). EDL muscles were dissected and incubated in collagenase type I (0.4% in DMEM, ThermoFisher Scientific) for 1 hour. Myofibers were separated using trituration with a glass pipet and washed 3 times with DMEM. We cultured myofibers in DMEM (Dulbecco's modified Eagle's medium; high glucose, L-glutamine with 110 mg/ml sodium pyruvate) containing 20% FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin and 2.5 ng/ml bFGF. See Table 2-12 for growth medium components and concentrations. Following culture, myofiber fixation was performed using 2% PFA (Cedarlane) in PBS for for 5-10 mins. Samples were permeabilized in 0.1% Triton, 0.1 M glycine in PBS at for 5 mins prior to immunostaining protocol (See 2.12 for immunostaining protocol).

Product	Source	Identifier	Final Concentration
DMEM	Gibco	11320082	-
DMEM	Life Technologies	11995073	
Ham's F-10	Wisent	318-051-CL	
Collagenase Type I	ThermoFisher Scientific	8504	0.4% in DMEM
Collagenase Type I	Sigma	C0130-1g	0.4% in DMEM
Fetal Bovine Serum	GE Life Sciences	SH30396.03	20% in DMEM
Fetal Bovine Serum	Wisent	098-150	20% in DMEM
Chicken Embryo Extract	MP Biomedicals	92850145	2% in DMEM
Chicken Embryo Extract	Cedarlane	C3999(US)	2% in DMEM
bFGF (human recombinant)	Cedarlane	GF003AFMG(CH)	2.5 ng/ml
Penicillin Streptomycin	Wisent	450-201-EL	1000 µg/ml
Penicillin/Streptomycin (100x)	GIBCO	15140122	1%
Lipofectamine RNAimax	Lifetechnologies	11668019	-
DsiRNA - KDR	Integrated DNA Technologies	mm.Ri.Kdr.13	10 nM
DsiRNA – Dag1	Integrated DNA Technologies	mm.Ri.Dag1.13	10 nM
DsiRNA - DMD	Integrated DNA Technologies	mm.Ri.Dmd.13	10 nM
EGF	Miltenyi Biotec	130-093-825	100 ng/ml
Wnt7a	R&D Systems	3008WN025/CF	50 ng/ml
VEGF-E	Cedarlane	CYT-263-2	100 ng/ml

Table 2-12. Single Myofiber Isolation and Culture Reagents

2.11 siRNA Transfection

We performed RNA interference using Lipofectamine RNAimax (Life technologies) with predesigned dicer-substrate short interfering RNA strands (Integrated DNA Technologies). Two-hit siRNA transfections or scrambled controls were administered at 10 nm concentrations to culture medium at 4h and 16h followed by culture medium refresh at 22h from the start of culture.

Product	Source	Identifier	Final Concentration
Lipofectamine RNAimax	Lifetechnologies	11668019	-
DsiRNA - KDR	Integrated DNA Technologies	mm.Ri.Kdr.13	10 nM
DsiRNA – Dag1	Integrated DNA Technologies	mm.Ri.Dag1.13	10 nM
DsiRNA - DMD	Integrated DNA Technologies	mm.Ri.Dmd.13	10 nM
VEGF-E	ProSpec	CYT-263-2	100 ng/ml

Table 2-13 RNA Interference Reagents

2.12 Immunostaining, Antibodies, and PLA

Sample fixation was performed using 2% PFA (Cedarlane) for myofibers, or 4% PFA (Cedarlane) for cryosections for 5-10 mins. Samples were permeabilized in 0.1% Triton, 0.1M glycine in PBS at for 5 mins. Myofibers were blocked in PBS containing 5% horse serum, 1% BSA (Sigma), 0.5% Triton-X100 (Sigma-Aldrich) for 1-2h. Cryosections were blocked in 5% goat serum, 2% BSA, 0.3% Triton with MOM Blocking reagent in PBS.

Primary antibodies were diluted in blocking solution for 2h at room temperature or overnight at 4°C. Samples are washed 3 times with PBS. Samples were then incubated with secondary antibodies diluted in blocking solution for 2h at room temperature and washed 3 times with PBS. Nuclei were stained with DAPI or Hoescht for 10 min before washing 3 times with PBS before mounting with mounting medium (MJS Biolynx). See Table 2-15. Immunofluorescence Antibodies for complete list of antibodies and concentrations used.

Proximity ligation assays (PLA) were performed on EDL myofibers following fixation using Duolink (Sigma) PLA probes according to kit instructions. Briefly, samples were blocked with Duolink II Blocking solution (Sigma) for 1h at 37°C.

Samples were incubated overnight at 4°C with PLA probes (See Table 2-14. Immunostaining Reagents) diluted at 1:5 and primary antibodies diluted in Duolink II Antibody Diluent (Sigma) according to concentrations listed in Table 2-15. Samples were washed 2x with Wash Buffer A (Sigma) for 10 min at room temperature. A Ligation reaction was performed using a ligation mixture of Duolink II Ligation Stock (5x, Sigma) at 1:5 and ligase (Sigma) at 1:40 in water for a final volume of 100 µl/well. Samples were washed 2x with Wash Buffer A (Sigma) for 10 min at room temperature. An amplification step was performed using a mixture of Duolink II Amplification Stock (5x, Sigma) at 1:5, Duolink Polymerase (Sigma) at 1:80, combined with Alexa Fluor secondary antibodies 1:1000 for a final volume of 100ul/well. Samples were washed 2x with Wash Buffer B (Sigma) for 20 mins at room temperature. Samples were then mounted with Duolink II Mounting Medium with DAPI (Sigma).

Product	Source	Identifier	Final Concentration
8% Paraformaldehyde	Cedarlane	157-8	2%
Donor Equine Serum	GE Life Sciences	SH30074.03	5%
Bovine Serum Albumin (BSA)	Sigma	A7906-100G	1%
Triton-X100	Sigma-Aldrich	T8787-250ML	0.5%
Goat serum	Gibco	16210-64	5%
Glycine	Fisher Chemical	BP3815	0.1 M in PBS
MOM blocking reagent	MJS BioLynx	VECTMKB22131	1:40 dilution in PBS
DAPI	Sigma-Aldrich	D8417-5MG	0.4 µg/ml
Hoescht	Sigma	B2261-100MG	1:1000 in PBS
VECTASHIELD® Vibrance™ Antifade Mounting Medium with DAPI	MJS BioLynx	VECTH180010	-
Duolink Detection Reagents Red	Sigma-Aldrich	DUO92008-100RXN	-
Sterile Water	Wisent	809-115-CL	-
Duolink II Wash Buffers for Fluorescence Applications	Sigma-Aldrich	DUO82049-4L	-
Duolink In Situ Mounting Medium with DAPI	Sigma-Aldrich	DUO81040	-
Duolink In Situ Probemaker MINUS	Sigma (Olink Bioscience)	DUO92010	-
Duolink In Situ Probemaker PLUS	Sigma (Olink Bioscience)	DUO92009	-
Duolink In Situ PLA Probe Anti-Mouse PLUS	Sigma (Olink Bioscience)	DUO92001-100RXN	-
Duolink In Situ PLA Probe Anti-Mouse MINUS	Sigma (Olink Bioscience)	DUO92004-100RXN	-
Duolink In Situ PLA Probe Anti-Goat PLUS	Sigma (Olink Bioscience)	DUO92003-100RXN	-
Duolink In Situ PLA Probe Anti-Goat MINUS	Sigma (Olink Bioscience)	DUO92006-100RXN	-
Duolink In Situ PLA Probe Anti-Rabbit PLUS	Sigma (Olink Bioscience)	DUO92002-100RXN	-
Duolink In Situ PLA Probe Anti-Rabbit MINUS	Sigma (Olink Bioscience)	DUO92005-100RXN	-
Click-iT EdU Alexa Fluor 647 Imaging Kit	Invitrogen	C10340	-

Table 2-14. Immunostaining Reagents

Host	Antibody	Source	Identifier	Final Concentration
Mouse	Anti-Pax7 (IgG1)	DSHB	https://doi.org/10.1016/S0925-4773(97)00097-X	50%
Chicken	Anti-GFP	Abcam	ab13970	10 µg/ml
Goat	Anti-KDR	R&D Systems	AF644	4 µg/ml
Rabbit	Anti-KDR	Abcam	ab39256	1 µg/ml
Rabbit	Anti-DMD	Abcam	Ab1527	
Rabbit	Anti-Dag1	Sigma	D1945	
Chicken	Syndecan-4	Cornelison et al., 2004	https://doi.org/10.1101/gad.1214204	1:100
Rabbit	Anti-Ki67	Abcam	Ab155580	1 µg /ml
Mouse	Anti-MyoG	R&D Systems	MAB668761	10 µg /ml
Rat	Anti-Laminin	Millipore Sigma	L0663	
Goat	Anti-Mouse IgG1 488	Invitrogen	A-21121	2 µg/ml
Goat	Anti-Mouse IgG2b 488	Invitrogen	A-21141	2 µg/ml
Goat	Anti-Mouse IgG1 546	Invitrogen	A-21123	2 µg/ml
Goat	Anti-Mouse IgG2 546	Invitrogen	A-21133	2 µg/ml
Goat	Anti-Mouse IgG1 568	Invitrogen	A-21124	2 µg/ml
Goat	Anti-Chicken IgG 488	Invitrogen	A-11039	2 µg/ml
Goat	Anti-Chicken IgG 546	Invitrogen	A-11040	2 µg/ml
Goat	Anti-Chicken IgY 568	Invitrogen	A-11041	2 µg/ml
Goat	Anti-Chicken IgG 647	Invitrogen	A-21449	2 µg/ml
Goat	Anti-Rabbit IgG 546	Invitrogen	A-11010	2 µg/ml
Goat	Anti-Rabbit IgG 647	Invitrogen	A-21244	2 µg/ml
Goat	Anti-Rabbit IgG (H+L) 647	Invitrogen	A-32733	2 µg/ml
Donkey	Anti-Mouse IgG1 (H+L) 488	Invitrogen	A-21202	2 µg/ml
Donkey	Anti-Mouse IgG1 (H+L) 546	Invitrogen	A-10036	2 µg/ml
Donkey	Anti-Mouse IgG1 (H+L) 647	Invitrogen	A-21240	2 µg/ml
Donkey	Anti-Mouse IgG (H+L) 647	Invitrogen	A-31571	2 µg/ml
Donkey	Anti-Goat IgG (H+L) 488	Invitrogen	A-11055	2 µg/ml

Donkey	Anti-Goat IgG (H+L) 546	Invitrogen	A-11056	2 µg/ml
Donkey	Anti-Goat IgG (H+L) 568	Invitrogen	A-11057	2 µg/ml
Donkey	Anti-Chicken IgY 488	Cedarlane	703-545-155	2 µg/ml
Donkey	Anti-Chicken IgY 647	Cedarlane	703-606-155	2 µg/ml
Donkey	Anti-Chicken IgY 647	Sigma	AP194SA6	2 µg/ml
Donkey	Anti-Rabbit IgG (H+L) 488	Invitrogen	A-21206	2 µg/ml
Donkey	Anti-Rabbit IgG (H+L) 546	Invitrogen	A-10040	2 µg/ml
Donkey	Anti-Rabbit IgG (H+L) 647	Invitrogen	A-31573	2 µg/ml
Wheat Germ Agglutinin , Alexa Fluor 488 Conjugate	N-acetylglucosamine and N-acetylneuraminic acid	Invitrogen	W11261	5 µg/ml
Wheat Germ Agglutinin , Alexa Fluor 647 Conjugate	N-acetylglucosamine and N-acetylneuraminic acid	Invitrogen	W32466	5 µg/ml

Table 2-15. Immunofluorescence Antibodies

2.13 Image Acquisition

Immunostained samples were imaged on an Axio Observer.Z1 microscope equipped with a LSM510 META confocal laser scanner and a plan-Apochromat 63x/1.40 Oil DIC M27 objective or an Axioplan 2 microscope equipped with a plan-Neofluar 40x/1.30 Oil DIC and a plan-Neofluar 100x/1.30 Oil DIC objective. Images were processed and analyzed with Axiovision, Zen, and FIJI software. 3D z stack images were projected by maximum intensity using Fiji software (<https://fiji.sc/>).

2.14 Magnetic Activated Cell sorting (MACS)

Satellite cell suspensions were obtained from hindlimb muscles as previously described (Sincennes et al., 2017). Samples were digested in collagenase-dispase solution (10 g/L collagenase B, 4 g/L dispase I in HAM's F-10 media) for the duration of a custom gentleMACS™ dissociator program (approximately 35 mins). Cells were resuspended in MACS buffer (PBS, 0.5% BSA, 2 mM EDTA) prior to negative selection using the satellite cell cocktail (Miltenyi Biotec). Satellite cells were obtained using a positive selection anti-integrin $\alpha 7$ microbeads (Miltenyi Biotec).

We isolated mouse-derived brain endothelial cells using the Neural Dissociation Kit P (Miltenyi Biotec) following kit instructions. Samples were dissociated with warmed enzyme mix 1 (1900 μ L Buffer X, 50 μ L enzyme P per 400 mg tissue, Miltenyi Biotec). Samples were incubated in enzyme mix 1 for 15 min at 37°C. Samples were incubated in enzyme mix 2 (20 μ L Buffer Y, 10 μ L Enzyme A, Miltenyi Biotec) and mechanically dissociated using a glass pipette. Samples were incubated in enzyme mix 2 for 10 min at 37°C. Samples were strained through 70 μ m membrane with 10 ml HBSS solution (Miltenyi Biotec) and centrifuged at 300xg for 10 min. Cells were resuspended in 270 μ L PEB buffer (HBSS, 0.5% BSA, 2 mM EDTA) with 30 μ l of CD31 microbeads (Miltenyi Biotec) for positive selection of endothelial cells. See Table 2-16. MACS Reagents.

Product	Source	Identifier
gentleMACS™ Octo Dissociator with heaters	Miltenyi Biotec	130-096-427
Mini & MidiMACS™ Starting Kits	Miltenyi Biotec	130-091-632 130-091-501
LD Column	Miltenyi Biotec	130-042-901
LS Column	Miltenyi Biotec	130-042-401
MS Column	Miltenyi Biotec	130-042-201
PBS (1x)	Wisent	311-010-FL
EDTA (Final 2mM)	Invitrogen	AM9260G
BSA (Final 0.5%)	Sigma	A7906-100G
Satellite Cell Isolation Kit	Miltenyi Biotec	130-104-268
Anti-Integrin $\alpha 7$	Miltenyi Biotec	130-104-261
Neural Dissociation Kit P	Miltenyi Biotec	130-092-628
CD31 Microbeads	Miltenyi Biotec	130-097-418

Table 2-16. MACS Reagents

2.15 Fluorescence Activated Cell Sorting (FACS)

Single cell suspensions were obtained as described above in section 2.14:

Magnetic Activated Cell Sorting. Satellite Cell populations were resuspended in FACS buffer (PBS, 0.5% FBS, 2 mM EDTA) and sorted using a previously published strategy of $\alpha 7$ -integrin, and Lin⁻ markers, with the addition of VCAM1 and Integrin $\beta 1$ to enrich for satellite stem cell populations (Choo et al., 2017; Dumont et al., 2015a; Kuang et al., 2007; Rozo et al., 2016). Single cell suspensions from MDX mice were cleaned up by MACS Lineage negative selection (Satellite Cell Isolation Kit, Miltenyi Biotec) prior to FACS sorting. See Table 2-17. FACS Antibodies.

Antibody	Company	Identifier	Concentration
647 conjugated anti-mouse α 7-integrin	AbLab	67-0010-05	15:100
PE/Cy7 VCAM CD106	Cedarlane	105719 105720	1:20
BV421 Integrin β 1	BD Bioscience	564131	1:20
PE conjugated anti-mouse CD45	eBioscience	12-0451-82	1:50
PE conjugated anti-mouse CD11b	eBioscience	12-0112-81	1:50
PE conjugated anti-mouse Sca-1 (Ly-6A/E)	BD Pharmingen	553108	1:50
PE conjugated anti-mouse CD31 (PECAM1)	BD Pharmingen	553373	1:50

Table 2-17. FACS Antibodies

2.16 Histological Analysis of Muscle Section

TA samples were isolated and immersed in OCT compound before being frozen using an isopentane (Thermo Scientific) and liquid nitrogen bath. Samples were stored at -80°C until required. Samples were cut at 10 μ m sections. See Table 2-18. Cryosection Reagents.

Fiber ferret size and central nucleated fibers were analyzed using non-fixed samples washed with PBS and stained with Wheat Germ Agglutinin (Life Technologies) for 1h at room temperature. Samples were counterstained with DAPI and mounted with Permafluor. Minimum fiber ferret size measurement was performed using SMASH software in MATLAB 2020a as described previously (Smith and Barton, 2014). Samples with significant artifacts were excluded from automated analysis. Total number of myofibers in each tissue was manually verified. >95% of myofibers were quantified across each section.

Product	Source	Identifier
Tissue-Tek® O.C.T. Compound	Sakura® Finetek	25608-930
Superfrost™ Plus Microscope Slides	Fisher Scientific	12-550-15
Cover Glasses	Fisher Scientific	12-544D
2-Methylbutane (Certified ACS)	Thermo Scientific	O35514

Table 2-18. Cryosection Reagents

2.17 Statistical Analysis

Compiled data were expressed as mean $\pm$ standard error of the mean (SEM), and experiments were performed with a minimum of three biological replicates unless otherwise stated. Statistical comparisons of two conditions used a two-tailed Student's t-test. Paired tests were used for biologically matched samples (Figure 14, 19, 20, 22, 23, 25B, 25J). Unpaired tests were used to compare unrelated samples (Figure 26F). One-way ANOVA analysis was performed in experiments affected by one factor (i.e. time) (Figure 13, 18, 21). Two-way ANOVA analysis was performed in experiments affected by two or more factors (i.e. control vs treatment and injury vs non-injury) (Figure 16, 24, 25C-I, 26A-D, 26H-I). Multiple comparison tests were performed following ANOVA analysis. Tukey's multiple comparison test was performed when comparing every mean with every other mean, whereas Dunnett's multiple comparison test was performed when comparing a control mean with other means. No data were removed as outliers with graphic data point representation were included where appropriate. Experimental design incorporated user blind when possible. Statistical analysis was performed in GraphPad Prism or Excel. The level of significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Chapter 3. Results I. High Content Screen Development

All experiments were performed by the author, William Chen, except for the following.

Those presented in Table 3-1 were performed by Jaime Gutierrez. William Wang contributed to the design of the MATLAB script logic, programmed by Theodore A. Perkins represented in Figure 8.

3.1 High Content Screen Design

We designed and developed a novel screen combining lineage tracing, single myofiber culture, automated image acquisition, and bioinformatics. Our high-content screening platform is capable of detecting and quantifying changes in satellite stem cell asymmetric and symmetric divisions (**Figure 3A**).

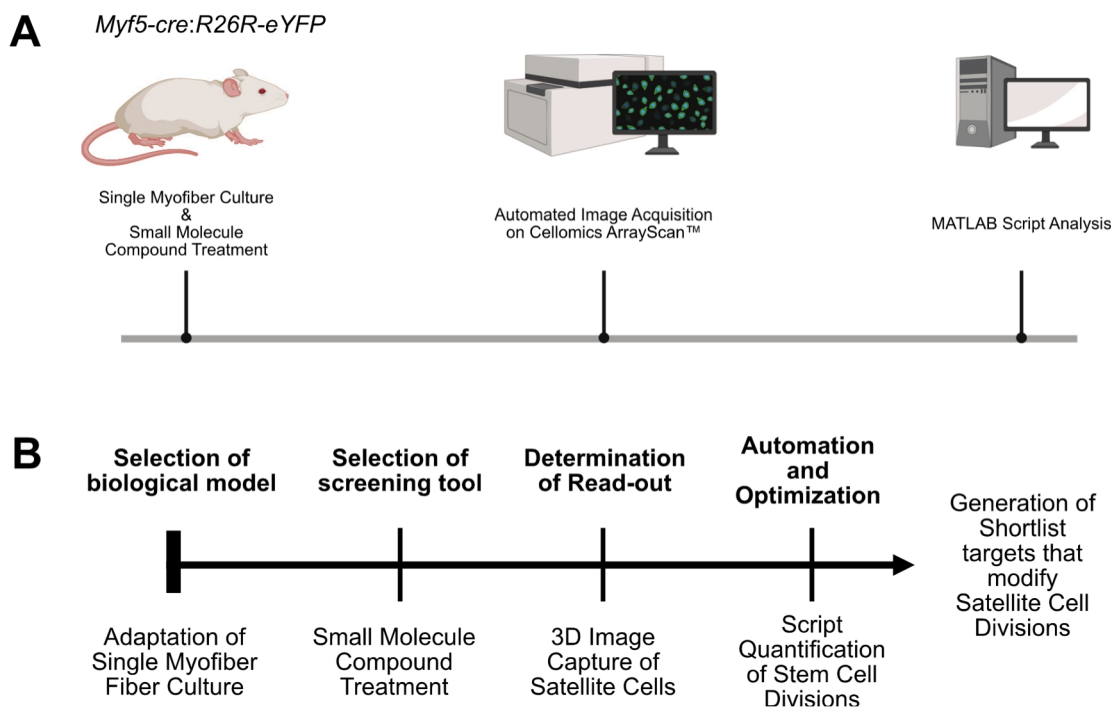


Figure 3. High Content Screen Design and Optimization Milestones

A. Workflow of High-content Analysis Screen. **B.** Milestones required for the development of a high content screen designed to identify cell signaling pathways involved in satellite stem cell symmetric and asymmetric division.

3.1.1 Selection of biological model: Adaptation of EDL Single Myofiber Culture for High Content Screening

The gold standard for quantifying changes in stem cell divisions combines the use of lineage tracing with single EDL myofiber culture (Kuang et al., 2007; Pasut et al., 2013). The transgenic mouse model *Myf5-Cre:R26R-eYFP* is used for the quantification of asymmetric and symmetric divisions. Within this system, committed Pax7⁺ satellite cells having expressed *Myf5* are marked by YFP expression allowing for direct visualization of symmetric and asymmetric satellite stem cell divisions.

We selected to use the FDB muscle group for our screening platform. FDB myofibers, compared to EDL myofibers, are considerably shorter in length compared to EDL myofibers with higher satellite cell density and greater proportions of satellite stem cells (**Table 3-1**). These two properties of FDB myofibers make it possible to culture a monolayer of 50-100 fibers within each well on a 96-plate for high content screening. Additionally, FDB myofibers possess a higher density of satellite stem cells compared to EDL myofibers, allowing for sufficient satellite cell division events and statistical power. Thus, we adapted the existing single myofiber culture to use the hindlimb muscle group *flexor digitorum brevis* (FDB) in lieu of the *extensor digitorum longus* (EDL).

	<i>Extensor Digitorum Longus (EDL)</i>	<i>Flexor Digitorum Brevis (FDB)</i>
Digestion Time	1h	3h
N fibers per mouse	300	1000
N of Pax7 ⁺ satellite cells per fiber	10-15	3-7
Myofiber Length (mm) (Tarpey et al., 2018)	5.44 ± 0.12	0.744 ± 0.01
Satellite cell density per mm	1.84 – 2.76	4.03 – 9.41
% Pax7 ⁺ Myf5 ⁻ stem cells per total Pax7 ⁺ satellite population [‡]	9 ± 2.6	18 ± 2.3

Table 3-1 Satellite Cell Properties of EDL vs FDB myofibers

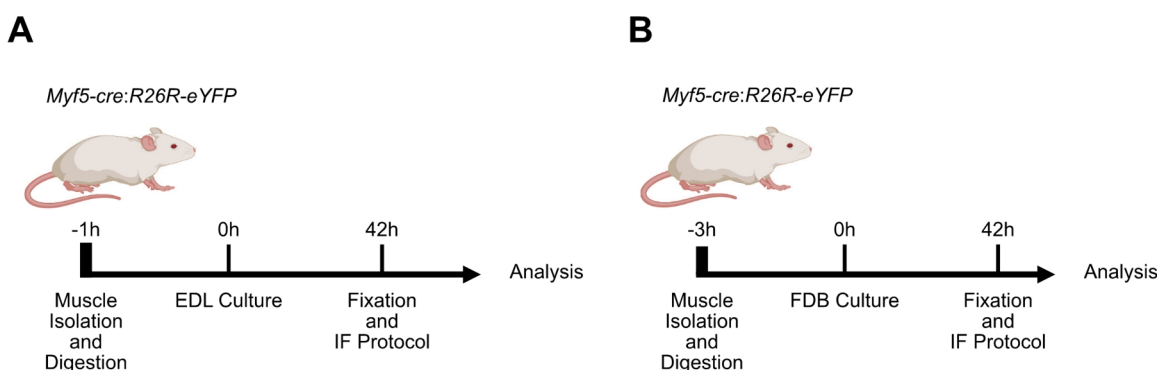


Figure 4. EDL and FDB Single Myofiber Culture

Single myofiber culture protocol of **A.** EDL (extensor digitorum longus) and **B.** FDB (flexor digitorum brevis) hindlimb muscle groups derived from *Myf5-Cre;R26R-eYFP* mice. Isolated muscle groups are digested in collagenase type I and cultured in vitro in DMEM supplemented with 20% FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin and 2.5 ng/ml bFGF. Myofibers are fixed at 42h for visualization and quantification of first satellite cell divisions following activation.

3.1.2 Selection of screening tool: Ontario Institute for Cancer Research (OICR)

Kinase Inhibitor Library

Stem cells and cancer cells share some notable similarities including the ability to self-renew and undergo unlimited divisions (Lechman et al., 2016; Lucey et al., 2009;

Shinin et al., 2006). Thus, to elucidate novel targets and pathways that modulate satellite stem cell division, we opted to screen the Ontario Institute for Cancer Research (OICR) kinase inhibitor library, consisting of 480 compounds. The targets of these compounds in this library are known, and most candidates have undergone clinical trials with some approved for treatment. Selection of a library with known targets allows for further investigation on potential novel pathways involved in stem cell division.

3.1.3 Readout

Our screen adapts the use of traditional myofiber culture with reporter alleles *Myf5-Cre:R26R-eYFP* combined with automated immunofluorescent imaging for high content analysis (Kuang et al., 2007). The raw readout is derived from immunofluorescent signal identifying satellite cells by Pax7⁺ expression, committed satellite cells by YFP⁺ expression, and x,y coordinates to identify and quantify the types of satellite cell divisions.

3.1.4 Cellomics™ Automation and Optimization

3.1.4.1 Microscope and Plate Position Optimization

The first optimization step for image acquisition is to create a plate profile to ensure the Cellomics Arrayscan has the right specifications for the plates used in our screen. Although the microscope is capable of adjusting its x,y,z coordinates for each new well imaged, establishing a plate profile with the specifications of our culture plate reduced the number of adjustments required as well as inefficiencies by minimizing the number of required fields to image an entire well. The plate profile specifications included the length, width, height of the plate, and depth of individual wells. The

microscope was optimized such that the initial start points of each well began centered at the bottom of the well (**Figure 5 B-D**). Although real-time small ad hoc adjustments may still be necessary due to manufacture variance, these optimization steps significantly reduced run times by hours.

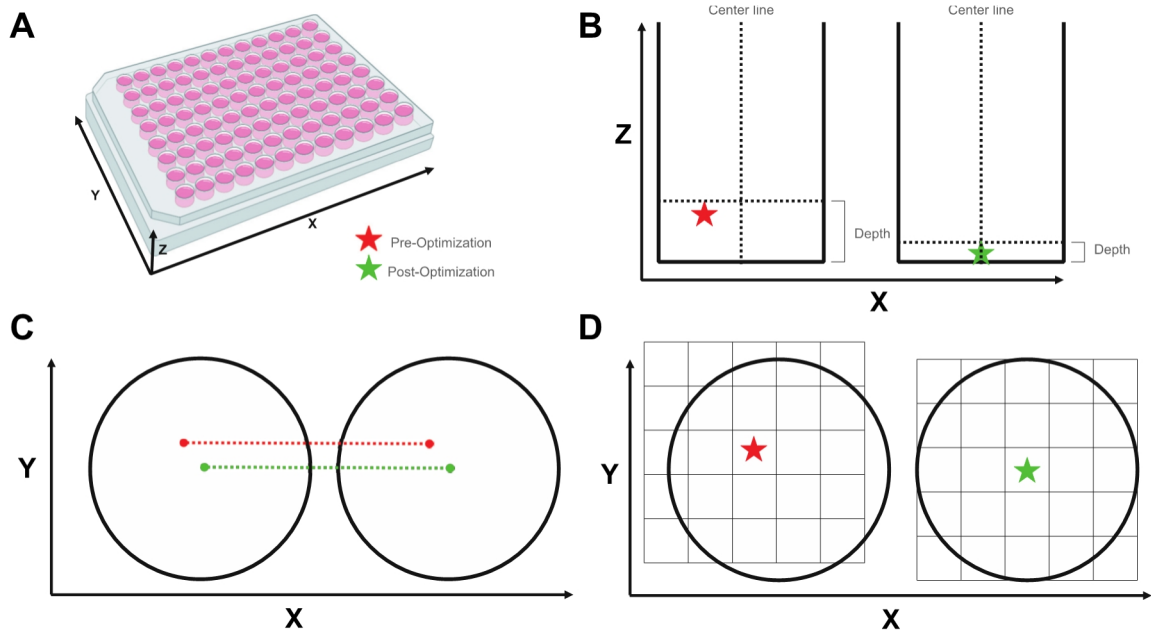


Figure 5. Plate and Well Optimization

A. Schematic of a 96-well plate. Screen optimizations of **B.** starting well depth (z-axis), **C.** centered start point (x,y, axis), and **D.** image field efficiency.

3.1.4.2 Image Acquisition and Background Subtraction Optimization

Satellite cells require 3-D imaging to visualize, as they are positioned at the periphery of FDB myofibers that are approximately 20-25 μm in diameter. To optimize the speed of image acquisition, an autofocus function is programmed to first scan for DAPI-positive cells within a field of depth of 91 μm by sampling seven (7) z-stacks at 13 μm intervals before image acquisition (**Figure 6A**). If no DAPI-positive cells are detected, then the microscope skips to the next field. If DAPI-positive cells were

detected, the projection direction is then centered, such that the median stacks for image acquisition will fall within the center of each myofiber. Image acquisition is then performed using seven (7) z-stacks spaced 6.5 μm apart for a total depth of field of 45 μm at 200x (Objective 20x) magnification (**Figure 6B**) to account for variability in plate level and thickness and potential overlapping myofibers.

To prevent artificial introduction of artifacts while maintaining efficient background subtraction, a maximum-minimum difference projection method was chosen. This method uses differences in pixel brightness between adjacent sources and z-stacks to reduce background (**Figure 6C**). Each channel was then individually optimized based on immunofluorescent signal pattern and its purpose within the screening protocol, discussed below.

This screen utilizes three (3) immunofluorescent channels to identify and locate bright objects (Pax7⁺ satellite cells) contrasted against a dark background. These channels are: Pax7 (Channel 1, CH1), YFP (Channel 2, CH2), and DAPI (Channel 3, CH3) (**Figure 6D**). The Cellomics™ Cell Profiling program provides a number of adjustable parameters to build a custom detection algorithm. The algorithm is divided into several processes: image preprocessing, object identification, object identification, object validation, and object selection. The full parameter values can be found in **Table 3-2**.

Pax7 (CH1) nuclear signal is used to identify satellite cells. A low pass filter was chosen because this channel is used to identify objects of similar size to measure the presence or absence of signal rather than intensity. Low pass filters can enhance small details obscured by noise and function by replacing a pixel value with the calculated average signal of a pixel its immediate neighbours (Buzzi et al., 1976). The low pass

filter used here selectively applies the low pass filter function to lower intensity pixels, leaving bright objects untouched. A conservative low pass filter with a radius of 20 pixels is applied to Pax7 (CH1), to remove image noise surrounding the cell that may interfere with object detection.

YFP (CH2) cytoplasmic signal is used to determine satellite cell commitment by measuring signal intensity for quantification. 3D surface fitting is a less invasive background subtraction algorithm designed to preserve the signal intensity of objects for analysis. A 3D surface background is constructed from the minimum intensity values within an area which is then subtracted from the original image. A 3D surface radius of 15 pixels was applied to remove background within the cytoplasm.

Although DAPI (CH3) is used to discriminate satellite cells from artifact and debris, and detects nuclear signal like Pax7 (CH1), we opted to adopt 3D surface fitting to preserve signal intensity. The low pass filter did not function as effectively compared to 3D surface fitting likely due to the small punctate signaling that accompanies DAPI staining. This led to over-aggressive background subtraction around puncta and blotchy signal. Thus 3D surface fitting was more effective at removing background while maintaining complete nuclei staining. A 10-pixel radius 3D surface fitting was applied to remove background surrounding DAPI-stained nuclei.

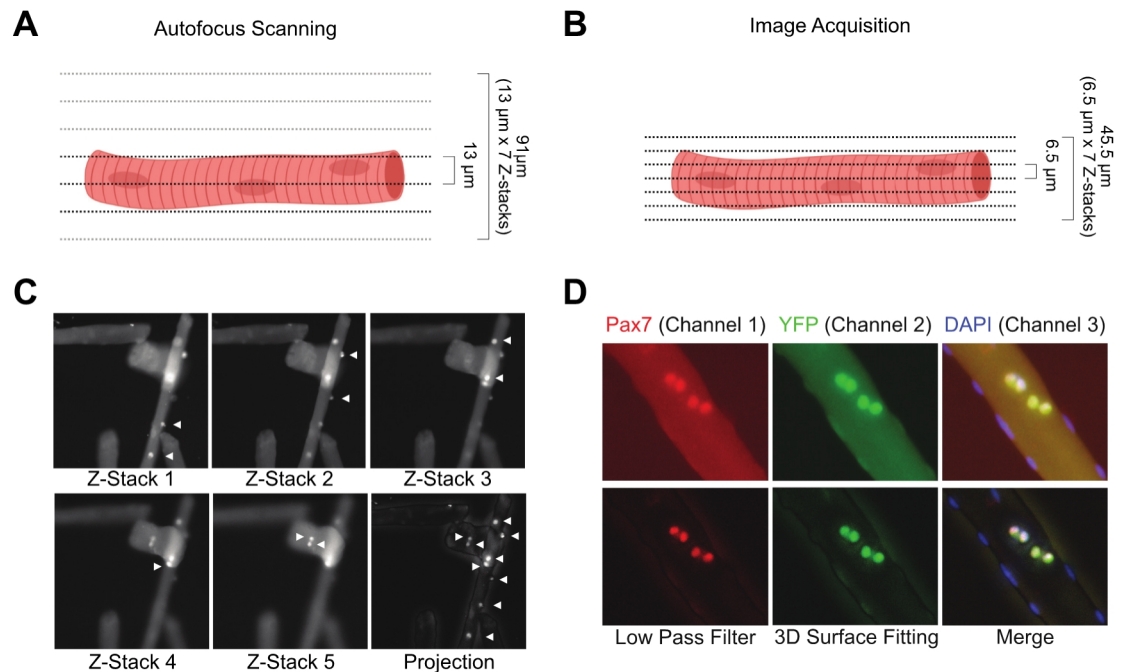


Figure 6. Image Acquisition Automation and Optimization

Image Automation was achieved using the Cellomic ArrayScan™ platform at 200x (Objective 20x) magnification. **A.** FDB myofibers are located by autofocus scanning. A depth field of 91 μm field is searched for DAPI⁺ cells using 7 z-stacks (dotted lines) acquired 13 μm apart. **B.** Image acquisition is performed at a depth of field of 45.5 μm using 7 z-stacks (dotted lines) acquired 6.5 μm apart. **C.** Z-stacks are combined into a projection image. **D.** Images are optimized for analysis using a max-min algorithm for background subtraction.

3.1.4.3 Cell Detection Optimization

Our screening algorithm identifies satellite cells by building an object selection mask based on Pax7 (CH1) signal. A threshold of intensity is used to determine a positive signal. This object selection mask then applies a smoothing algorithm to reduce the sharpness of intensity variations to the Pax7 (CH1) signal, to connect fragments of objects together. Conservative or stringent smoothing may break apart nuclei, whereas parameters that are too relaxed may not be stringent enough to accurately determine

nuclei numbers. An optimized smoothing algorithm follows the contours of the nuclei (Figure 7A). A segmentation algorithm separates peaks of intensity to resolve cells that are close together (i.e. a dividing couplet) (Figure 7B).

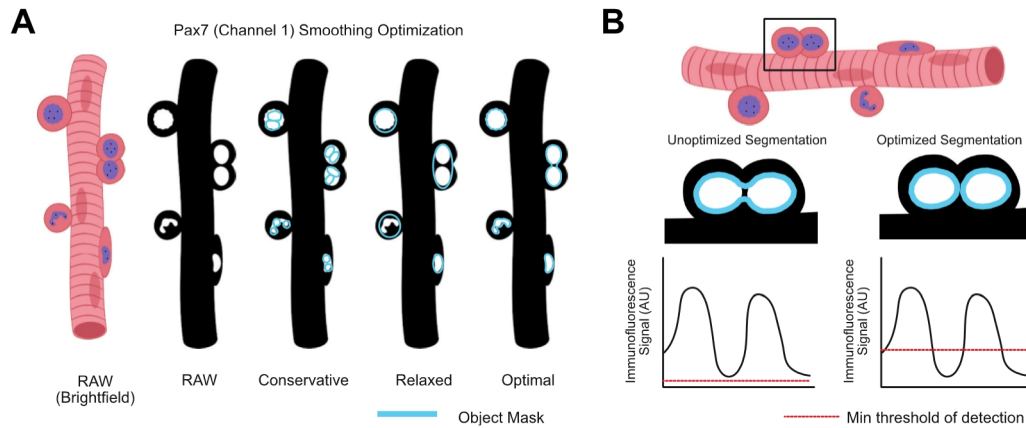


Figure 7. Satellite Cell Detection: Object Identification Optimization

A cartoon schematic of satellite cell detection optimization. An object mask is built to identify satellite cells based on Pax7 (CH1) signal. The detection parameters are further refined by a (A) smoothing and (B) segmentation algorithm applied to the object mask.

Once an object mask has been drawn, a number of parameters are optimized to validate and select satellite cells (Table 3-2C). Valid objects (satellite cells) must satisfy all the set parameters for Channel 1 (Pax7), based on area, shape, signal variance and average signal intensity. Channel 2 (YFP) and Channel 3 (DAPI) signal is then recorded. The Cellomics™ program exports the x-,y- coordinates of each object, and signal intensity of each channel. Channel 3 (DAPI) was used to further discriminate cell from debris while Channel 2 (YFP) was used to determine the commitment status of satellite cells at the next step using a MATLAB™ based script.

A. Image Preprocessing				
Channel	Object Type	Method	Set Value	Units
1	Bright	Low Pass Filter	20	Pixels
2	Bright	3D Surface Fitting	15	Pixels
3	Bright	3D Surface Fitting	10	Pixels

B. Object Identification				
Channel	Algorithm	Set Value	Max Range	Units
1	Smoothing	2	0-500	Pixel area
1	Thresholding	75	0-500	Pixels
1	Segmentation	2	1-246	Intensity

C. Object Validation and Selection				
Channel	Parameter	Set Range	Max Range	Units
1	Area	70 – 350	0 – 2000	Pixels ²
1	Shape (P2A)	0 – 1.28	0 – 2	Number
1	Shape (LWR)	0 – 1.76	0 – 3	Number
1	Variance Intensity	0 – 400	0 – 600	Intensity
1	Average Intensity	1800 – 6000	0 – 16384	Intensity
2	Average Intensity	0 – 15000	0 – 16384	Intensity
3	Average Intensity	400 – 9000	0 – 16384	Intensity

Table 3-2. Cellomics™ Script Parameters

Channels are identified as Pax7 (Ch1), YFP (Ch2), DAPI (Ch3). **A.** Image preprocessing and background subtraction methods are applied to each channel. Values are pixel radii of effect. **B.** Object identification is based on Pax7 (Ch1) using smoothing, thresholding and segmentation. Smoothing value is the area over which brightness is redistributed. Threshold values determine the minimum intensity to be considered an object. Segmentation is the minimum relative height of intensity peaks between objects to be segmented. **C.** Object validation and selection is determined by signal values that fall within the set range. Area is the pixel area of objects. Shape (P2A) is the ratio of perimeter squared to 4PI*area of object (n=1 for circular objects). Shape (LWR) is the shape based on ratio of length to width of an object. Variance Intensity is the standard deviation of intensity of pixels within an object mask. Average intensity is the mean average of intensity of pixels within an object mask.

3.2 MATLAB Script Couplet Detection

3.2.1 MATLAB Script Design

A MATLAB-based script was designed to quantify the commitment status of non-dividing and dividing satellite cell couplets using the Cellomics output (**Figure 8**). The Cellomics outputs data on every identified Pax7 (CH1) cell. This output includes the X,Y-coordinates and the average intensity detected in each channel: Pax7 (CH1), YFP (CH2) and DAPI (CH3).

Thresholds of detection are first determined before quantification. Since all objects output by the Cellomics are Pax7-positive, only thresholds for YFP (CH2) and DAPI (CH3) need to be determined. The YFP (CH2) threshold is set by building a histogram of all detected Pax7 (CH1) objects, yielding a bimodal distribution of negative (YFP-) and positive (YFP+) objects. The minimum threshold of detection is determined as the point of separation between these two peaks: objects whose signal is below the threshold will be determined to be negative, while objects whose signal is above the threshold will be determined to be positive. The thresholds for DAPI are determined as the points that separate low and high-expressing outliers. Thresholds of detection for YFP (CH2) and DAPI (CH3) must be determined with each new run due to variances in immunofluorescence signal.

The script logic begins by first verifying real cells based on DAPI (CH3) signal. False positives (objects too bright) and negative cells (objects too dim) are excluded from analysis based on DAPI (CH3) signal. If a satellite cell is determined to be a real (DAPI+), then its commitment status (YFP) will be determined based on the threshold set for Channel 2.

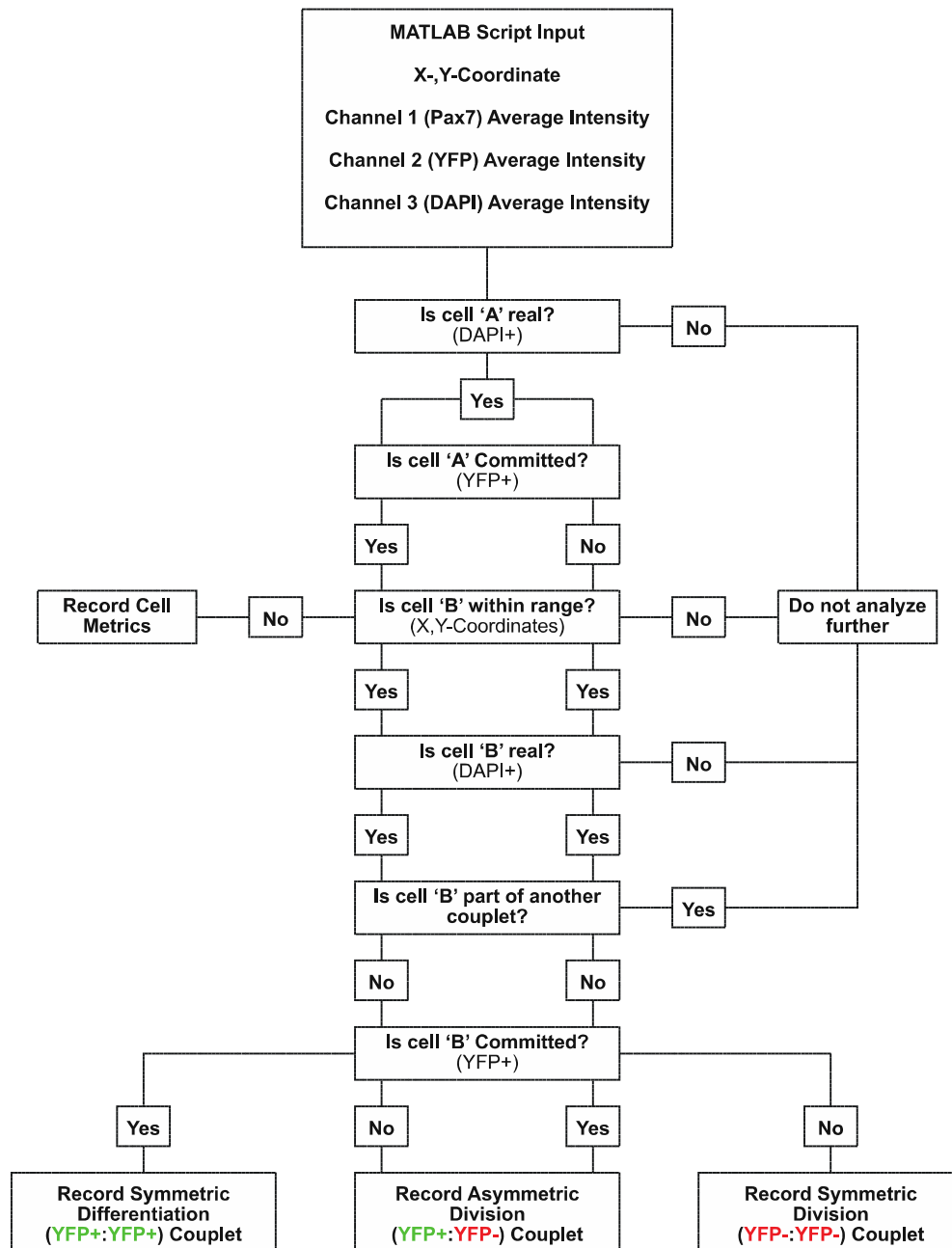


Figure 8. MATLAB Script Logic

A schematic of the MATLAB script logic designed to quantify satellite cell couplets. The Cellomics output is used as the MATLAB input. Each satellite cell is verified to be real based on DAPI (CH3) signal. Commitment status is determined based on YFP (CH2) signal. Couplets are satellite cells within 17.6µm, or 45 pixels at 200x (Objective 20) magnification.

Although wild-type satellite cells do migrate post-division, dividing couplets derived from *Myf5-Cre:R26R-eYFP* myofibers stay coupled for the duration of the first division (24-42h) (Kuang et al., 2007). We determined this distance to be 17.6 μm or 45 pixels, measured from the center of each nucleus at 200x magnification. This allows for the identification of couplets based on proximity to other satellite cells. The MATLAB script determines if cell 'A' is part of a dividing couplet by identifying the next closest cell partner using the X-,Y-coordinates data. If cell 'A' is not within range of a secondary cell 'B' to be considered a dividing couplet, the script will record the cell metrics (committed vs non committed, non-dividing satellite cell) and move on. If cell, 'B' is detected within range, that is not part of another couplet, the script will identify the type of couplet based on the YFP (CH2) signal of both cells (see **Table 3-3**). Cells that are closest together are prioritized as couplets. To avoid false positives, couplets will not be recorded within cell clusters of 3+ satellite cells, as cell division origins are impossible to determine.

Couplet Types	YFP+	YFP-
YFP+	Symmetric Differentiation (YFP+:YFP+)	Asymmetric Division (YFP+:YFP-)
YFP-	Asymmetric Division (YFP+:YFP-)	Symmetric Division (YFP-:YFP-)

Table 3-3. MATLAB Script Couplet Identification.

The MATLAB script identifies three possible satellite cell couplet combinations based on YFP (CH2) signal. A symmetric differentiation couplet is identified as two YFP+ cells (YFP+:YFP+). A satellite stem symmetric division is identified as two YFP- cells (YFP-:YFP-). A satellite stem asymmetric division is identified as one YFP+ and one YFP- cells (YFP+:YFP-).

3.2.2 MATLAB Script Validation

We then validated the precision and accuracy of our platform by determining the Z-factor for both satellite cell detection and couplet recognition of our scripts (**Figure 9B**). We compared four replicates of manual and script quantifications to determine the mean detection scores (μ_p), false positives (μ_n), and their respective standard deviations (σ_p, σ_n) (**Figure 9C-F**). The Z-factor score for satellite cell and couplet quantifications are determined to be 0.93 and 0.81 respectively.

Our high content analysis platform is able to maintain a high satellite cell detection rates of 91% and low false positive rates of 1.41% (**Figure 9D**). Couplet recognition is also detected at a rate of 78% and false positive rate of 1.16% (**Figure 9F**). We found no differences in detection rates of all three couplet types compared to manual quantification (**Figure 9G**).

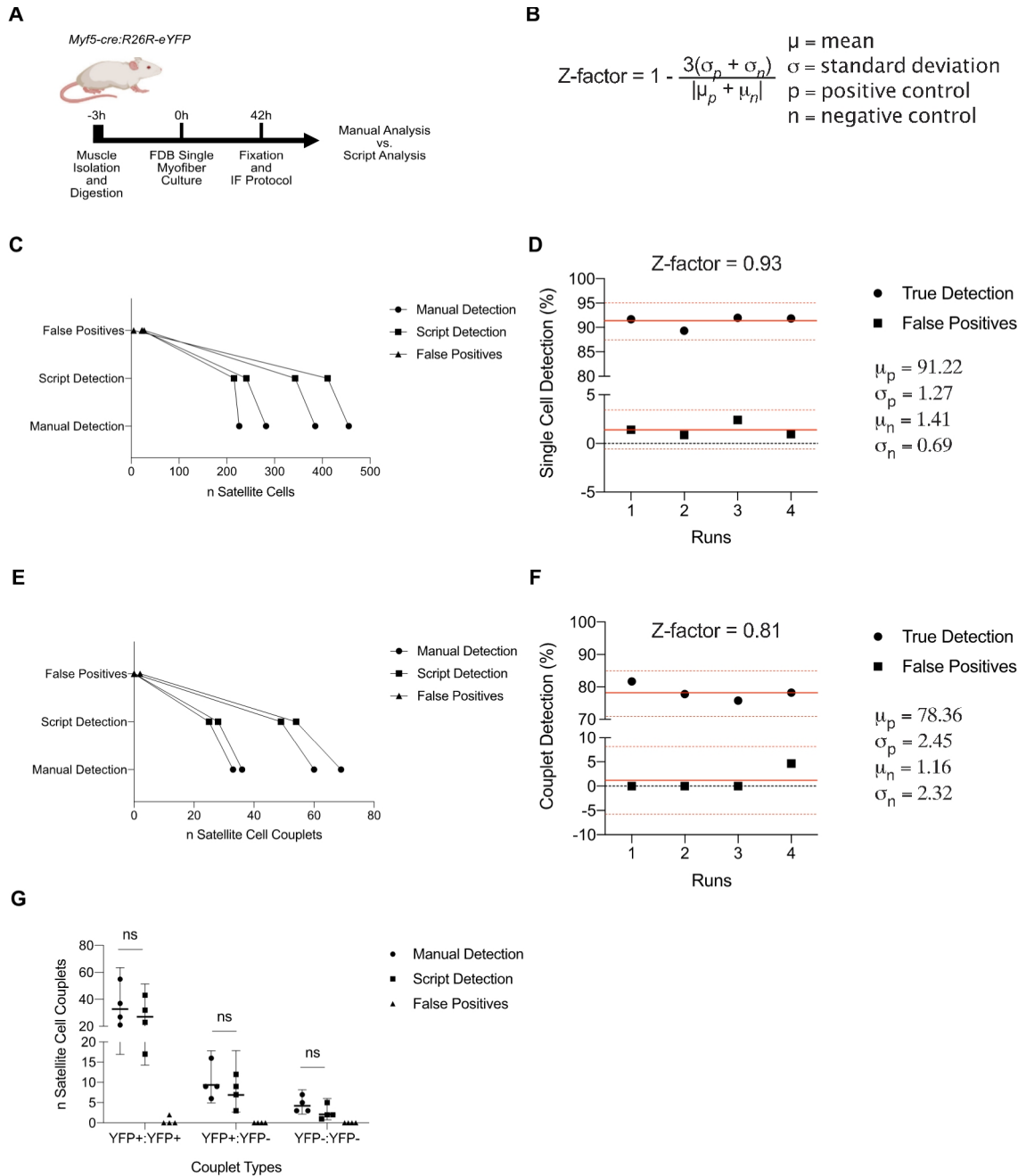


Figure 9. Screen Validation and Z-factor Calculation

A. *Myf5-Cre;R26R-eYFP* FDB single myofibers were isolated and cultured in DMEM supplemented with 20% FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin and 2.5 ng/ml bFGF for 42h. **B.** Z-Factor equation. **C.** Comparison of manual vs. script satellite cell quantification. Each line is a separate technical run (n=4). **D.** Satellite Cell Detection rate and Z-factor score visualization. Mean detection rates are solid red lines.

Standard deviation multiplied by 3 are dotted red lines. **E.** Comparison of manual vs. script couplet quantification. Each line is a separate run. N=4. **F.** Couplet Detection rate and Z-factor score visualization. Mean detection rates are solid red lines. Standard deviation multiplied by 3 are dotted red lines. **G.** Comparison of manual vs. script quantification of couplet types. Data are geometric means with 95% CI (n=4). Student's paired t-test.

3.3 FDB Culture and Fixation Optimization

The traditional myofiber culture growth medium was originally optimized for EDL myofiber culture. To ensure optimal satellite cell divisions, we optimized the growth media components for FDB single myofiber culture.

The base growth medium used in EDL myofiber culture is DMEM. We compared various mixtures of DMEM and F12 medium to determine a more suitable growth medium base for single myofiber culture than traditional DMEM alone. A 80:20 percent mixture of DMEM:F10 demonstrated a minute trend of stimulating satellite cell numbers and stem cell couplets (**Figure 10**). However, we determined the batch-to-batch variability in mixing base media did not outweigh the minute gains in satellite cell expansion. To maintain consistency, we opted to continue using DMEM as a base medium for single FDB myofiber culture.

Myofiber culture medium is supplemented with Fetal Bovine Serum (FBS) to provide various growth factors that stimulate satellite cell division. A concentration between 10-20% FBS yielded the greatest numbers of satellite cells per fiber (**Figure 11B**), while total stem cell numbers (YFP-) increased up until 30% compared to 0-10% FBS (**Figure 11C**). Total satellite cell divisions and stem cell divisions did not demonstrate a notable increase past 20% FBS (**Figure 11D-F**). Given the expense of FBS

and similar proportions of satellite stem cell divisions observed at 20%+ FBS, we opted to continue supplementing growth medium with 20% FBS for FDB myoculture.

Myofiber growth medium is further supplemented with basic fibroblast growth factor (bFGF) to enhance the division of satellite cells. The optimal concentration of bFGF supplementation was tested to determine the lowest concentration required to stimulate satellite cell divisions (**Figure 12**). We observed a final bFGF concentration of 2.5 ng/ml to be the minimal concentration required to stimulate total satellite cell numbers and divisions (**Figure 12B-D**). Concentrations of bFGF above 5 ng/ml diminished the numbers of satellite stem cell divisions stem cell divisions (**Figure 12EF**). Thus our findings determined that a final bFGF concentration of 2.5 ng/ml was optimal in culturing single FDB myofibers.

The final optimized growth medium was determined to consist of DMEM supplemented with final concentrations of 20% Fetal Bovine Serum (FBS), 2.5 ng/ml basic fibroblast growth factor (bFGF), 1% Chicken Embryo Extract (CEE), 1% penicillin/streptomycin.

Traditional EDL myofiber culture with *Myf5-Cre:R26R-eYFP* are typically fixed at 42h to capture dividing satellite cell couplets. Given the slight differences in satellite cell density and fiber size, we performed a time point experiment to confirm the optimal fixation time for capturing satellite cell couplets on FDB myofibers (**Figure 13**).

Fibers fixed at 45h demonstrated increased cell clusters, defined as 3 or more cells together (**Figure 13B**) with greater YFP⁺ populations leading to higher total satellite cell numbers without changes in YFP⁻ stem cell populations (**Figure 13C, Figure 13D**). No differences in satellite cell couplet types were found between time points (**Figure 13F-**

H). These results confirmed that a 42h fixation time point was optimal in capturing satellite cell couplets on FDB myofibers.

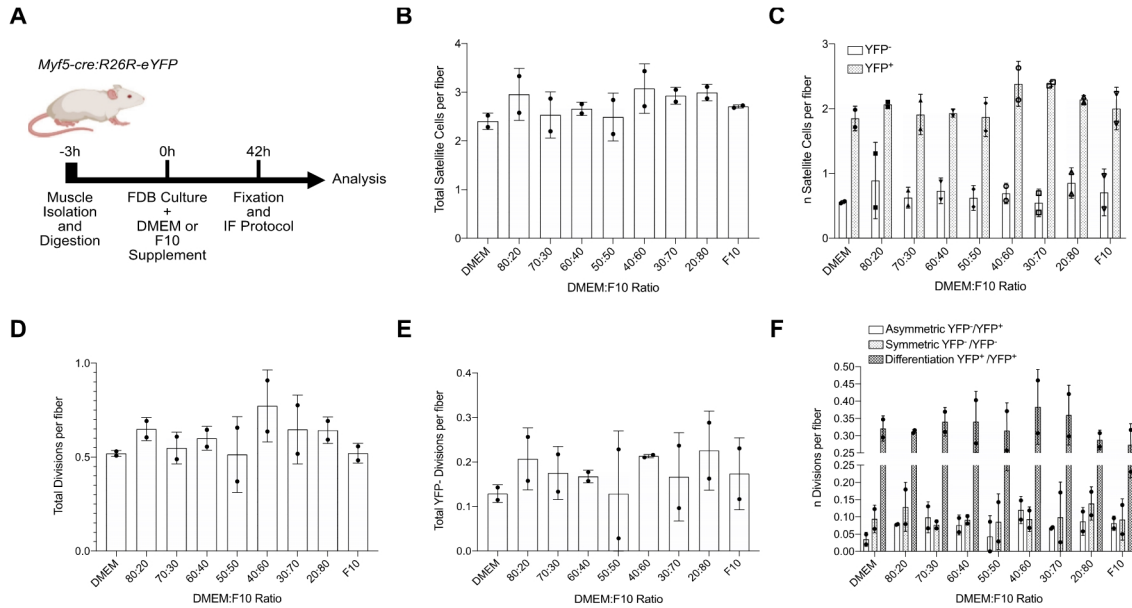


Figure 10. Growth Medium Base Optimization

A. *Myf5-Cre:R26R-eYFP* FDB single myofibers were isolated and cultured with a growth medium base of DMEM, F10, or percent ratios of the two medium. Growth media was then supplemented with 20% FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin and 2.5 ng/ml bFGF. **B.** Total Satellite Cells. **C.** Quantification of YFP⁺ and YFP⁻ populations. **D.** Total division counts. These counts include differentiation YFP⁺/YFP⁺, asymmetric YFP⁺/YFP⁻ and symmetric YFP⁻/YFP⁻ divisions. **E.** Total divisions containing YFP⁻. **F.** Quantification of Asymmetric YFP⁺/YFP⁻, Symmetric YFP⁻/YFP⁻, stem cell divisions and YFP⁺/YFP⁺ satellite differentiation divisions. All results were normalized to fiber count. N=2, 77-190 fibers per condition. Data are mean ± standard deviation.

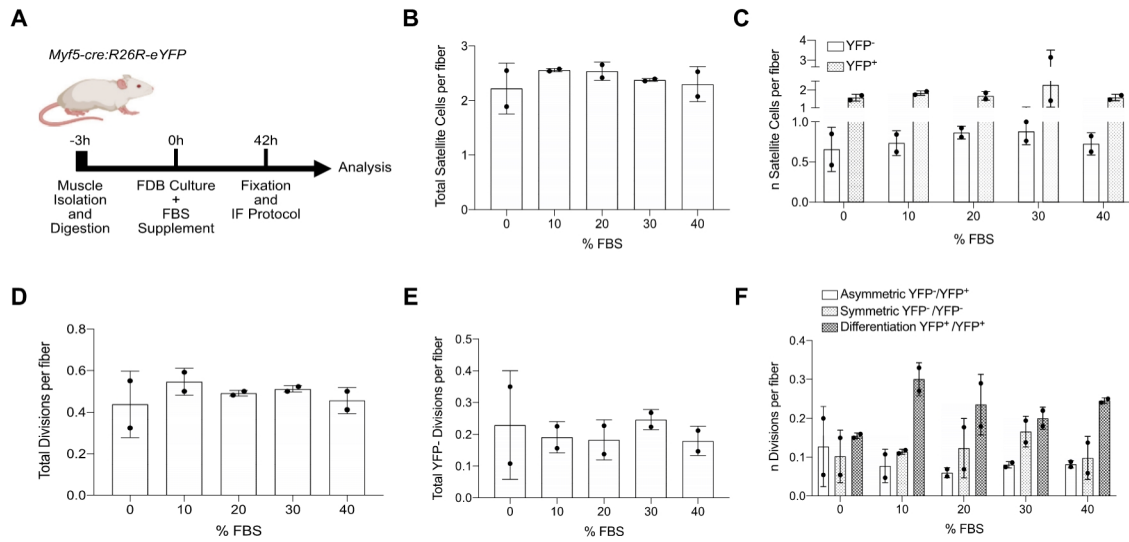


Figure 11. Growth Medium Serum Optimization

A. Schematic of experimental design. *Myf5-Cre:R26R-eYFP* FDB single myofibers were isolated and cultured in DMEM, supplemented with FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin, 2.5 ng/ml bFGF. **B.** Total Satellite Cells. **C.** Quantification of YFP+ and YFP- populations. **D.** Total division counts. These counts include differentiation YFP+/YFP+, asymmetric YFP+/YFP- and symmetric YFP-/YFP- divisions. **E.** Total divisions containing YFP-. **F.** Quantification of Asymmetric YFP+/YFP-, Symmetric YFP-/YFP-, stem cell divisions and YFP+/YFP+ satellite differentiation divisions. All results were normalized to fiber count. N=2, 77-190 fibers per condition. Data are mean $\pm$ standard deviation.

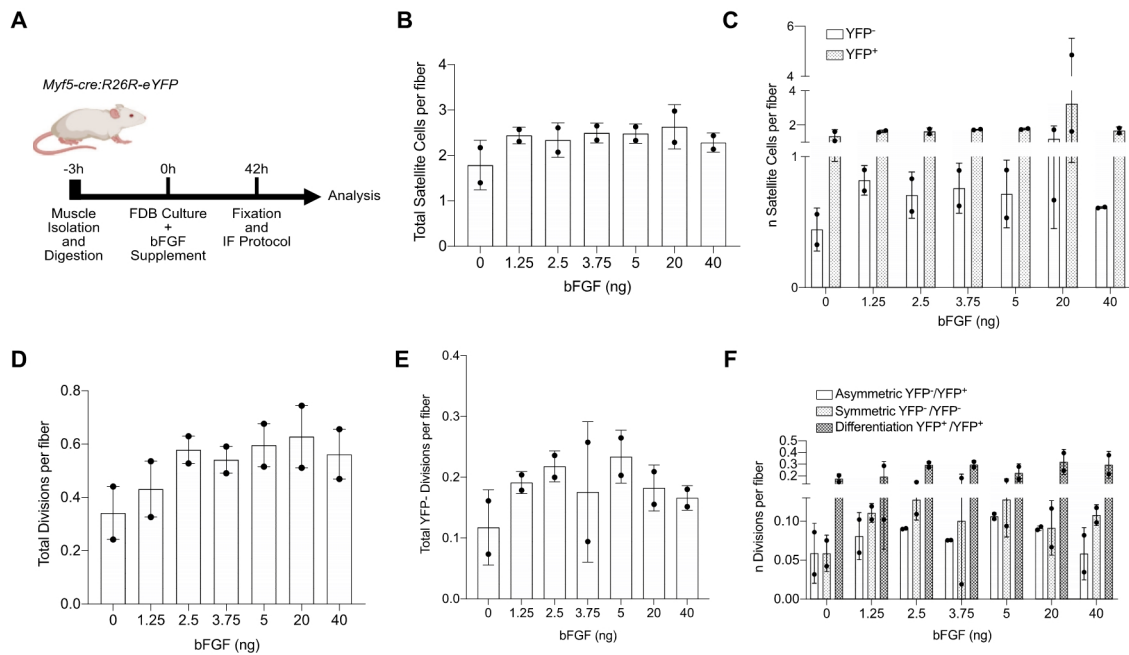


Figure 12. Growth Medium bFGF Optimization

A. Schematic of experimental design. *Myf5-Cre:R26R-eYFP* FDB single myofibers were isolated and cultured in DMEM, supplemented with FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin, and various concentrations of basic fibroblast growth factor (bFGF). **B.** Total Satellite Cells. **C.** Quantification of YFP+ and YFP- populations. **D.** Total division counts. These counts include differentiation YFP+/YFP+, asymmetric YFP+/YFP- and symmetric YFP-/YFP- divisions. **E.** Total divisions containing YFP-. **F.** Quantification of Asymmetric YFP+/YFP-, Symmetric YFP-/YFP-, stem cell divisions and YFP+/YFP+ satellite differentiation divisions. All results were normalized to fiber count. N=2, 77-190 fibers per condition. Data are mean $\pm$ standard deviation.

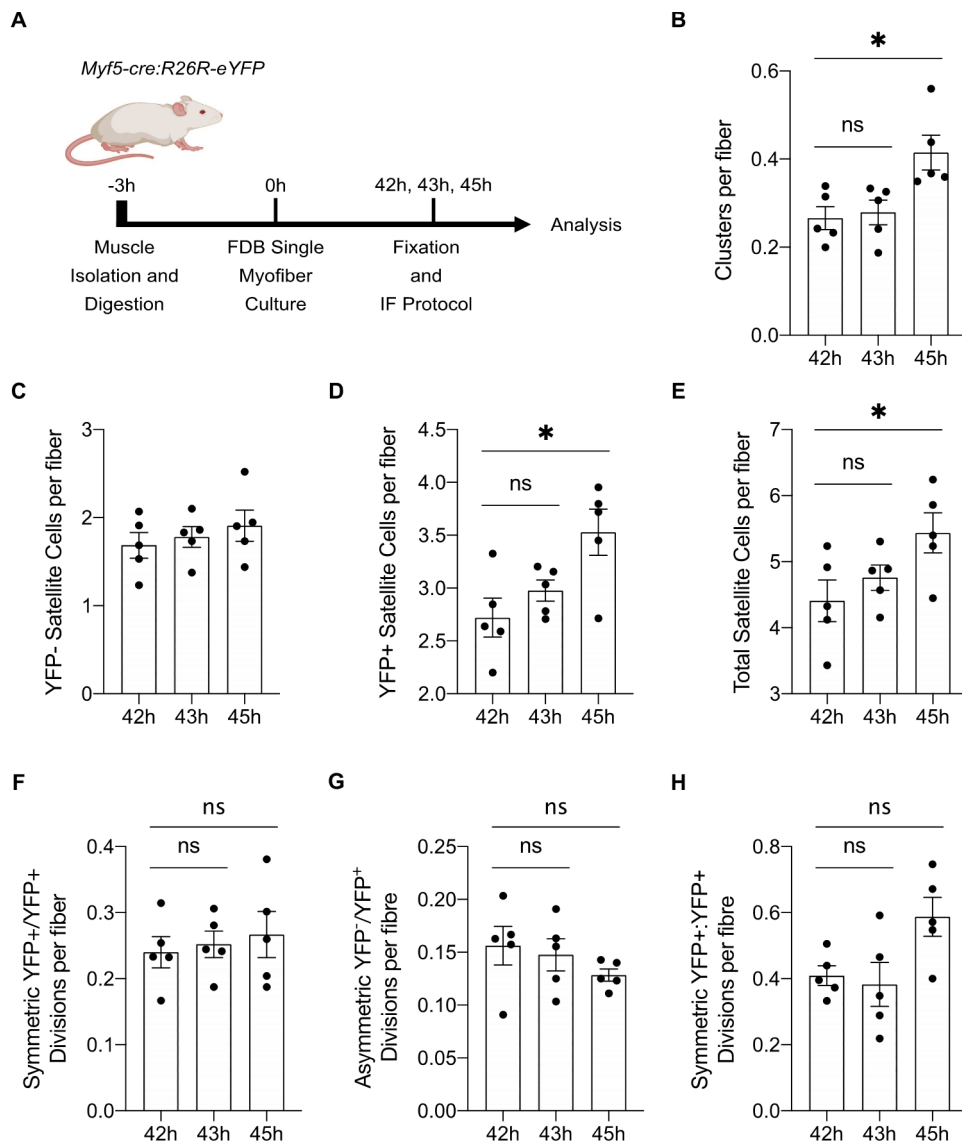


Figure 13. Fixation Time Point Optimization

A. Schematic of experimental design. *Myf5-Cre:R26R-eYFP* FDB single myofibers were isolated and cultured for 42h. Growth medium is DMEM supplemented with 20% FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin, 2.5 ng/ml bFGF. Changes in **B.** satellite cell clusters of 3+ cells, **C.** YFP- population, **D.** YFP+ population, **E.** Total satellite cell population, **F.** Symmetric YFP+:YFP+ divisions, **G.** Asymmetric YFP+:YFP- divisions, **H.** Symmetric YFP+:YFP+ divisions were quantified and compared at 42h, 43h, 45h fixation time points. N=5, 273-330 fibers per condition. Data are mean $\pm$ SEM. *p<0.05; One-way ANOVA with Dunnett's multiple comparison test.

3.4 Asymmetric and Symmetric Satellite Cell Division Positive Control

We selected EGF and Wnt7a as positive controls for asymmetric and symmetric satellite stem cell divisions, respectively. These controls are used to identify small molecule compounds that moderate satellite stem cell divisions. The EGF and Wnt7a ligands were chosen because they activate endogenous pathways of satellite stem cell asymmetric and symmetric division (Grand et al., 2009; Wang et al., 2019). Comparison of EGF and Wnt7a controls allow for the detection of novel modulators of satellite stem cell division with physiological relevance.

Traditional myofiber culture normalises satellite cell division quantifications per fiber. The Cellomics platform at the time of screening was unable to incorporate quantification of fiber numbers precisely. As a workaround, we opted to normalise changes in satellite cell divisions to the total number of stem cell divisions: divisions containing a YFP⁺ cell. Positive controls for satellite stem cell asymmetric (EGF) and symmetric division (Wnt7a) were consistent with previously published findings, demonstrating a 1.3-1.5 fold change in divisions (**Figure 14**). As previously described, our platform adapts the use of *flexor digitorum brevis* (FDB) muscles which are more appropriate for screening due to their size and satellite cell density (Shefer and Yablonka-Reuveni, 2005; Wang et al., 2019). Our novel screening platform was able to quantify changes in both asymmetric and symmetric stem cell fate decisions because it is based on visual detection by image rather than PCR detection of Cre-mediated deletions (Wang et al., 2019).

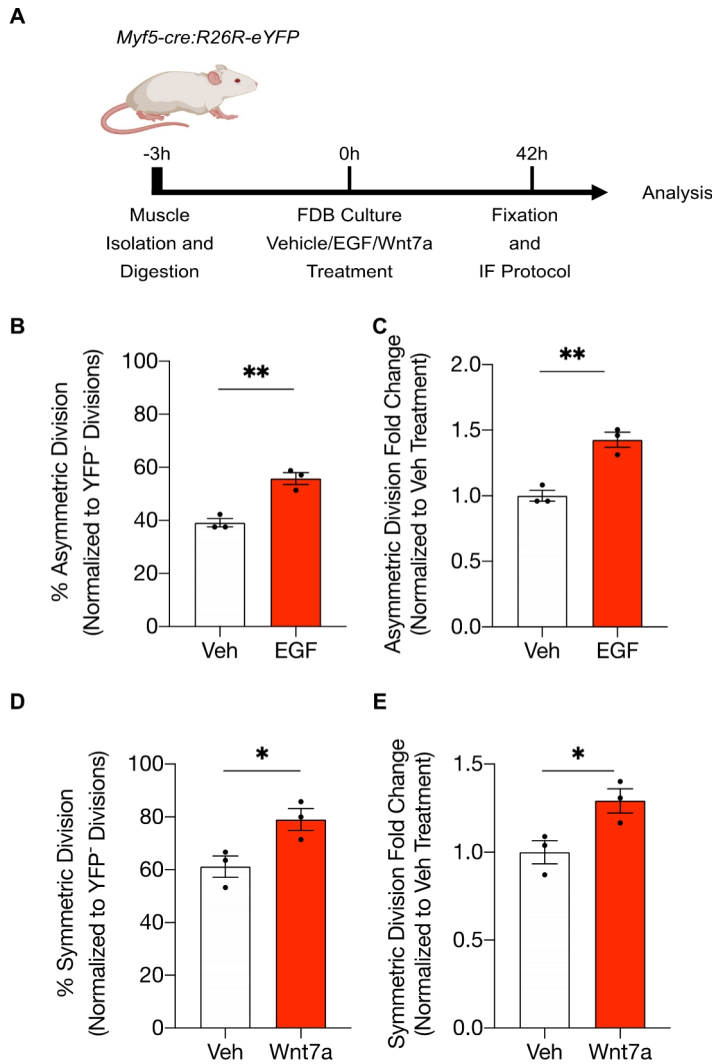


Figure 14. Cellomics EGF and Wnt7a Positive Control Validation

A. Schematic of experimental design. *Myf5-Cre:R26R-eYFP* FDB single myofibers were isolated and cultured for 42h. Growth medium is DMEM supplemented with 20% FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin, 2.5 ng/ml bFGF. Test conditions were further supplemented with PBS (Vehicle), EGF (100 ng/ml) or Wnt7a (50 ng/ml). EGF effect on satellite stem cell asymmetric division was quantified as **B.** percent (%) change normalised to total YFP⁺ divisions, or **C.** fold change normalised to vehicle treatment. Wnt7a effect on satellite stem cell symmetric division was quantified as **D.** percent (%) change normalised to total YFP⁺ divisions, or **E.** fold change normalised to vehicle treatment. Data are presented as mean. Error bars are $\pm$ SEM (n=3). *p<0.05; **p<0.01; Paired Student's t-test.

3.5 Identification of KDR by High Content Screening as a Modulator of Satellite Stem Cell Asymmetric Division

The satellite cell is extremely sensitive to its microenvironment. Changes to the satellite cell niche lead to changes in satellite cell identity in merely hours (Machado and Relaix, 2016; Velthoven et al., 2017). Therefore, we devised a novel image-based high content analysis platform to quantify satellite stem cell fate decisions using *ex vivo* culture of single myofibers to preserve the satellite cell niche. We combined the *Myf5-Cre* (Tallquist et al., 2000) and *R26R-eYFP* (Srinivas et al., 2001) alleles to visualize stem cell fate decisions as previously described (Kuang et al., 2007; Wang et al., 2019) (**Figure 15A**). This imaging approach allows for lineage tracing of muscle stem cells (*Myf5*⁻ and *eYFP*⁻) that commit (*Myf5*⁺ and *eYFP*⁺) to the myogenic lineage (**Figure 15B**).

We screened the Ontario Institute for Cancer Research (OICR) kinase inhibitor library containing over 480 kinase inhibitors using Wnt7a and EGF as positive controls for stimulating symmetric and asymmetric divisions, respectively (Grand et al., 2009; Wang et al., 2019). The targets affecting muscle stem cell divisions were analysed using Gene Ontology enrichment analysis to help determine which pathways may be involved (**Figure 15F**). We required an additional method of assessing positive hits to account for the caveats due to the resource limitations of our screen. For example, we screened compounds at 1 μ M, while many of the compounds were designed to specifically inhibit several targets at various concentrations. To accommodate these limitations while limiting subjectivity, we devised a weighted scoring system to identify compound inhibitors that affected muscle stem cell division (**Figure 15E**): multiple inhibitors with IC₅₀ values closer to the

screening concentration of 1 μ M were weighted more heavily than targets with fewer inhibitors and IC50 values further from 1 μ M (**Figure 15C-F**). Using this asymmetric scoring system, we evaluated the effect of small molecule compounds on muscle stem cell division. Previously identified small molecule inhibitors that stimulate muscle stem cell symmetric division (EGFR-AurkA and P38 α/β MAPK signaling) demonstrated an expected decrease in asymmetric score validating the reliability of our platform to quantify asymmetric stem cell division (Bernet et al., 2014; Chang et al., 2018; Wang et al., 2019). Although the OICR kinase inhibitor library did not contain inhibitors targeting Fzd7, the receptor for non-canonical Wnt7a-driven symmetric stem cell division, inhibitors of downstream effectors of PCP signaling (i.e ROCK signaling) and cross talk pathways demonstrated an expected increase in asymmetric score (**Figure 15D**) (Bentzinger et al., 2014; Grand et al., 2009; Maltzahn et al., 2011, 2012a, 2012b). Together, these results demonstrate our high content analysis platform capable of identifying modulators of both asymmetric and symmetric stem cell divisions. Notably, we identified the receptor tyrosine kinase KDR as a positive regulator of muscle asymmetric division (**Figure 15C**).

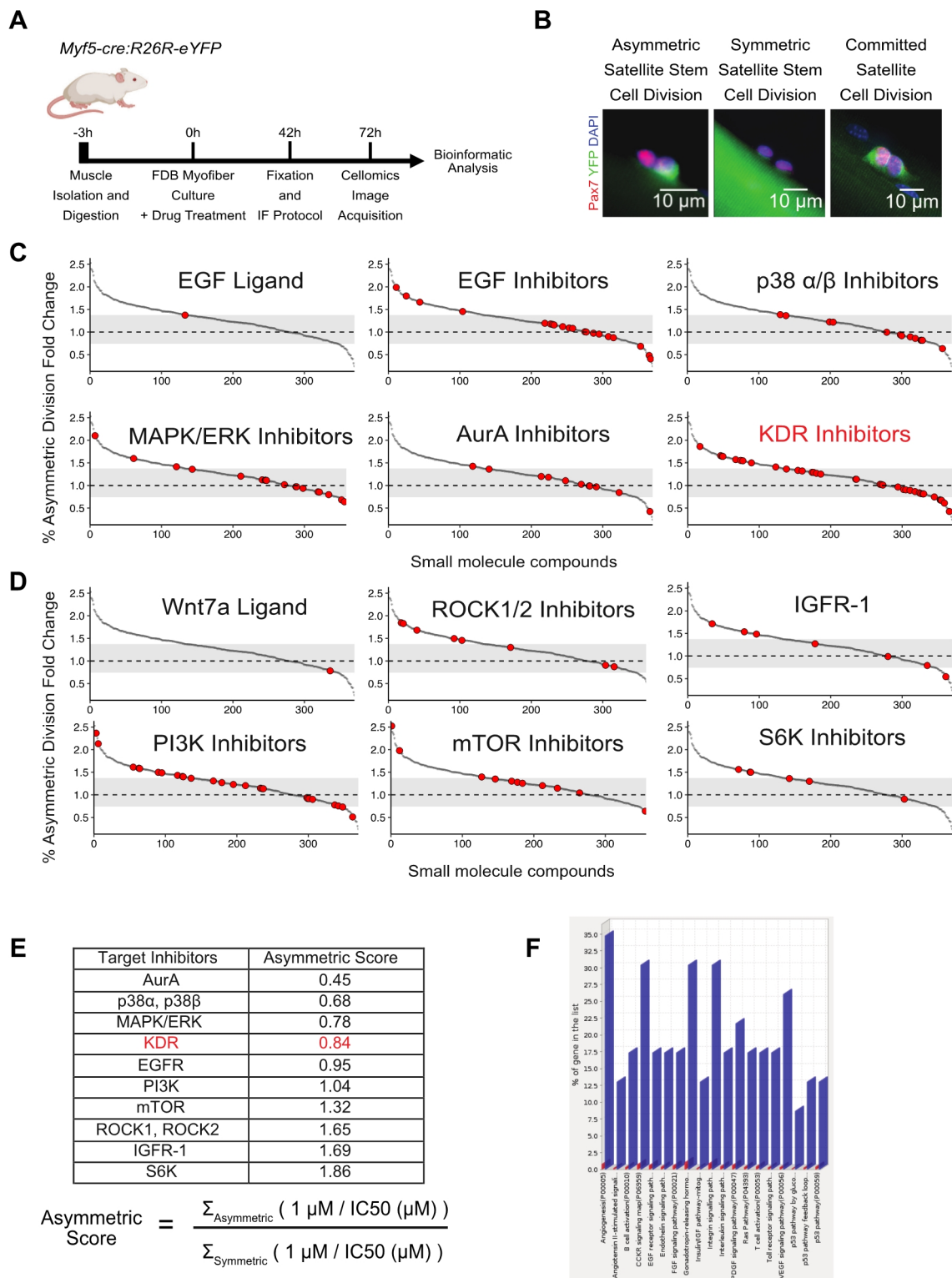


Figure 15. High Content Screen Results

A. Schematic of high content screen experimental design. *Myf5-Cre:R26R-eYFP* FDB single myofibers were isolated and cultured for 42h. Growth medium is DMEM

supplemented with 20% FBS, 2% Chicken Embryo Extract, 1% Penicillin/Streptomycin, 2.5 ng/ml bFGF. Vehicle controls were supplemented with 0.1% DMSO in PBS and positive controls for asymmetric and symmetric division with EGF (100 ng/ml) or Wnt7a (50 ng/ml), respectively. Small molecule compounds were screened in technical duplicates at 1 μ m. Results were analyzed using a custom MATLAB-based script.

B. Visual readout of satellite cell couplets quantified.

C-D. Readout of small molecule screen with inhibitors that **C.** decrease asymmetric division or **D.** increase asymmetric division. Positive controls for asymmetric and symmetric division are EGF and Wnt7a ligand, respectively. Each grey dot represents a single compound. Red dots indicate the compounds specified. Data is represented as mean of technical duplicates. Data is plotted as fold change in percent (%) asymmetric division compared to vehicle treatment, normalized to YFP- divisions.

E. Asymmetric scoring results and formula used based on number of inhibitors and IC50 values.

F. GO Terms analysis of top hits and enriched pathways.

Chapter 4. Results II. Investigation of KDR as a Modulator of Muscle Satellite Stem Cell Asymmetric Division

All experiments were performed by the author, William Chen, except for the following. Morten Ritso performed half the cardiotoxin injuries presented in Figure 25, Figure 26, Figure 27 and Figure 28.

4.1 Kinase Domain Receptor Expression Validation

Given the scarcity of satellite cells and difficulty in maintaining stem cell identity in culture, we validated KDR expression by quantitative digital droplet PCR (ddPCR) of freshly sorted satellite cells (FSC) from uninjured hindlimb *tibialis anterior* (TA) and *gastrocnemius* (GA) muscle, and proliferating myogenic precursor cells (PMC) isolated from hindlimb TA and GA muscle at 3 days post cardiotoxin injection (DPI) using *Pax7-nGFP* mice (Sambasivan et al., 2009) in both wild type and crossed with the *mdx* strains (**Figure 16A**). We followed the same gating strategy as previously reported and validated, with the addition of positive markers β 1-Integrin (CD29) and VCAM1 (CD106) to further enrich for the stem cell population (Choo et al., 2017; Kuang et al., 2007; Rozo et al., 2016). Robust expression of *Pax7* and the myogenic regulatory factors (MRF) confirmed that myogenic cells were highly enriched in these isolations (**Figure 16B**).

Our ddPCR results confirm the expression of *Kdr* mRNA within satellite cells with greater expression in FSC relative to low expression in PMC populations in both wild type and *mdx* (**Figure 16C**). These results corroborate our transcriptome data on satellite cells and others (Dumont et al., 2015a; Micheli et al., 2020). KDR protein expression was confirmed by immunofluorescence on satellite cells cultured on myofibers for 24h (**Figure**

17). Interestingly, KDR expression appears to be upregulated upon re-entry into the cell-cycle during activation of satellite cells at 24h during culture. We observed KDR expression in a subset of satellite cells at 24h, which increased to nearly all satellite cells by 42h (**Figure 18**) Expression of *KDR* does not appear to be expressed in progenitors (**Figure 16C**), and mRNA and protein expression in primary myoblasts was below the limit of detection (not shown). We also confirmed the expression of the KDR ligand *Vegfa* in both FSC and PMC populations, corroborating previously published findings on VEGFA secretion by satellite cells, suggesting a potential autocrine source for KDR signaling in satellite cells (**Figure 16D**) (Verma et al., 2018).

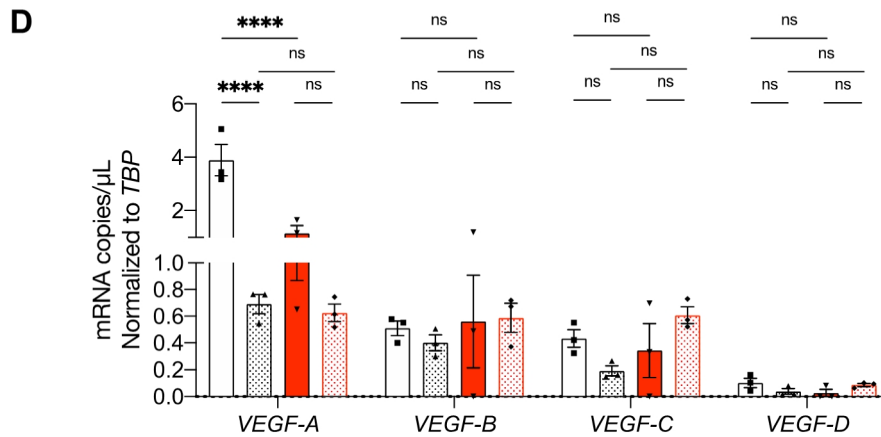
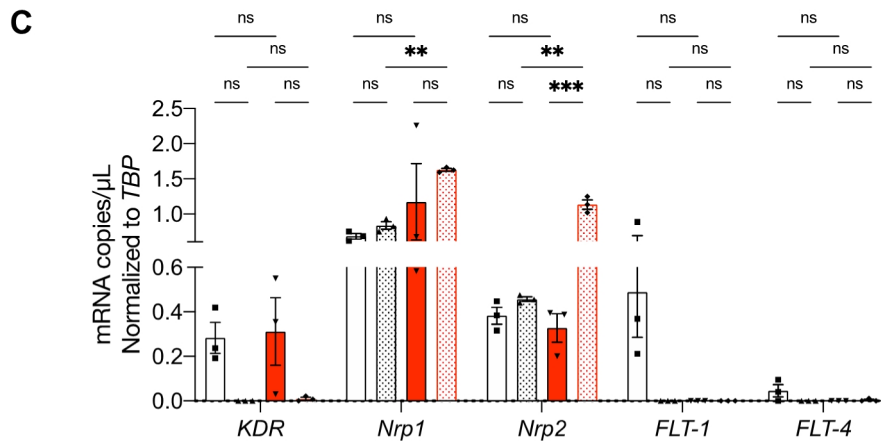
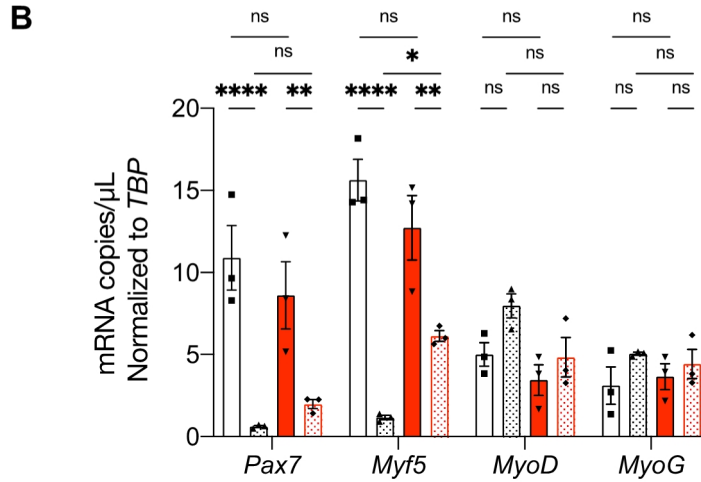
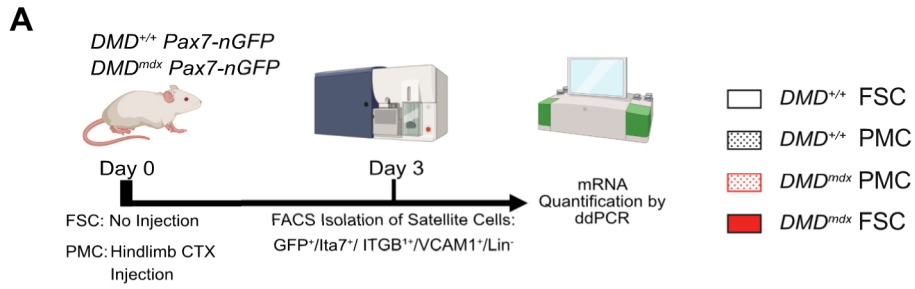


Figure 16. KDR is expressed in satellite cells

A. Schematic of satellite cell isolation by FACs experimental design. Positive markers for satellite cells include GFP, α 7-integrin (ITGA), β 1-integrin (ITGB1), and VCAM1. Negative lineage markers (Lin-) include CD45, CD11b, Sca-1 and CD31.

B. Expression of myogenic regulatory factors (MRFs) normalized to TBP. Data are presented as mean. Error bars are $\pm$ SEM (n=3). ***p<0.001, **** p<0.0001; two-way ANOVA with Tukey's multiple comparisons test.

C. Receptor expression of KDR (VEGFR2), Nrp1, Nrp2, FLT-1 (VEGFR1), FLT-4 (VEGFR3) normalized to TBP. Data are presented as mean. Error bars are $\pm$ SEM (n=3). **** p<0.0001; two-way ANOVA with Tukey's multiple comparisons test.

D. Ligand expression of VEGFA, VEGFB, VEGFC, VEGFD normalized to TBP. Data are presented as mean. Error bars are $\pm$ SEM (n=3). **** p<0.0001; two-way ANOVA with Tukey's multiple comparisons test.

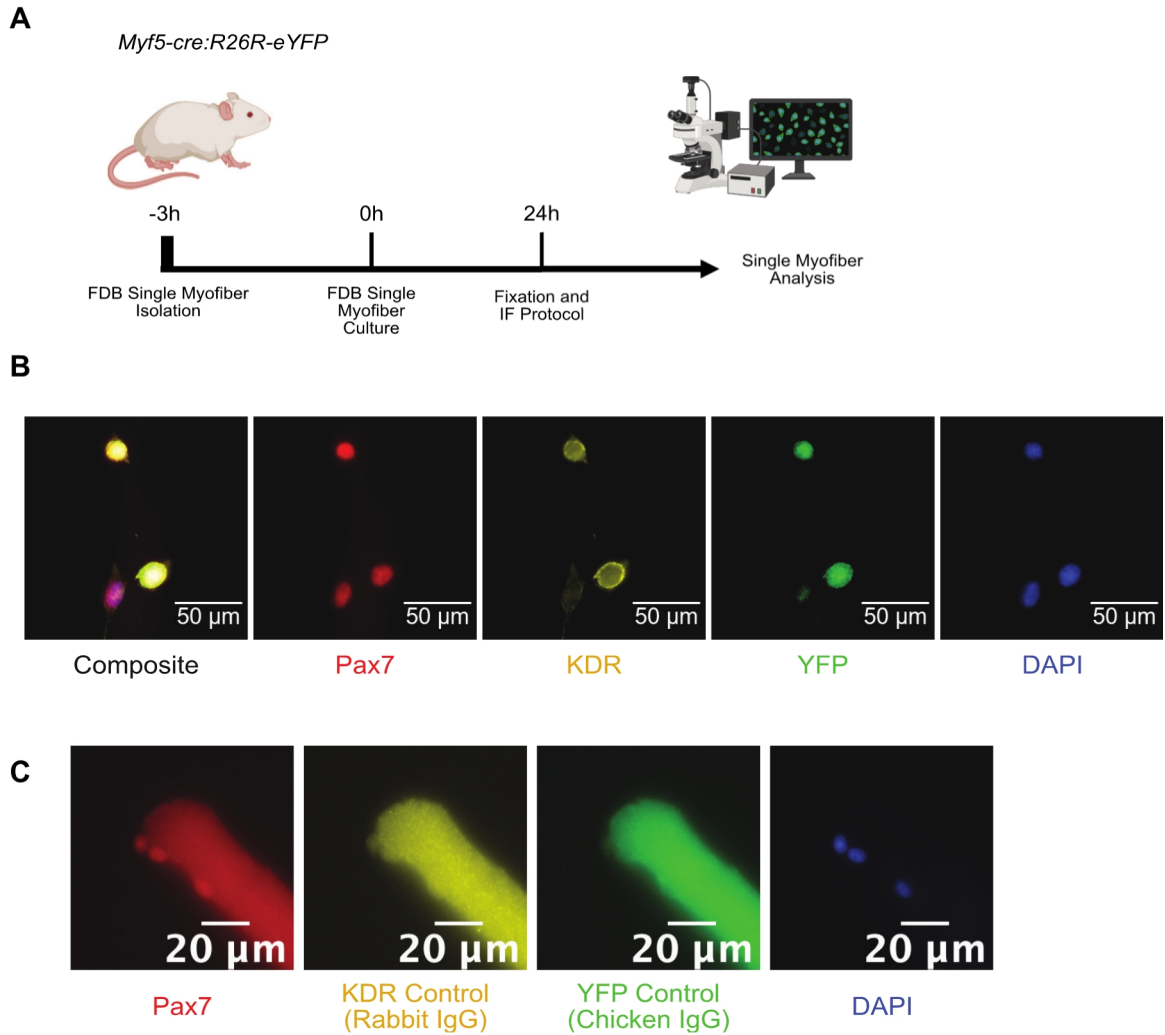


Figure 17. KDR Protein Validation

A. Schematic of FDB single fiber culture experimental design.

B. Representative immunofluorescence (IF) images of KDR expression (yellow) in PAX7+ (red) satellite eYFP- and eYFP+ (green) cells on FDB single myofibers isolated from *Myf5-Cre:R26R-eYFP* mice. Nuclear DAPI (blue) stain was used.

C. Pax7+ (red) satellite cells stained with IgG controls for KDR (rabbit, yellow), GFP (chicken, green) and nuclear DAPI (blue).

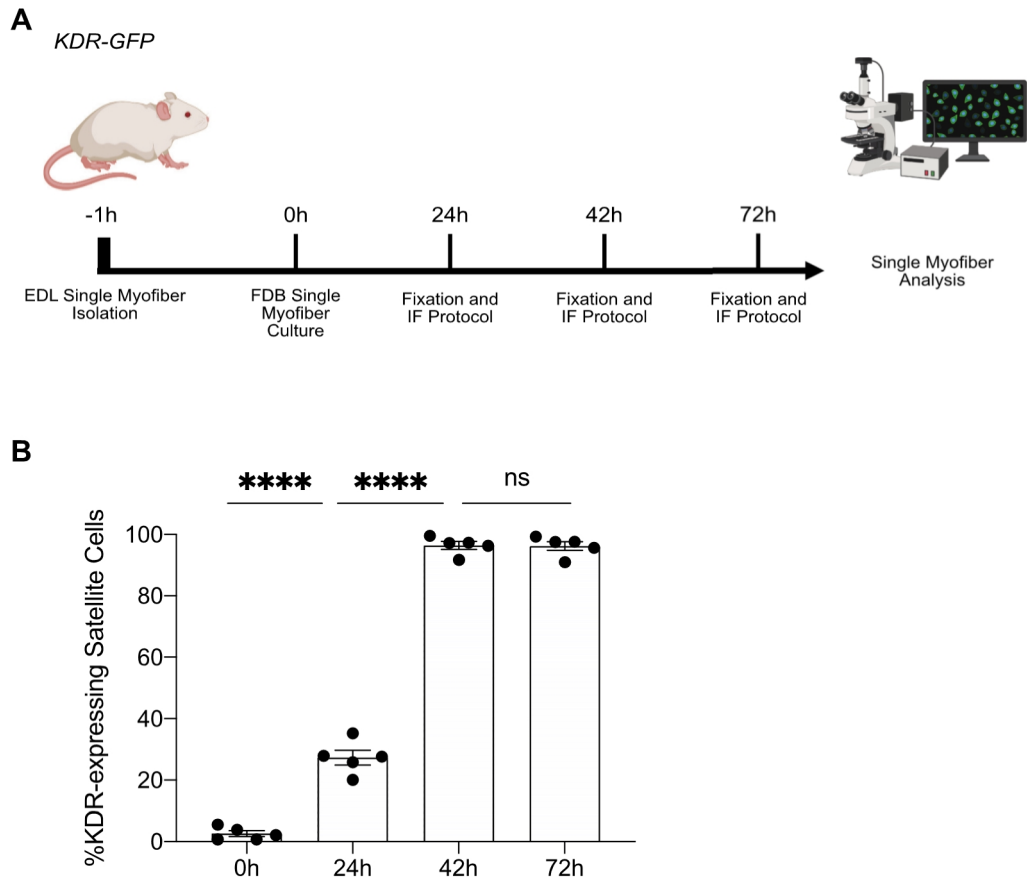


Figure 18. KDR is expressed in activated satellite cells

A. Schematic of EDL single fiber culture experimental design.

B. Quantification of GFP-expressing cells from KDR-GFP myofibers. Data are presented as mean. Error bars are $\pm$ SEM (n=5). **** $p < 0.0001$; one-way ANOVA with Tukey's multiple comparisons test.

4.2 KDR signaling modulates stem cell asymmetric division

To validate the effect of KDR signaling on stem cell asymmetric division and exclude possible off-target effects from small molecule compounds, we quantified satellite stem cell divisions following siRNA knockdown of KDR on extensor digitorum longus (EDL) myofibers from *Myf5-Cre:R26R-eYFP* mice (**Figure 20**). We confirmed the siRNA knockdown of *Kdr* using pre-validated commercially available qPCR and ddPCR primers on mouse-derived endothelial cells and by immunofluorescence (**Figure 19**).

Upon siRNA-mediated knockdown of *Kdr*, we observe a seven-fold decrease in satellite stem cell asymmetric divisions without a significant effect on the numbers of symmetric stem cell divisions (**Figure 20B, Figure 20C**). The impairment of asymmetric division following siKDR treatment corroborates our findings from pharmacological inhibition of KDR (**Figure 15C, Figure 15E**). This effect is specific to eYFP⁻ satellite stem cell divisions, as we observed no changes to numbers of eYFP⁺ satellite myogenic cell divisions (**Figure 20D**). Asymmetric stem cell divisions generate one stem (eYFP⁻) and one committed (eYFP⁺) daughter cell (Kuang et al., 2007). Consistent with this, we observed no change in the number of total Pax7⁺ satellite cells nor the number of eYFP⁻ satellite stem cells after the first round of divisions after 42h of culture (**Figure 20E, Figure 20F**).

To further understand the role of KDR signaling in muscle stem cell asymmetric division, we explored the effects of activating the KDR signaling pathway. Given the promiscuous binding of VEGF ligands and isomers to multiple VEGF receptors, we selected the use of Orf virus-derived VEGFE for its specificity in binding to KDR only

(Ogawa et al., 1998; Vieira et al., 2010). VEGFE has previously been demonstrated to be able to bind to KDR homodimers and heterodimers with co-receptors Nrp1/Nrp2, with equal potency compared to VEGFA, without binding to decoy receptors VEGFR1 (also known as Flt1) or soluble VEGFR1 (sFlt1) (**Figure 20G, Figure 16C**) (Kawamura et al., 2008; Ogawa et al., 1998; Vieira et al., 2010). Stimulation of the KDR signaling pathway with VEGFE ligand treatment increased the numbers of asymmetric divisions nearly 3-fold, corroborating the siRNA knockdown results, confirming that KDR signaling positively modulates asymmetric muscle stem cell division (**Figure 20H**). Again, KDR signaling is specific to asymmetric stem cell division with no changes in symmetric satellite stem cell divisions, satellite myogenic cell divisions, total satellite cell numbers and satellite stem cell populations (**Figure 20I-L**).

Taken together, our data here suggests that KDR signaling specifically stimulates satellite stem cell asymmetric divisions. Interestingly, KDR signaling does not affect satellite stem cell fate decisions to undergo symmetric division, meaning satellite stem cell fate decisions are not necessarily binary.

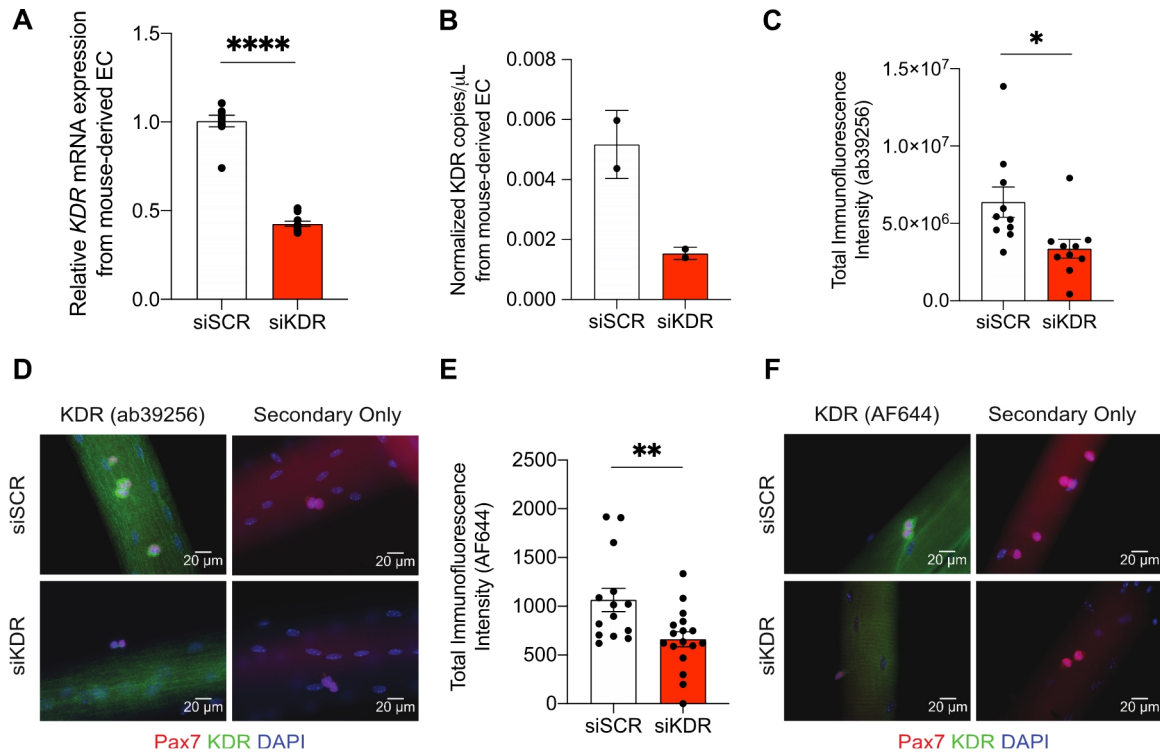


Figure 19. KDR siRNA Validation

A. Validation of siKDR (10 nM) on wild type mouse-derived endothelial cells (EC) by qPCR. Data are presented as mean. Error bars are $\pm$ SEM (n=5 technical replicates). ****
p<0.0001; Student's paired t-test.

B. Validation of siKDR (10 nM) on wild type mouse-derived endothelial cells (EC) by ddPCR using pre-validated KDR probes. Data are presented as mean. Error bars are $\pm$ SD (n=2).

C. Validation of KDR antibody (ab39256) by siKDR (10nM) knockdown. Data are presented as mean. Error bars are $\pm$ SEM (n=10 cells per condition). *p<0.05; Student's paired t-test.

D. Representative immunofluorescence (IF) images of Pax7+ (red) satellite cells, KDR (green, ab39256), secondary only controls (Alexa Fluor 488) and DAPI (blue) nuclear stain on EDL single myofibers isolated from wild-type mice treated with siSCR control or siKDR.

E. Validation of KDR antibody (AF644) by siKDR (10nM) knockdown. Data are presented as mean. Error bars are $\pm$ SEM (n=14-17 cells per condition) **p<0.01; Student's paired t-test.

F. Representative immunofluorescence (IF) images of Pax7⁺ (red) satellite cells, KDR (green, AF644), secondary only controls (Alexa Fluor 488) and DAPI (blue) nuclear stain on EDL single myofibers isolated from wild-type mice treated with siSCR or siKDR.

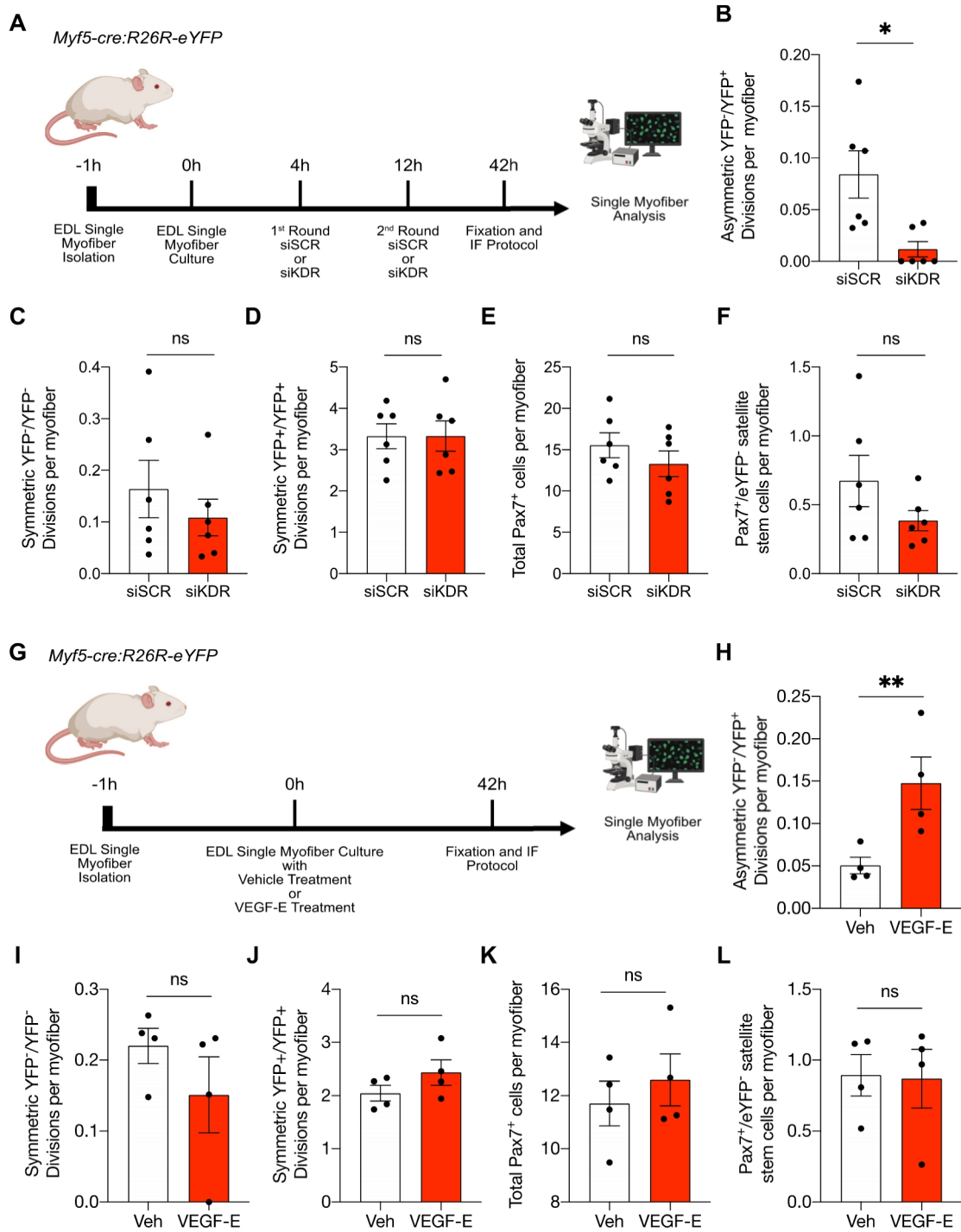


Figure 20. KDR modulates satellite stem cell asymmetric division

A. Schematic of (B-F) siRNA knockdown experiments on EDL single myofiber isolated from Myf5-Cre:R26-eYFP treated with control (siSCR, 10 nM) or KDR knockdown (siKDR, 10 nM). Analysis was performed after 42h of culture. Data are presented as

mean. Error bars are $\pm$ SEM (n=6). *p<0.05; Student's paired t-test.

B. Numbers of Asymmetric YFP-/YFP+ divisions per myofiber.

C. Numbers of Symmetric YFP-/YFP- divisions per myofiber.

D. Numbers of Symmetric YFP+/YFP+ divisions per myofiber.

E. Numbers of Pax7+ satellite cells per myofiber.

F. Numbers of Pax7+/eYFP- satellite stem cells per myofiber.

G. Schematic of (H-L) VEGFE ligand treatment experiments on EDL single myofiber

isolated from Myf5-Cre:R26-eYFP treated with vehicle (Veh) or VEGFE (100 ng/ml).

Analysis was performed after 42h of culture. Data are presented as mean. Error bars are $\pm$ SEM (n=4). *p<0.05; Student's paired t-test.

H. Numbers of Asymmetric YFP-/YFP+ divisions per myofiber.

I. Numbers of Symmetric YFP-/YFP- divisions per myofiber.

J. Numbers of Symmetric YFP+/YFP+ divisions per myofiber.

K. Numbers of Pax7+ satellite cells per myofiber.

L. Numbers of Pax7+/eYFP- satellite stem cells per myofiber.

4.3 KDR signaling requires the dystrophin glycoprotein complex

Given the specificity of KDR signaling and its role in asymmetric but not symmetric division, we explored the role of KDR signaling in the context of DMD using *mdx* mice. Previously, we showed that satellite cells lacking dystrophin (*Dmd*) or dystroglycan (*Dag1*) exhibit an inability to establish polarity leading to a significant reduction in numbers of asymmetric divisions and failure to generate sufficient numbers of committed progenitors for efficient muscle regeneration (Dumont et al., 2015a).

To investigate whether KDR is closely associated with the DGC complex, a requirement for protein-protein interaction, we performed a proximity ligand assay (PLA) on KDR and two components of the DGC complex: *Dmd* and *Dag1* in wild type satellite cells. Interestingly, we observed an increase in PLA signal with both components of the

DGC complex, indicating close proximity between KDR and the DGC complex in satellite cells (**Figure 21**).

To investigate KDR signaling in the DMD context, we isolated and cultured single EDL myofibers from *mdx* mice containing the *Myf5-Cre:R26R-eYFP* allele for quantification of satellite stem cell asymmetric divisions. Intriguingly, we observed no effect on asymmetric stem cell divisions following treatment of *mdx* single myofibers with VEGFE (**Figure 22B**). Moreover, VEGFE treatment of *mdx* myofibers did not affect satellite stem cell symmetric divisions, satellite myogenic cell divisions, or changes in satellite cell populations (**Figure 22C-F**).

To further investigate whether KDR signaling is overstimulated or impaired in *mdx* satellite cells, we performed siRNA-mediated knockdown of *Dmd* on single myofibers isolated from *Myf5-Cre:R26R-eYFP* mice treated with VEGFE to mimic the *mdx* in an acute context (**Figure 22G**). We utilized a previously reported and validated siRNA knockdown of *Dmd* and reconfirmed its effect by immunofluorescence and on myoblasts after 2-days of differentiation by ddPCR using pre-validated commercially available *DMD* primers (**Figure 23**) (Dumont et al., 2015a).

VEGFE treatment was unable to rescue asymmetric divisions of satellite stem cells in conjunction with siRNA-mediated knockdown of *Dmd* (**Figure 22H**). In addition, no changes were observed in satellite stem cell symmetric divisions (**Figure 22I**). Consistent with previous findings in *mdx* mice, we observed fewer numbers of eYFP⁺ satellite myogenic cell divisions resulting in reduced numbers of total satellite cells following knock down of *Dmd* (**Figure 22J, Figure 22K**) (Dumont et al., 2015a). Moreover, we did

not observe any change in numbers of eYFP⁺ satellite stem cells after 42h of culture (**Figure 22L**).

Our findings here show that the KDR-mediated satellite stem cell asymmetric division axis requires the DGC to function and the loss of DGC cannot be rescued by ligand stimulation of KDR.

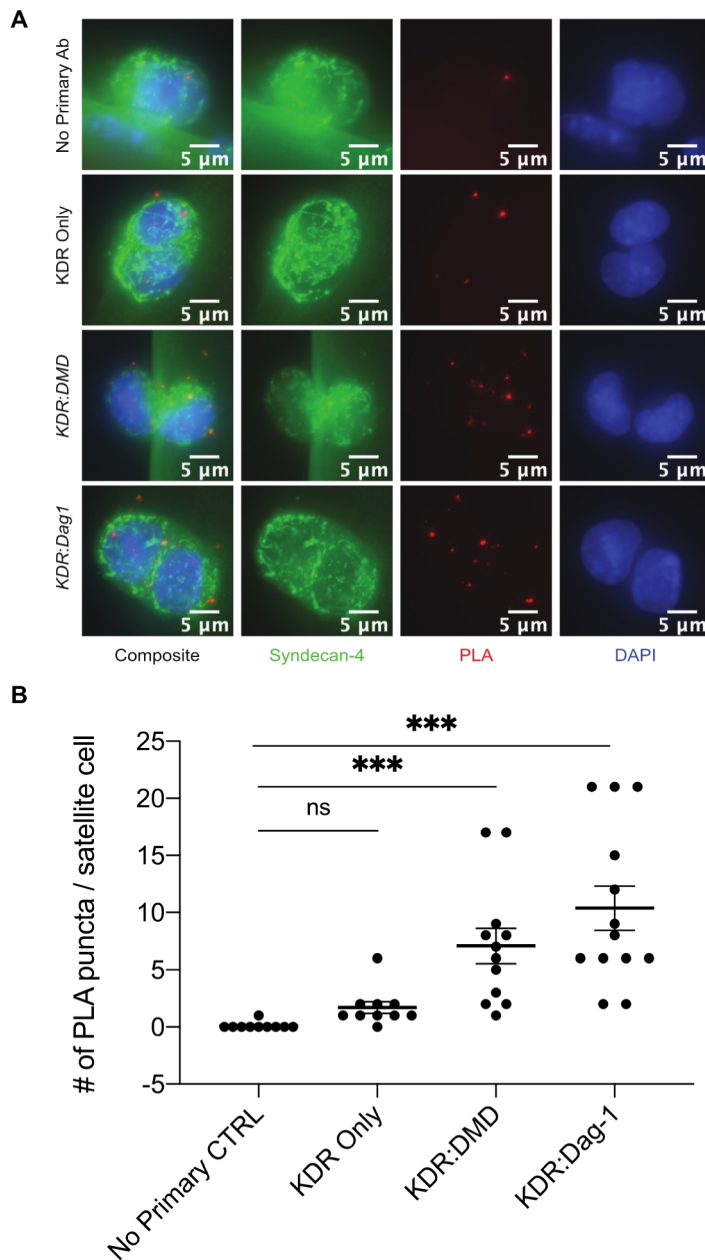


Figure 21. KDR is closely associated to the DGC

A. Representative immunofluorescence (IF) images of proximity ligand assay (PLA) punctate signal (red) of KDR:Kag1 and KDR:DMD antibodies on wild-type Syndecan-4+ (green) satellite cells and DAPI (blue) nuclear stain. Control conditions are secondary PLA probes only (No Primary Ab) or single primary KDR antibody (KDR only).

B. Quantification of PLA signal in single primary antibody controls or KDR:DMD and KDR:Kag1. Data are presented as mean. Error bars are $\pm$ SEM (n=11-13 cells per

condition). ** $p < 0.001$, *** $p < 0.0001$; One-way Anova with Dunnett's multiple comparison test.

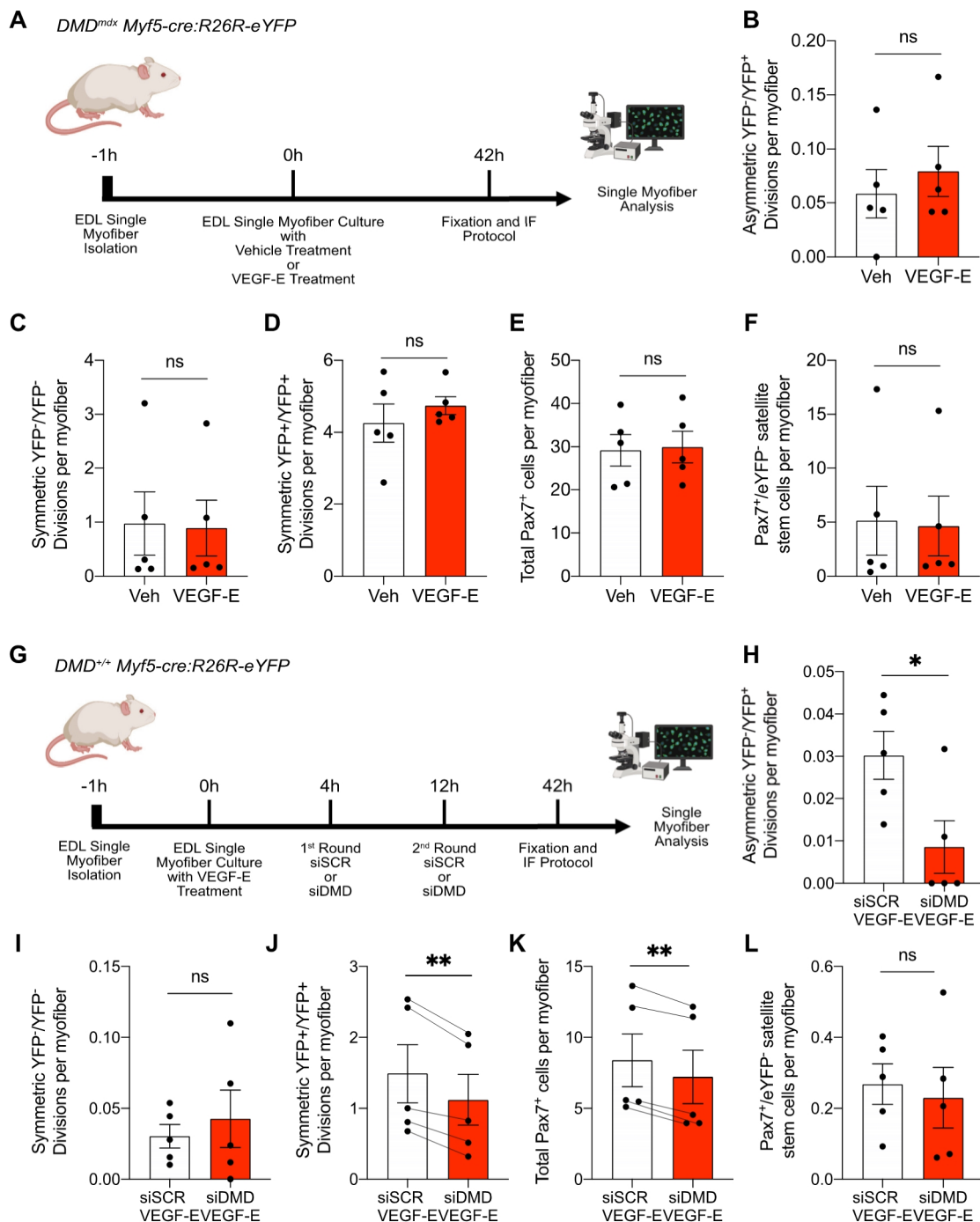


Figure 22. MuSC KDR signaling requires the dystroglycan complex

A. Schematic of (**B-F**) ligand treatment with vehicle (Veh) or VEGFE (100 ng/ml) on EDL single myofiber isolated from *mdx Myf5-Cre:R26-eYFP*. Analysis was performed after 42h of culture. Data are presented as mean. Error bars are $\pm$ SEM (n=5); Student's paired t-test.

B. Numbers of Asymmetric YFP-/YFP+ divisions per myofiber

- C.** Numbers of Symmetric YFP-/YFP- divisions per myofiber.
- D.** Numbers of Symmetric YFP+/YFP+ divisions per myofiber.
- E.** Numbers of Pax7+ satellite cells per myofiber.
- F.** Numbers of Pax7+/eYFP- satellite stem cells per myofiber.
- G.** Schematic of (H-L) VEGFE (100ng/ml) treatment on control (siSCR, 10 nM) or siDMD (10 nM) treated EDL single myofibers isolated from *Myf5-Cre:R26-eYFP*. Analysis was performed after 42h of culture. Data are presented as mean. Error bars are $\pm$ SEM (n=5).
*p <0.05, **p<0.001; Student's paired t-test. See also Figure S3C-E.
- H.** Numbers of Asymmetric YFP-/YFP+ divisions per myofiber
- I.** Numbers of Symmetric YFP-/YFP- divisions per myofiber.
- J.** Numbers of Symmetric YFP+/YFP+ divisions per myofiber.
- K.** Numbers of Pax7+ satellite cells per myofiber.
- L.** Numbers of Pax7+/eYFP- satellite stem cells per myofiber.

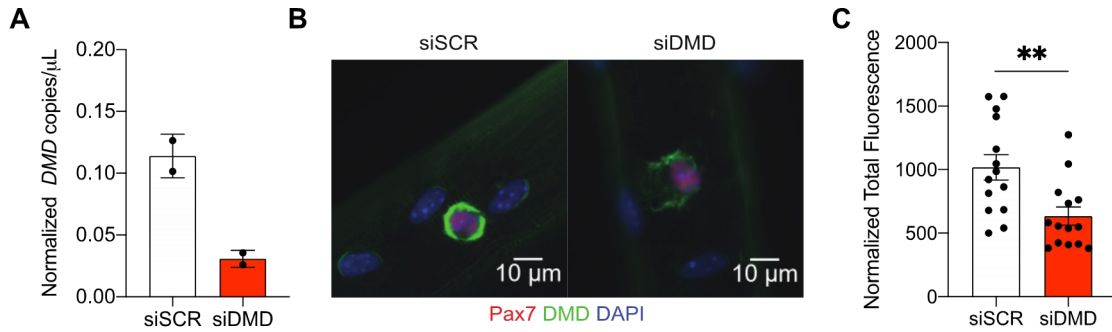


Figure 23. siDMD Validation

A. Validation of siDMD on wild-type myoblasts in 2-Day differentiation medium treated with either control siSCR (10 nM) or siDMD (10 nM) by ddPCR using pre-validated DMD probes. Data is presented as mean. Error bars are $\pm$ SD (n=2).

B. Representative immunofluorescence (IF) images of Pax7+ (red) satellite cells, DMD (green) and DAPI (blue) nuclear stain on EDL single myofibers isolated from wild-type mice treated with siSCR (10 nM) control or siDMD (10 nM).

C. Quantification of D and validation of siDMD (10 nM) by immunofluorescence. Data is presented as mean. Error bars are $\pm$ SEM (n=14 cells per condition). **p<0.001; Student's paired t-test.

4.4 Reduced asymmetric divisions in KDR-deficient MuSC leads to fewer progenitors on cultured myofibers

Defects in asymmetric division can lead to cascading effects on the generation of progenitor cell populations. Following the first round of division around 42h, satellite cells rapidly undergo multiple rounds of division, forming clusters of satellite cells (Yin et al., 2013). To investigate these early effects following asymmetric division impairment, we crossed *Pax7^{CreER}* (Murphy et al., 2011) with *KDR^{flx/flx}* (Hooper et al., 2009) to delete *Kdr* specifically in Pax7-expressing satellite cells, hereafter referred to as KDR-KO. Findings in our datasets using KDR-KO mice were compared to control *Pax7^{CreER}* littermates. These mice were injected with tamoxifen for 5-days before EDL myofiber isolation and cultured

with hydroxy tamoxifen (4-OHT). After culturing for 47h or 71h, myofibers were incubated with EdU one hour prior to fixation (**Figure 24A**). Syndecan-4 (Sdc4) was used to mark satellite cells to allow for the comparison of MyoG-expressing differentiating satellite cells to total satellite cells in this data set, as MyoG and Pax7 expression are mutually exclusive.

As expected, we observed a two-fold increase in satellite cell numbers at 72h compared to 48h in both control and KDR-KO fibers (**Figure 24B**). However, deficits in asymmetric division should be reflected in the population of early proliferating myogenic progenitors. Indeed, KDR-KO myofibers showed a 30% significant reduction in the numbers of Sdc4⁺ satellite cells at 72h and a similar trend at 48h, indicating a reduced generation of early myogenic cells (**Figure 24B**). We also observed a marked reduction in the number of cell clusters containing >3 cells in close proximity at both 48h and 72h time points, corroborating downstream deficits in asymmetric stem cell division (**Figure 24C**).

Quantification of the number of dividing Sdc4⁺EdU⁺ satellite cells revealed a 50% reduction in the numbers of dividing satellite cells at 48h in KDR-KO compared to control dividing satellite cells (**Figure 24D**). To determine whether the changes in Sdc4⁺ cell numbers were due to loss of asymmetric division or impaired proliferation, we then looked at the proportions of EdU⁺ actively dividing cells. We found no changes in total proportions of actively dividing EdU⁺ cells at both 48h and 72h time points suggesting the rate of satellite cell proliferation does not change, and that the reduction KDR-KO EdU⁺ dividing cells at 48h is due to impaired asymmetric division limiting the numbers of cells available for division (**Figure 24E**). Thus, KDR signaling is not involved in satellite cell proliferation kinetics.

MyoG is expressed in myogenic cells that have withdrawn from the cell cycle and committed to terminal differentiation. Satellite cell derived progenitors expressing MyoG in KDR-KO cells were also reduced in number at 72h, with about half the number of MyoG-expressing cells present per fiber (**Figure 24F**). However, analysis of the proportions of MyoG⁺ cells relative to the total number of cells revealed no difference both at 48h and 72h, demonstrating the rate of satellite cells undergoing differentiation does not change (**Figure 24G**). Together, these findings are consistent with the notion that the reduced levels of asymmetric divisions early in regeneration in the absence of KDR significantly reduce the generation of progenitors as reflected by the reduced numbers of MyoG-expressing progenitors undergoing commitment to differentiation.

Our results here provide further evidence that KDR signaling does not affect the rate of proliferation and differentiation of satellite cells. Rather, impairment of asymmetric division results in the reduced number of proliferative progenitors that are available to participate in repair.

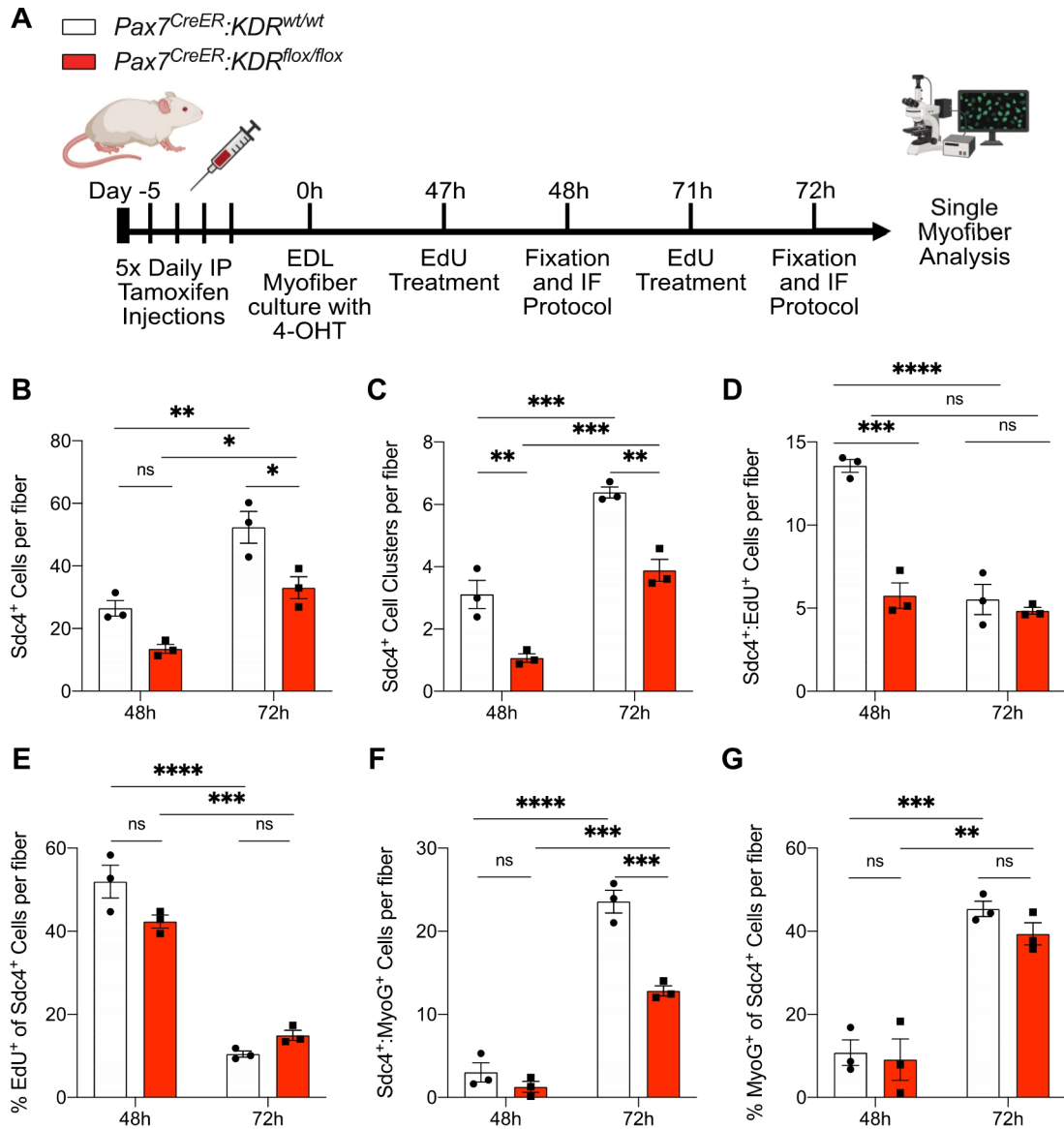


Figure 24. MuSC KDR-KO asymmetric division deficits limit satellite cell progenitors *ex vivo*

- A.** Schematic of (B-I) EdU treatment on EDL single myofiber isolated from *Pax7^{CreER}:KDR^{wt/wt}* (white) and *Pax7^{CreER}:KDR^{flox/flox}* (red) mice cultured for 48h and 72h. Data are presented as mean. Error bars are $\pm$ SEM (n=3). *p<0.05, **p<0.01, ***p<0.001; two-way ANOVA with Dunnett's multiple comparison test.
- B.** Total number of syndecan-4+ (Sdc4+) satellite cells per fiber.
- C.** Number of Sdc4+ satellite cell clusters (3+ cells).
- D.** Number of double positive Sdc4+:EdU+ cells per fiber.
- E.** Proportion of EdU+ cells of total Sdc4+ cells normalized per fiber.

F. Number of double positive Sdc4⁺:MyoG⁺ cells per fiber.

G. Proportion of MyoG⁺ cells of total Sdc4⁺ satellite cells normalized per fiber.

4.5 KDR-mediated asymmetric division is required for maintenance and repair

Thus far, we have demonstrated KDR impairment leads to reductions in asymmetric divisions, and that these effects can be seen during early regeneration (**Figure 20, Figure 24**). To investigate whether the effects of disrupting KDR-mediated asymmetric division persists throughout regeneration, we performed an *in vivo* injury experiment on KDR-KO and control *Pax7^{CreER}* littermates. Cardiotoxin (CTX) injections were administered to injure one TA, while the undamaged TA in the contralateral leg was used as a control. The mice were allowed to recover for 10 days to enable quantification of dividing Ki67 satellite cells, MyoG⁺ progenitors, and fiber Feret's minimal diameter (**Figure 25A**). After 10 days post injury (10-DPI), we found no difference in body weights or injured TA to body weight ratios (**Figure 25B, Figure 25C**).

Interestingly, we observed that KDR deletion resulted in a significant 2-fold decrease in satellite cell numbers that corresponded with a trend of increased MyoG progenitors and centrally nucleated fibers in uninjured KDR-KO TA muscles at 10 days, after tamoxifen treatment suggesting a propensity for fusion (**Figure 25D, Figure 25H, Figure 26C, Figure 27, Figure 28**). At 10 days following cardiotoxin induced injury, we observed a 1.6-fold decrease in numbers of Pax7⁺ satellite cells in the TA from KDR-KO mice compared to *Pax7^{CreER}* control counterparts (**Figure 25D**). Consequently, we also

observed a reduction in the numbers of actively dividing Ki67⁺ satellite cells (**Figure 25E, Figure 25F**).

To investigate whether the reduction in satellite cell numbers is due to reduced proliferation rate or cascading effects in population from impaired asymmetric division, we compared both the proportion of Ki67⁺ satellite cells and population change in response to injury and found no difference between KDR-KO and *Pax7^{CreER}* control mice (**Figure 25G, Figure 25J**). These findings confirm the rate of proliferation in both KDR-KO and control littermates are comparable, and that the deficits seen in satellite cell numbers 10 days post injury are due to reduced generation of proliferating progenitors as a consequence of the reduction in asymmetric satellite stem cell divisions.

To investigate differentiation kinetics, we quantified the numbers of myogenic cells expressing MyoG in both KDR-KO and *Pax7^{CreER}* control mice (**Figure 25H, Figure 28**). As expected, MyoG⁺ satellite cells increased dramatically at 10 days following injury (**Figure 25H**). In uninjured TA muscle, we observed no significant difference in the numbers of MyoG-expressing cells when comparing KDR-KO to *Pax7^{CreER}* control mice (**Figure 25H**). In line with our previous results on KDR-KO myofibers, we observed a 2-fold decrease in the numbers of MyoG⁺ cells in injured KDR-KO compared to *Pax7^{CreER}* control TA (**Figure 24F, Figure 25H**). Again, KDR signaling does not affect the rate of differentiation even at 10 days post injury despite fewer MyoG⁺ cells, as we observed no difference when comparing the proportion of MyoG⁺ cells in injured TA of KDR-KO and *Pax7^{CreER}* mice (**Figure 25I**). These findings are consistent with our earlier observations that KDR does not affect the rate of differentiation, and that deficits seen in progenitor populations stem from a reduced number of asymmetric satellite stem cell divisions.

To determine the impact of the loss of KDR and asymmetric division on muscle regeneration, we analyzed the number and size of myofibers (**Figure 26, Figure 28**). We observed no significant difference in the total number of fibers or centrally nucleated fibers in KDR-KO TA muscle (**Figure 26A-D**). However, quantification of Feret's fiber diameter revealed a significant increase in the numbers of smaller myofibers (**Figure 26E-I**). Indeed, the distribution of the Feret's fiber diameter shows a significant leftward shift indicating the increase in numbers of smaller sized fibers (**Figure 26F, Figure 26G**).

Our evidence demonstrates that KDR-signaling is required for stimulation of asymmetric division and its loss handicaps the early generation of committed satellite cells required for the exponential expansion of myogenic progenitors for repair. However, KDR-signaling does not affect the downstream rate of myogenic proliferation and differentiation during regeneration. This cascading effect on regeneration demonstrates the importance of asymmetric division in muscle repair as well as maintenance.

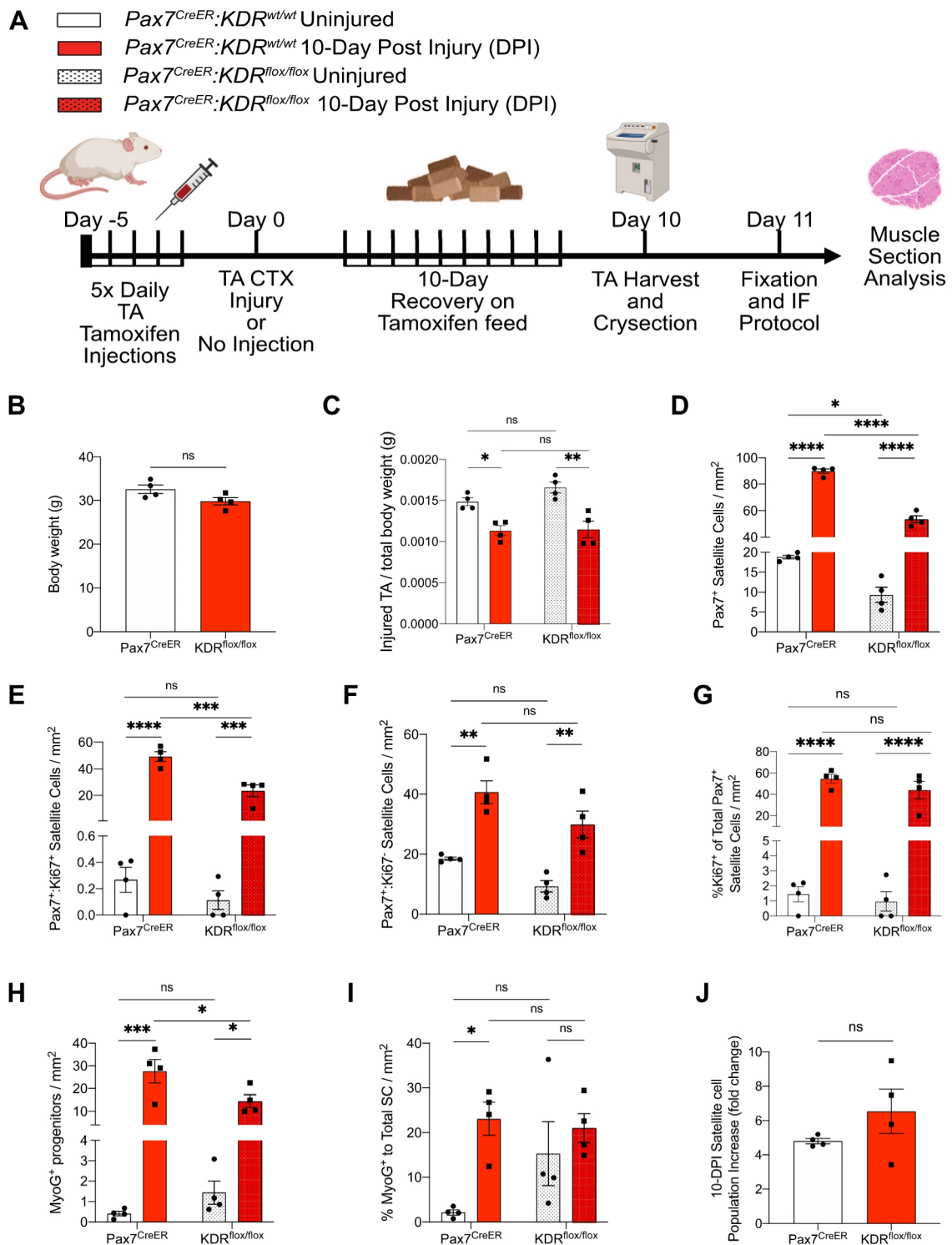


Figure 25. MuSC KDR-KO asymmetric division deficits limit satellite cell progenitors *in vivo*

A. Schematic of (B-G) Tibialis Anterior (TA) Cardiotoxin (CTX) injury experiment on *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flx/flx}* mice. Data are presented as mean. Error

bars are $\pm$ SEM (n=4). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; Student's paired t-test (B, J) and two-way ANOVA with Dunnett's multiple comparison (C-I).

B. Body weights (g) of *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flox/flox}* mice (n=4).

C. Ratio of Injured TA weight over total body weight (g) of *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flox/flox}* mice (n=4).

D. Quantification of Pax7⁺ satellite cell numbers normalized to area in uninjured vs injured TA sections from *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flox/flox}* mice.

E. Quantification of Pax7⁺Ki67⁺ satellite cell numbers normalized to area in uninjured vs injured TA sections from *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flox/flox}* mice.

F. Quantification of Pax7⁺Ki67⁻ satellite cell numbers normalized to area in uninjured vs injured TA sections from *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flox/flox}* mice.

G. Proportion of Pax7⁺Ki67⁺ satellite cells normalized to area in uninjured vs injured TA from *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flox/flox}* mice. Data is presented as mean. Error bars are $\pm$ SEM (n=4). **** p<0.0001.

H. Quantification of MyoG⁺ cell numbers normalized to area in uninjured vs injured TA sections from *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flox/flox}* mice

I. Proportion of Pax7⁺ satellite cells that are MyoG⁺ normalized to area in uninjured vs injured TA serial sections from *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flox/flox}* mice.

J. Fold change of satellite cell numbers in 10-days post injured TA compared to uninjured TA of *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{flox/flox}* mice (n=4).

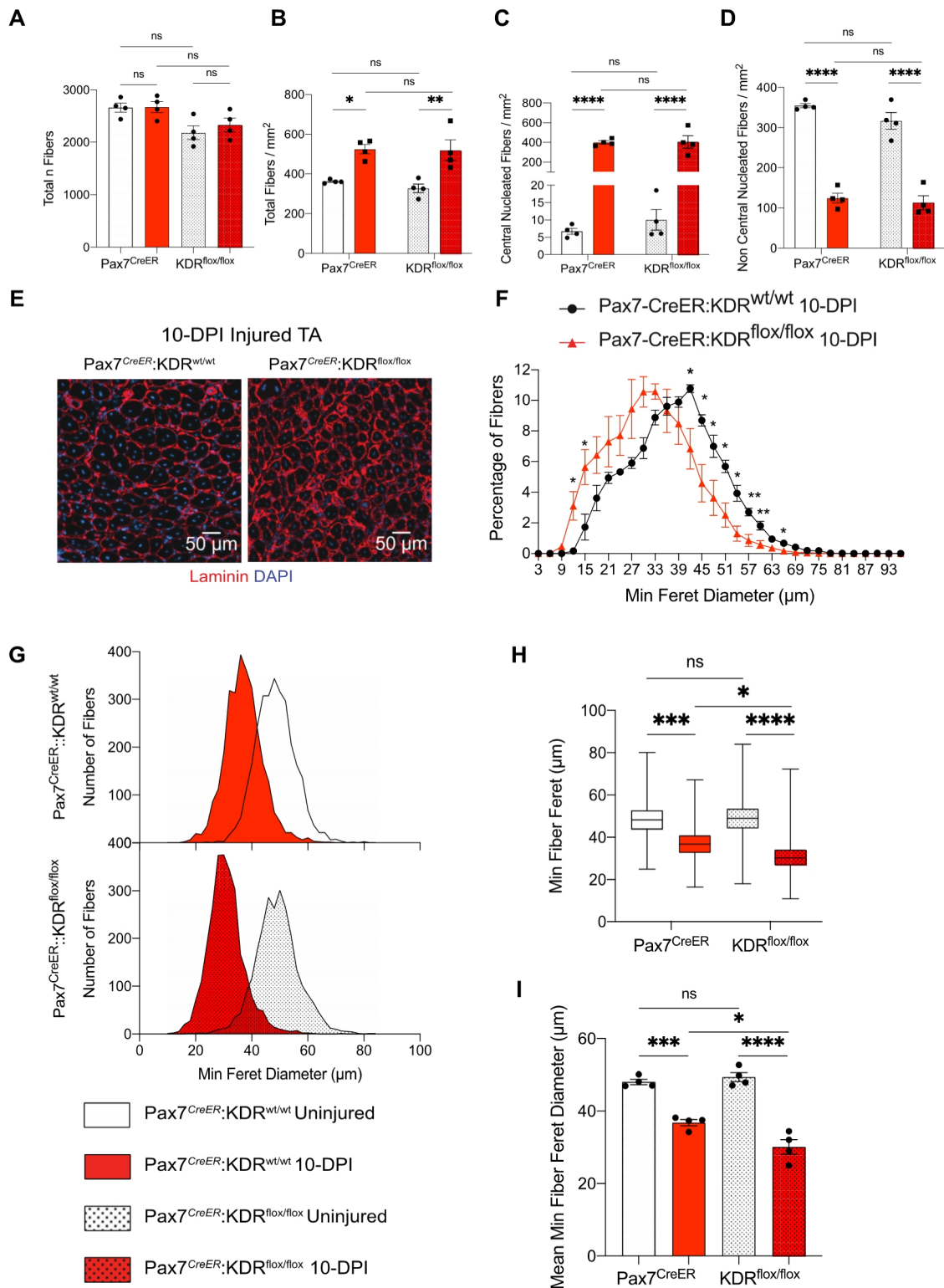


Figure 26. MuSC KDR-KO perturbs muscle regeneration

A. Total fiber count of TA sections in uninjured vs injured TA from *Pax7^{CreER}·KDR^{wt/wt}* and *Pax7^{CreER}·KDR^{lox/lox}* mice. Data are presented as mean. Error bars are $\pm$ SEM (n=4); two-way ANOVA with Dunnett's multiple comparison test.

B. Quantification of fiber numbers normalized to area in uninjured vs injured TA from *Pax7^{CreER}·KDR^{wt/wt}* and *Pax7^{CreER}·KDR^{lox/lox}* mice. Data are presented as mean. Error bars are $\pm$ SEM (n=4). *p<0.05, **p<0.01; two-way ANOVA with Dunnett's multiple comparison test.

C. Quantification of central nucleated fibers normalized to area in uninjured vs injured TA from *Pax7^{CreER}·KDR^{wt/wt}* and *Pax7^{CreER}·KDR^{lox/lox}* mice. Data are presented as mean. Error bars are $\pm$ SEM (n=4). ****p<0.0001; two-way ANOVA with Dunnett's multiple comparison test.

D. Quantification of non-central nucleated fibers normalized to area in uninjured vs injured TA from *Pax7^{CreER}·KDR^{wt/wt}* and *Pax7^{CreER}·KDR^{lox/lox}* mice. Data are presented as mean. Error bars are $\pm$ SEM (n=4). ****p<0.0001; two-way ANOVA with Dunnett's multiple comparison test.

E. Representative immunofluorescence (IF) images of 10-Day post injured (10-DPI) TA muscle sections from *Pax7^{CreER}·KDR^{wt/wt}* and *Pax7^{CreER}·KDR^{lox/lox}* mice stained with Laminin (red) and DAPI (blue).

F. Histogram overlay of minimum fiber ferret diameter (μ m) comparing 10-DPI *Pax7^{CreER}·KDR^{wt/wt}* vs *Pax7^{CreER}·KDR^{lox/lox}*. Data are presented as mean. Error bars are $\pm$ SEM (n=4). *p<0.05; Paired Student's t-test.

G-H. Distribution of minimum fiber ferret diameter (μ m) comparing uninjured vs injured TA from *Pax7^{CreER}·KDR^{wt/wt}* and *Pax7^{CreER}·KDR^{lox/lox}* mice. Data are presented as mean. (n=4). *p<0.05, ***p<0.001, ****p<0.0001; two-way ANOVA with Dunnett's multiple comparison test.

I. Mean comparisons of fiber ferret diameter (μ m) in uninjured vs injured TA from *Pax7^{CreER}·KDR^{wt/wt}* and *Pax7^{CreER}·KDR^{lox/lox}* mice. Data are presented as mean. (n=4).

* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$; two-way ANOVA with Dunnett's multiple comparison test.

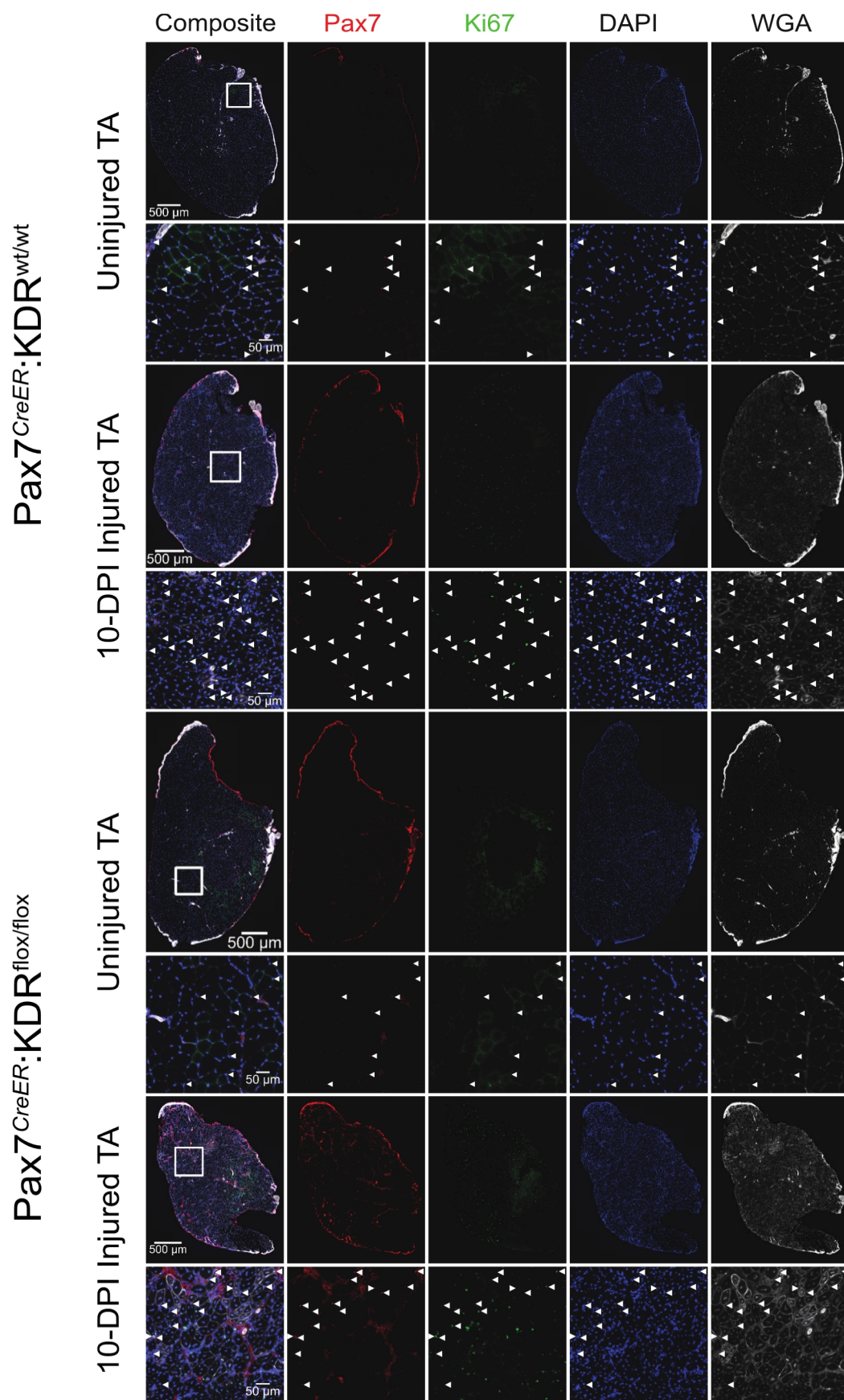


Figure 27. Representative immunofluorescent images of *in vivo* satellite cell populations

Representative immunofluorescence (IF) images of uninjured and 10-DPI injured TA muscle sections from *Pax7^{CreER}:KDR^{wt/wt}* and *Pax7^{CreER}:KDR^{lox/lox}* mice showing Pax7⁺ (red), Ki67⁺ (green), Wheat Germ (white), DAPI (blue). Arrows indicate Pax7⁺ cells.

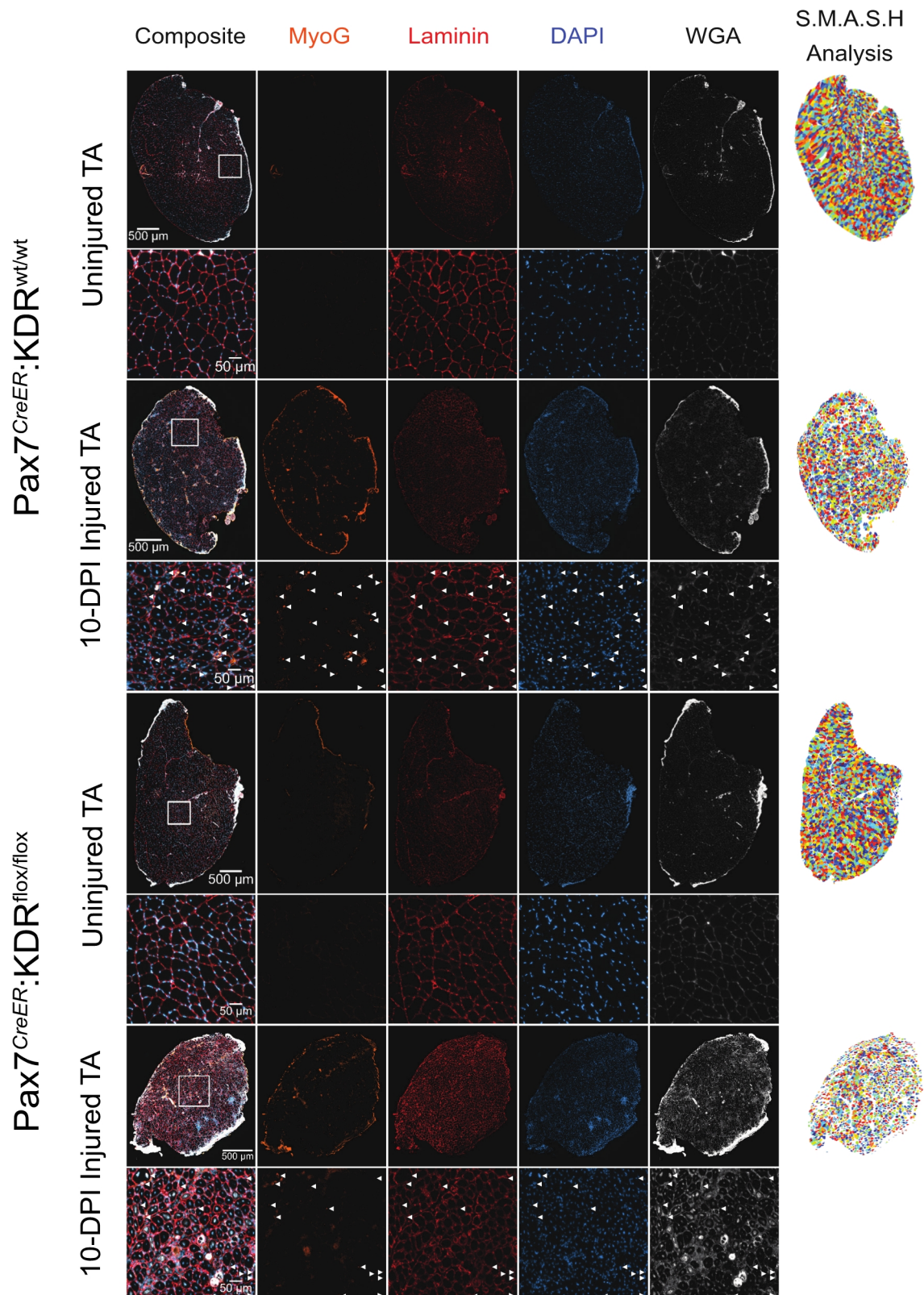


Figure 28. Representative immunofluorescent images of *in vivo* progenitors and SMASH analysis

Representative immunofluorescence (IF) images of uninjured and 10-DPI injured TA muscle sections from *Pax7^{CreER}·KDR^{wt/wt}* and *Pax7^{CreER}·KDR^{flox/flox}* mice showing MyoG⁺ (orange), Laminin (red), Wheat Germ (white), DAPI (blue). Arrows indicate MyoG⁺ cells. Far right column contains representative SMASH (semi-automatic muscle analysis)-generated mask of TA cross-sections used for analysis of fiber ferret diameter.

Chapter 5. Discussion

5.1 Small molecule screen design and optimization

We report the first image-based high content analysis platform using myofiber culture with a physiological read-out that preserves the satellite cell niche (**Figure 3**, **Figure 4**). Optimization of every component is integral to efficient screen design, and even small reductions may save hours in total screen run time. To that end, we optimized plate form factor, image acquisition, satellite cell detection, and script logic to achieve a robust screening platform with comparable detection to manual fiber analysis (**Figure 5**, **Figure 6**, **Figure 7**, **Figure 8**). Traditional myofiber analysis is performed on EDL myofibers (Dumont et al., 2015a; Grand et al., 2009; Kuang et al., 2007; Wang et al., 2019). However, EDL myofibers are unsuitable for screening purposes due to size, yield, and density of satellite cells (**Table 2-1**). Logistically, EDL fibers are too long in length to maintain a monolayer culture in a small form factor. Further, the yield of fiber numbers and satellite cell density is too low for a cost-effective screen. For these reasons, we chose to adopt FDB myofiber culture for screening purposes, and optimized culture conditions (**Figure 9**, **Figure 10**, **Figure 11**, **Figure 12**, **Figure 13**). Notably, our platform achieved satellite cell and couplet detection rates comparable to manual fiber analysis, and was able to identify changes in muscle stem cell divisions when stimulating previously reported endogenous asymmetric and symmetric stem cell division pathways using EGF and Wnt7a ligands (**Figure 14**) (Grand et al., 2009; Wang et al., 2019).

5.2 Adaptation of manual fiber culture for high content analysis

Our screening platform was able to perform unbiased analysis with a significant reduction in labor and processing time, particularly when performing parallel experimental conditions. Traditional EDL myofiber analysis typically requires 3-10 days to complete, depending on the number of conditions and biological replicates (**Figure 4**). In comparison, our screening platform was designed to perform up to 96 conditions in parallel in under 5 days (**Figure 15A**). The increase in throughput primarily stems from the increase in numbers of conditions that may be performed simultaneously, and the automated processes of image acquisition and quantification of satellite cell couplets.

The caveat of a physiological readout such as satellite cell couplets on cultured myofibers is the cost of reagents, mice, and the complexity of satellite cell culture readout limits the maximum throughput possible. The availability of mice limits the number of conditions that may be screened at once, as each 96-well plate requires approximately 6-10 FDB muscle isolations from 3-5 mice. Further, increases in technical and biological replicates significantly reduce the throughput in numbers of compounds that may be screened. To accommodate for these limitations, our platform screened the small molecule compound library in technical duplicates at one concentration (1 μ M).

However, the unique advantage of our high content platform is the preservation of the satellite cell micro niche. Myofiber culture maintains the satellite cell position between the basal lamina and sarcolemma, and is critical in maintaining stemness and cell identity (Baghdadi et al., 2018a; Verma et al., 2018; Yin et al., 2013). Thus, the

maintenance of satellite cell niche allows for physiologically relevant findings. This platform may be easily adapted to screen any number of compound libraries (i.e. inhibitors, metabolites, siRNA, ligands) and utilize other reporter alleles to investigate muscle satellite cell biology.

5.3 High content results and identification of KDR as a modulator of satellite asymmetric stem cell division

To assess the results of small molecule screening, we considered the variability introduced due to limited resources and time: we were limited to screening compounds in technical duplicates, at one biological replicate pooled from several mice, and at a concentration of 1 μ M. Further, the OICR library contains compounds that often inhibit more than one target kinase at differing IC₅₀ values. To account for increased variability under these limitations, we developed a weighted scoring system of asymmetric stem cell division to accurately identify modulators of asymmetric stem cell division. Compounds with an IC₅₀ value closer to the screening concentration (1 μ M) were given greater weight. Additionally, greater weight was also given to effects on stem cell divisions where multiple compounds produced similar effects. Using this system, we successfully identified targets of stem cell divisions previously published. We were able to identify EGF inhibitors that act contrarily to our positive EGF ligand control, as well as previously reported asymmetric satellite stem cell division pathways AurkA and p38- α/β MAPKs (**Figure 15C**, **Figure 15E**) (Chang et al., 2018; Wang et al., 2019). Our small

molecule library did not contain inhibitors against the Wnt7a receptor, Fzd7. However, we were able to identify changes in satellite cell divisions upon inhibition of related crosstalk and downstream Wnt7a signaling molecules in myoblasts, such as IGFR-1, PI3K, mTOR and ROCK that corresponded with decreased asymmetric divisions (**Figure 15D, Figure 15E**) (Maltzahn et al., 2011, 2012b; Siegle et al., 2018).

Notably, KDR (VEGFR2) was identified as a modulator of asymmetric stem cell division, and gene ontology analysis of targets screened as modulators for asymmetric division provided further confirmation, revealing an enrichment in angiogenesis and VEGF signaling pathways (**Figure 15E, Figure 15F**). Interestingly, ROCKI and ROCK II are negative regulators of VEGF signaling in endothelial angiogenesis, and pharmacological inhibition of ROCKI and ROCKII have been demonstrated to increase VEGF-mediated angiogenesis (Bryan et al., 2010; Kroll et al., 2009). Consistent with these findings, our screen reported an increase in asymmetric stem cell division upon treatment with ROCKI and ROCKII inhibitor compounds, suggesting a downstream cross-talk with non-canonical Wnt7a signaling (**Figure 15D, Figure 15E**). Together with these findings, we selected KDR for further investigation. Additionally, KDR is a transmembrane receptor, and provides a tangible target for manipulation.

5.4 KDR signaling in muscle satellite stem cells

This is the first report of KDR-mediated signaling and its physiological role in muscle satellite stem cells. In this study, we show how KDR signaling is a critical

component of stem cell fate decisions to undergo asymmetric division and that it requires a polarized intrinsic cell environment established by the DGC complex (Dumont et al., 2015a). KDR signaling is specific to asymmetric division and its manipulation does not affect satellite stem cell symmetric division nor downstream rate of progenitor cell proliferation and differentiation (**Figure 20, Figure 24**). Notably, we observed a 2-fold decline in the numbers of satellite cells following tamoxifen treatment suggesting that KDR signaling may also have a role in preventing precocious differentiation of satellite cells in quiescence (**Figure 25D**). Our study provides important new insight into the regulation of stem cell asymmetric divisions in muscle maintenance, repair, and disease states.

5.5 Satellite stem cell fate decision-making may not be binary

The prevailing theory on stem cell fate is that asymmetric versus symmetric division is a binary decision. Indeed, past investigations on cell polarity pathways have demonstrated increased stem cell asymmetric or symmetric division when the other is perturbed (Bentzinger et al., 2013; Kuang et al., 2007; Wang et al., 2019). This study provides the first evidence of a signaling pathway that exclusively affects asymmetric division without impacting symmetric division (**Figure 20B, Figure 20C, Figure 20H, Figure 20I**). These findings provide an important and novel understanding of muscle stem cell fate decision making. Although the binary decision of satellite stem cell divisions do not change (to either divide symmetrically or asymmetrically), perturbing or promoting one division type does not automatically push these cells to divide the opposite way. This

finding is a significant advance in our understanding of the role of asymmetric division in disease and aging, as previously reported asymmetric signaling pathways have been coupled with effects on symmetric division and quiescence (Bernet et al., 2014; Dumont et al., 2015a; Wang et al., 2019).

5.6 VEGF receptors and ligands expressed in satellite stem cells

VEGF receptors preferentially form homodimers, but may also form heterodimers with other VEGF receptors and its signaling can be modulated by coreceptors such as neuropilins and heparan sulphate proteoglycans (Olsson et al., 2006). Here we identify KDR and possible co-receptors Nrp1/Nrp2 expression in satellite stem cells (**Figure 16C**, **Figure 16D**, **Figure 17**). The identity and potential role of these coreceptors in relation to asymmetric division remains to be investigated. It is possible that VEGF coreceptors and their distribution within the cell may be regulated by cell polarity to modulate KDR signaling during asymmetric division.

Asymmetric divisions occur during the first few rounds of division (Feige et al., 2018; Kuang et al., 2007; Yin et al., 2013). Accordingly, we show KDR expression is upregulated upon activation in a subset of satellite cells, and persists throughout early regeneration (**Figure 16C**, **Figure 18**). VEGFR1 (Flt-1), a known decoy receptor that competitively and preferentially binds to VEGFA ligand over KDR (Tanaka et al., 1997), also demonstrated a similar expression profile to KDR and was downregulated in proliferating committed satellite cells (**Figure 16C**). Intriguingly, we did not detect Flt-1

expression by ddPCR from sorted *mdx* satellite cells (**Figure 16C**). Downregulation of Flt-1 in *mdx* satellite cells may be due to an attempt to compensate for impaired KDR signaling by the removal of VEGFR1-mediated negative feedback on KDR signaling.

Our gene expression analysis indicates that VEGFA is the primary ligand expressed in satellite cells (**Figure 16D**). Other sources of VEGFA in the muscle include myofibers, endothelial cells, and FAPs (Blackburn et al., 2019; Verma et al., 2018). The KDR signaling pathway is tightly controlled with numerous negative feedback loops to prevent overactivation (Simons et al., 2016). Given the abundant availability and redundant expression of VEGFA from multiple niche sources combined with the expression of KDR, its decoy receptor VEGFR1, and VEGFA ligand within satellite cells, it is likely that KDR-mediated asymmetric signaling occurs primarily in an autocrine fashion (i.e. satellite cell-satellite cell), or short-range paracrine signaling (i.e. myofiber-satellite cell signaling). Future work involving cell-specific KDR-KO in other satellite cell niche partners such as FAPs, endothelial cells, and fibroblasts may provide further insight on VEGF signaling in the muscle stem cell niche.

5.7 KDR signaling requires the DGC complex

Dystrophin, a critical component of the DGC, is required for establishing cell polarity in muscle stem cells, important for asymmetric division (Dumont et al., 2015a). Our findings show that KDR is closely associated with the DGC complex, and that impaired KDR-signaling in DMD cannot be rescued by ligand treatment, suggesting KDR

signaling requires the formation of the DGC complex (**Figure 22, Figure 21**). These findings give rise to two possible models for how KDR signaling may modulate asymmetric stem cell division (**Figure 29**). KDR signaling may be downstream of DGC formation, and require the establishment of cell polarity to facilitate its signaling in modulating asymmetric stem cell division. Alternatively, KDR may play a role in DGC assembly and establishment of cell polarity by binding to dystrophin to facilitate phosphorylation of PAR complex proteins, required for its polar segregation from the DGC. A PLA assay targeting KDR, dystrophin, and polarity proteins in satellite cells undergoing mitosis during the first wave of division may provide further insight on whether KDR itself is polarized, and when it is associated with dystrophin and the PAR complex during the establishment of cell polarity.

Given the complexity of receptor tyrosine kinase activity and cross talk potential, KDR signaling may be interacting with other asymmetric stem cell signaling pathways. Indeed, studies in cancer biology have demonstrated VEGF promotes self-renewal in cancer stem cells through KDR/STAT3 signaling, and stimulate phosphorylation of p38a MAPK, two known pathways of muscle stem cell asymmetric division (Bernet et al., 2014; Masson-Gadais et al., 2003; Nilsson et al., 2016; Price et al., 2014; Saulle et al., 2009; Troy et al., 2012; Zhao et al., 2015).

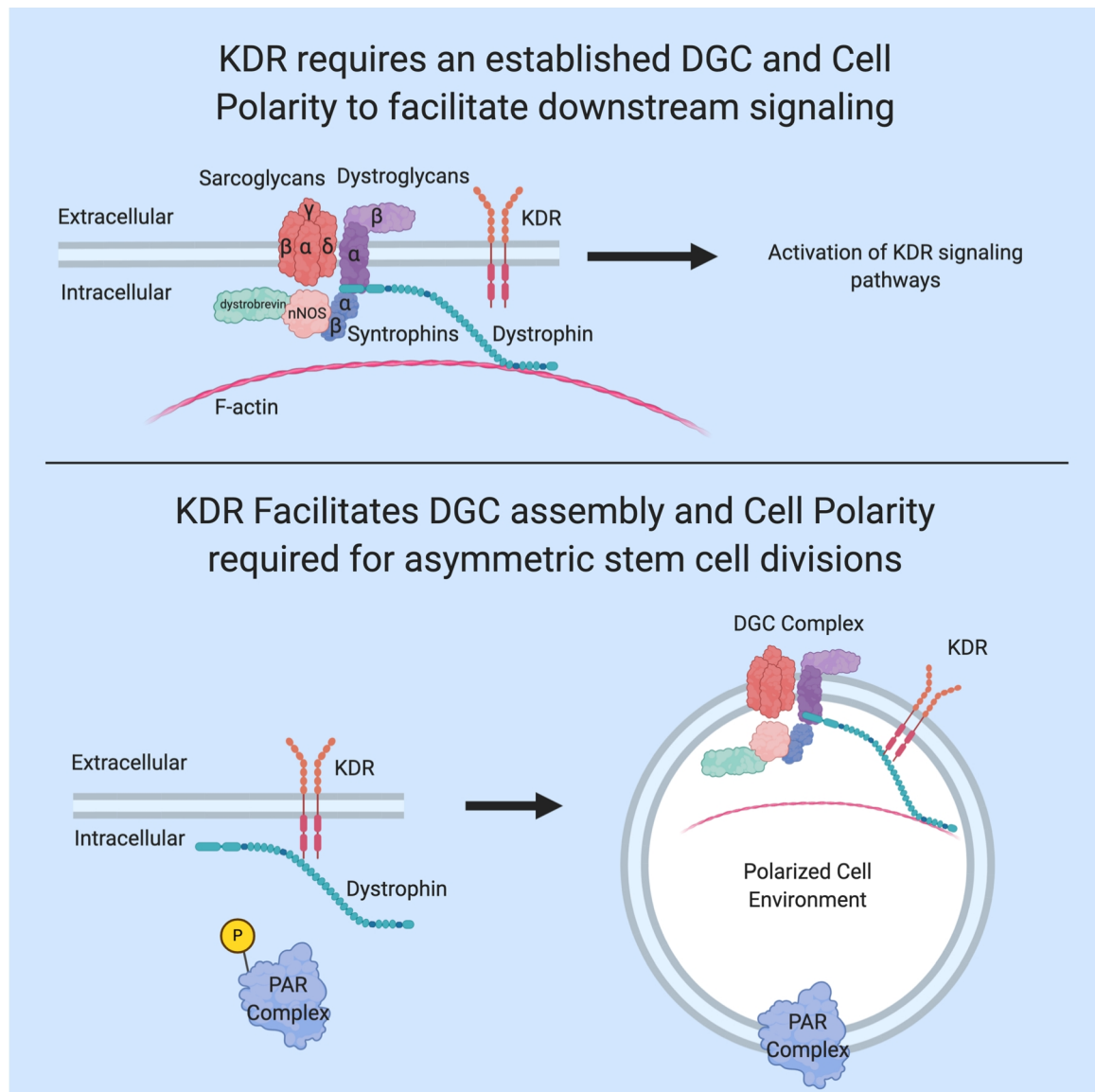


Figure 29. Models for KDR-dystrophin modulation of asymmetric stem cell division

Two models are proposed for how KDR and dystrophin modulate muscle asymmetric stem cell division. **TOP:** KDR binds to dystrophin to facilitate downstream KDR signaling to promote asymmetric stem cell division following the establishment of cell polarity. **BOTTOM:** KDR binding to dystrophin phosphorylates PAR complex proteins to facilitate intracellular polarization, and assembly of the DGC.

5.8 KDR signaling is required for homeostatic satellite cell maintenance

Our *ex vivo* and *in vivo* observations on KDR-KO mice suggest that an impairment of asymmetric division leads to a decline in the numbers of early myogenic progenitors, with no effect on proliferation and differentiation kinetics (**Figure 24, Figure 25, Figure 27, and Figure 28**). Analysis of KDR-KO on non-injured TA muscles revealed a 50% decline in satellite cell numbers 10 days following tamoxifen treatment, suggesting the onset in satellite cell number decline begins during steady state (**Figure 25D**). Corroborating with our findings of reduced satellite cell numbers in non-injured TA muscle, we observed that KDR-KO exhibited a trend of increased MyoG numbers, proportion of MyoG-expression satellite cells and centrally nucleated fibers that (**Figure 25H, Figure 25I, Figure 26C, Figure 26H**). These observations suggest that satellite cell specific loss of KDR in uninjured muscle promotes premature fusion through a break in quiescence.

The mechanism of action behind this decline may be associated with the chemoattractant functions of VEGFA signaling. Satellite stem cell derived VEGFA secretion has recently been reported to recruit endothelial cells, required for maintaining stem cell quiescence via Notch signaling (Verma et al., 2018). Similarly, myofiber secretion of VEGFA under homeostatic conditions may act as a chemoattractant to maintain quiescence by securing satellite cells within their niche (Baghdadi et al., 2018a; Blackburn et al., 2019). Observations on satellite cell numbers following satellite cell

specific VEGFA-KO complement our findings in KDR-KO mice, and demonstrate a similar loss in early myogenic numbers (Verma et al., 2018).

The similarities between KDR-KO and *mdx* phenotype include impaired asymmetric division and precocious activation of satellite cells. However, the *mdx* phenotype exhibits stem cell hyperplasia not present in KDR-KO, and cell division defects that are not limited to satellite asymmetric stem cell division (Dumont et al., 2015a). The similarities in muscle phenotype between KDR-KO and *mdx* reinforces the importance of asymmetric stem cell division in muscle repair, while the differences suggest other compensatory mechanisms are at play in the context of chronic injury.

5.9 KDR signaling is required for efficient muscle regeneration

Our results provide compelling evidence that KDR-mediated signaling is restricted to satellite stem cell fate decision and does not impact downstream proliferation or differentiation kinetics of progenitors (**Figure 24, Figure 25, Figure 27, Figure 28**). These findings suggest that the role of asymmetric division in response to acute injury is to generate sufficient numbers of satellite myogenic cells that are then able to undergo multiple but limited rounds of division. In accordance with our results, muscle satellite cell specific VEGFA knockout mice show reduced numbers of Pax7-expressing cells up to 12 weeks post injury (Verma et al., 2018). Thus, the marked reduction in satellite stem cell asymmetric divisions results in the generation of insufficient numbers of proliferating

myogenic progenitors to efficiently undertake the regeneration of muscle (**Figure 25, Figure 26**).

Our results show that KDR signaling is critical for asymmetric cell division and requires dystrophin to function, suggesting that this signaling pathway may require the establishment of the DGC complex, which is perturbed in DMD patients (**Figure 30**). Furthermore, KDR-mediated stimulation of asymmetric division is important for both maintaining satellite cell numbers, and the initial generation of proliferating committed cells that is required for efficient muscle repair in response to acute injury.

Future studies will elucidate the role of KDR signaling in larger context of stem cell polarity and the potential for small molecule activation of KDR as a therapeutic modality for enhancing muscle repair.

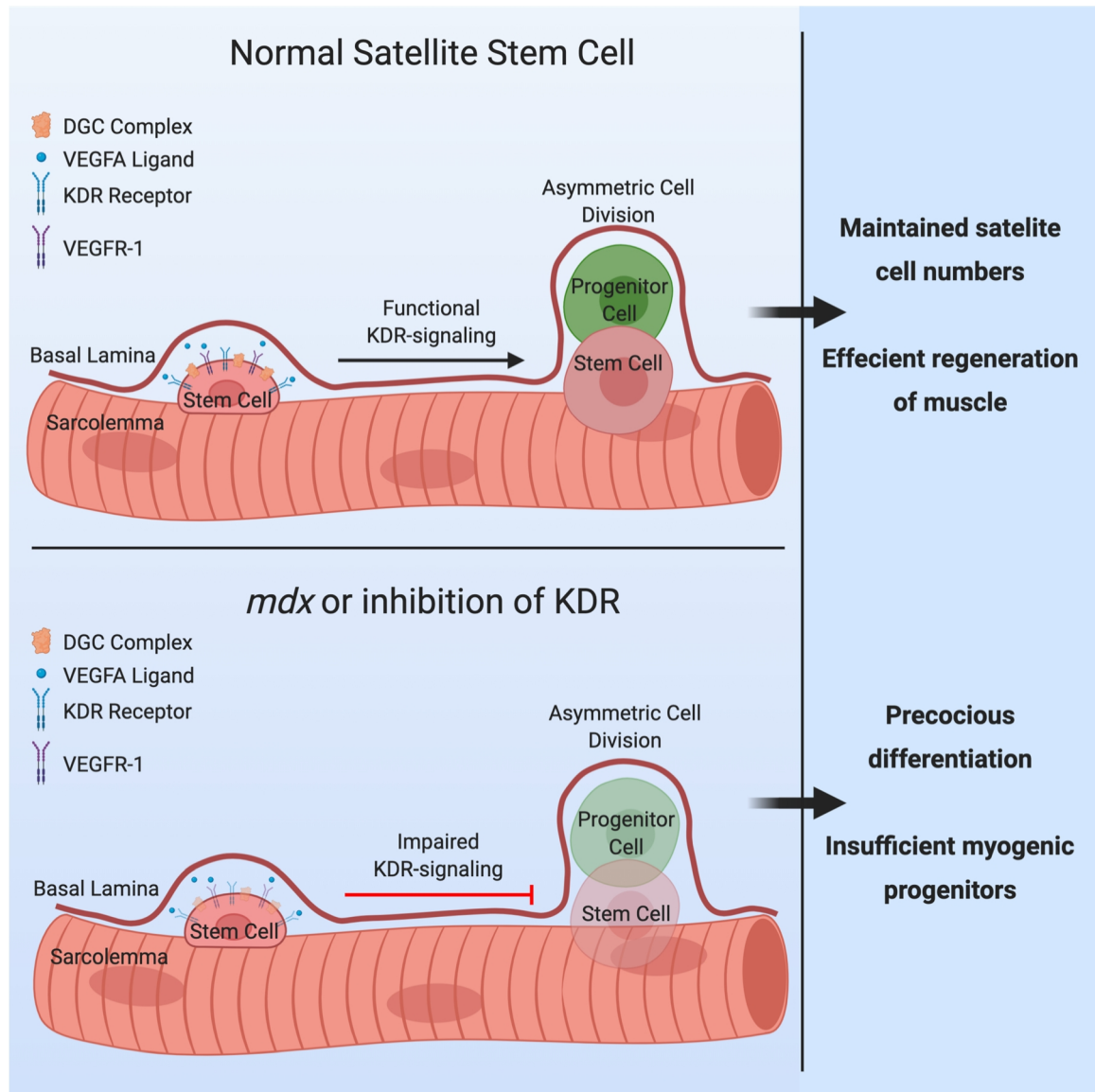


Figure 30. KDR signaling requires the dystroglycan complex (DGC) to modulate muscle stem cell asymmetric stem cell division.

5.10 KDR and dystrophin interactions in endothelial cells

Hypovascularization and ischemia is well documented in chronic muscle dystrophies including DMD and BMD, and novel therapeutic strategies aimed at improving angiogenesis and vasculature have demonstrated improved muscle function (Ennen et al., 2013; Shimizu-Motohashi and Asakura, 2014; Thomas, 2013). DMD-related ischemia has been attributed to the absence of nitric oxide synthase (NOS) from the sarcolemma, resulting in increased vasoconstriction leading to impaired blood flow (Rando, 2001). Additionally, satellite cells modulate vasculature growth through the secretion of VEGF to attract endothelial cells to the niche, and dystrophin-deficient satellite cells secrete less VEGF, exhibiting an impaired capacity for promoting angiogenesis (Rhoads et al., 2009, 2013; Verma et al., 2018).

However, the relationship between dystrophin and KDR in endothelial cells (ECs) has yet to be explored, despite expression of both genes in smooth muscles and blood vessels (Miyatake et al., 1989; Palladino et al., 2013; Shimizu-Motohashi and Asakura, 2014). Endothelial dystrophin forms a complex with endothelial NO synthase and caveolin-1, and dystrophin-deficient ECs demonstrate reduced NO production, cGMP formation, and impaired angiogenic properties (Palladino et al., 2013). We show that KDR modulation of satellite stem cell asymmetric division requires dystrophin (**Figure 21, Figure 22**). It would be interesting to investigate whether KDR/dystrophin also interact to establish cell polarity in endothelial cells. Indeed, KDR/Notch regulates endothelial cell polarity during angiogenic branching, and whether KDR/dystrophin

interact in this context to aid the establishment of cell polarity is an additional unexplored molecular mechanism that may contribute to ischemia in DMD muscle vasculature (Blanco and Gerhardt, 2013; Costa et al., 2017; Hayashi et al., 2013; Hellström et al., 2007).

5.11 Conclusion and Significance

The regulation of muscle stem cell (MuSC) asymmetric division plays an essential role in controlling the growth and repair of skeletal muscle. We discover kinase domain receptor (KDR) as a positive modulator of MuSC asymmetric division using an *in-niche* high-content screen and confirmed its expression in satellite cells by ddPCR and immunofluorescence. Knockdown of KDR significantly reduces the numbers of asymmetric divisions, whereas ligand stimulation of KDR increases the numbers of asymmetric divisions. KDR signaling is impaired in dystrophin-deficient satellite cells and requires a polarized cell environment established by the dystrophin glycoprotein complex (DGC) to direct asymmetric division. Mice lacking KDR in MuSCs exhibit reduced numbers of satellite cells due to precocious differentiation, and deficits in regeneration consistent with impaired asymmetric division and reduced generation of progenitors. Therefore, our experiments identify KDR signaling as playing an essential role in MuSC function and muscle regeneration.

Chapter 6. Appendices

6.1 List of OICR Compound Inhibitors

Formula	Mol Weight	Name	Target
C26H30N6O3	474.555	PHA-739358, Danusertib	ABL1 (ABL), RET, TRKA (TRK), AurA (AuroraA), FGFR1 (FLT2)
C21H15Cl2FN4O	429.274	PD-180970	ABL1 (ABL), SRC (c-SRC)
C27H32ClN5O5	542.026	AZD0530, Saracatinib, NSC-735464	ABL1 (ABL), SRC (c-SRC), KIT (c-KIT)
C22H21BrCl2N6O	536.252	ABT-702 dihydrochloride	AK (Adenosine Kinase)
C11H13IN4O4	392.15	5-Iodotubercidin	AK (Adenosine Kinase), CK1a (Casein kinase 1, CK1)
C20H26Cl2N2O	381.339	10-DEBC hydrochloride	AKT1 (PKBa)
C11H9Cl2CuN3O2S	381.725	FPA 124	AKT1 (PKBa)
C13H16N6O4	320.304	API-2, Triciribine, NSC154020, TCN, Tricibine, VQD-002	AKT1 (PKBa)
C24H25Cl2N5O	470.394	A-443654, A-654	AKT1 (PKBa)
C25H23Cl2N5O	480.389	MK-2206	AKT1 (PKBa)
C22H30N6	378.514	Akt-I-1	AKT1 (PKBa)
C21H27N7O3	425.484	GSK690693, GSK-690693	AKT1 (PKBa)
C36H30F3N7O3	665.664	Akt 1/2 Kinase inhibitor	AKT1 (PKBa), AKT2 (PKBb)
C23H22ClN3	375.894	Akt-I-1,2	AKT1 (PKBa), AKT2 (PKBb)
C40H43N7O2	653.815	Merck-22-6	AKT1 (PKBa), AKT2 (PKBb)

C24H32ClN5O2	457.996	GDC-0068, RG-7440	AKT1 (PKBa), AKT2 (PKBb), AKT3 (PKBg)
C20H20ClN3	337.846	AT7867, AT-7867	AKT1 (PKBa), AKT2 (PKBb), AKT3 (PKBg), p70S6K (S6K, S6K1)
C21H25ClN6O2	428.915	AZD-5363, AZD5363	AKT1 (PKBa), AKT2 (PKBb), AKT3 (PKBg), p70S6K (S6K, S6K1)
C32H29N5O2	515.605	Enzastaurin, LY-317615	AKT1 (PKBa), PKCb, p70S6K (S6K, S6K1), GSK3B
C30H40ClN7O3S	614.202	TAE-684	ALK
C30H34N4O2	482.617	CH5424802, AF-802, CH-5424802	ALK
C29H40N8O3S	580.745	ASP-3026	ALK, EGFR (ERBB1, HER1)
C21H22Cl2FN5O	450.337	PF-02341066, PF-2341066, Crizotinib	ALK, MET (c-MET, HGFR)
C25H22N6	406.482	LDN193189, LDN-193189, DM-3189	ALK2, BMPR1A (ALK3)
C20H12N2O3S	360.386	A 769662, A-769662	AMPK
C24H25N5O	399.488	6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine, Dorsomorphin, BML-275, AMPK Inhibitor, Compound C	AMPK, ALK2, BMPR1A (ALK3), BMPR1B (ALK6)
C24H27Cl2N5O	472.41	Dorsomorphin dihydrochloride, BML-275, AMPK Inhibitor, Compound C	AMPK, ALK2, BMPR1A (ALK3), BMPR1B (ALK6)
C30H33N3O5S	547.665	KU-60019	ATM
C17H15N7O2	349.347	CP466722, CP-466722	ATM
C21H17NO3S2	395.495	KU-55933, KU-0064	ATM, PI3K
C18H16N4O3S	368.41	VE-821	ATR
C23H18Cl3FN4O3S	555.836	CGK 733	ATR, ATM
C27H20ClFN4O4	518.924	MLN-8237	AurA (AuroraA)
C25H31N7O6	525.557	ENMD-2076, ENMD-981693	AurA (AuroraA)
C19H29ClN6O	392.926	TC-A 2317	AurA (AuroraA)
C31H31ClFN7O2	588.075	hydrochloride	AurA (AuroraA)
		Aurora A Inhibitor I	AurA (AuroraA)

C22H21ClFN3O3S	461.937	MK-5108, VX-689	AurA (AuroraA)
C25H15ClF2N4O2	476.862	MLN8054, MLN-8054	AurA (AuroraA) AurA (AuroraA), AurB (AuroraB), AurC (AuroraC)
C29H37N5O7	567.633	ZM-447439	AurA (AuroraA), AurB (AuroraB), AurC (AuroraC)
C18H15ClN6OS2	430.934	SNS-314	AurA (AuroraA), AurB (AuroraB), AurC (AuroraC)
C28H21N7OS	503.578	AMG-900	AurA (AuroraA), AurB (AuroraB), AurC (AuroraC)
C23H25ClN8OS	497.016	CCT129202, CCT-129202	AurA (AuroraA), AurB (AuroraB), AurC (AuroraC)
C28H35N7O2	501.623	PHA-680632	AurA (AuroraA), AurB (AuroraB), AurC (AuroraC), ABL1 (ABL), FLT3 (STK1, FLK2), JAK2
C23H28N8OS	464.586	VX-680, MK-0457, Tozasertib, VX6	AurA (AuroraA), AurB (AuroraB), AurC (AuroraC), CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B
C17H15F2N7O3S	435.408	JNJ-7706621	AurA (AuroraA), AurB (AuroraB), AurC (AuroraC), FLT3 (STK1, FLK2)
C26H31BrN8O	551.481	CCT137690	AurA (AuroraA), AurB (AuroraB), JAK2, JAK3, ABL1 mutant(ABL mutant), FLT3 (STK1, FLK2)
C19H23N7O2	381.432	AT9283, AT-9283	AurA (AuroraA), AurB (AuroraB), KDR (VEGFR2, VEGFR, FLK1)
C18H20N6OS	368.456	CYC-116	AurB (AuroraB)
C26H31FN7O6P	587.54	AZD-1152, Barasertib AZD-1152-HQPA,	AurB (AuroraB)
C26H30FN7O3	507.56	Barasertib	AurB (AuroraB)
C29H32N4O3S	516.654	Hesperadin	AurB (AuroraB), Tyrosine Kinase inhibitor

			AXL (Ark), KIT (c-KIT), FLT3 (STK1, FLK2), MET (c-MET, HGFR), PDGFRa (PDGFR2), RET, RAD51 expression inhibitor
C23H21N5O3S	447.51	MP-470, Amuvatinib	
C21H14Cl2N4O3	441.267	PD173955-Analogue 1 Imatinib Mesylate, Gleevec, Glivec, CGP- 57148B, STI-571	BCR, ABL1 (ABL) BCR, ABL1 (ABL), PDGFRb (PDGFR, PDGFR1), KIT (c-KIT)
C30H35N7O4S	589.708		BCR, ABL1 (ABL), PDGFRb (PDGFR, PDGFR1), KIT (c-KIT)
C28H22F3N7O	529.516	Nilotinib, Tasigna, AMN-107	BCR, ABL1 (ABL), SRC (c-SRC)
C21H16Cl2N4OS	443.349	PD173955, PD-173955	BCR, ABL1 (ABL), SRC (c-SRC), EphA1 (EPH), FYN (p59fyn), LCK (p56lck), PDGFRb (PDGFR, PDGFR1), YES, Erk2 (ERK, p38)
C22H26ClN7O2S	488.006	Dasatinib, BMS-354825, Sprycel, NSC-732517	BCR, ABL1 (ABL), SRC (c-SRC), FGR (SRC2), LYN
C26H29Cl2N5O3	530.446	Bosutinib, SKI-606, Bosulif	BCR, ABL1 (ABL), SRC (c-SRC), FLT3 (STK1, FLK2), KIT (c-KIT), RET, PDGFRb (PDGFR, PDGFR1), FGFR1 (FLT2), FGFR2, FGFR3
C29H27F3N6O	532.559	AP-24534, Ponatinib	
C17H14ClF2N3O3 S	413.826	PLX-4720, Raf Kinase Inhibitor V GDC-0879, AR- 00341677	BRAF (B-raf)
C19H18N4O2	334.372		BRAF (B-raf)
C27H27N5O2	453.536	SB590885, SB-590885 PLX-4032, Vemurafenib, Zelboraf, RG-7204, RO-5185426	BRAF (B-raf)
C23H18ClF2N3O3 S	489.922		BRAF (B-raf), RAF1 (c- Raf)
C27H25N5O2	451.52	AZ628, AZ-628	
C23H22FN7O2	447.465	PTK6 CompoundA PTK6 Inhibitor	BRK (PTK6)
C23H23FN8O	446.48	CompoundB	BRK (PTK6)
C11H8Br2N2O2	360.001	LFM-A13	BTK (Bruton's tyrosine kinase)

C25H24N6O2	440.497	PCI-32765, Ibrutinib	BTK (Bruton's tyrosine kinase), LCK (p56lck), LYN
C43H68ClNO11	810.453	Pimecrolimus, Elidel, SDZ-ASM-981	Calcineurin (CN, PP-2B)
C44H69NO12	804.018	FK-506, Tacrolimus, Fujimycin, Prograf	Calcineurin (CN, PP-2B), INS expression inhibitor, FKBP12 (Rotamase)
C21H14N2O5	374.346	STO-609 acetic acid	CAMKK2 (CAMKK)
C38H35N5O6S2	721.844	KN-62	CAMKK2 (CAMKK)
C19H25Cl2N7	422.355	CGP-74514A hydrochloride	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B
C16H19ClN6O	346.815	Compound 52, NG-52	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B
C18H22N6O3S	402.471	NU-6102	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A
C15H13F2N7O2S2	425.436	Cdk1/2 Inhibitor III	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A
C20H28N6O3	400.475	CVT-313	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E
C18H24N6O	340.423	Bohemine	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E
C16H10N2O2	262.263	Indirubin, NSC-105327, Couroupitine B, Indigo red, Indigopurpurin	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK4/Cyclin D1, CDK5, GSK3B
C19H31N5O2	361.482	PHA-793887	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK4/Cyclin D1, CDK5/p25
C19H25ClN6O	388.894	Purvalanol A, NG 60	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK4/Cyclin D1,

			CDK5/p35, DYRK1A (DYRK)
			CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK4/Cyclin D1, CDK5/p35, GSK3B
C16H10N2O5S	342.326	Indirubin-5-sulfonic acid	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK4/Cyclin D1, CDK6/Cyclin D3, CDK7, CDK9/Cyclin T1, Bcl-2, Mcl-1, Survivin, XIAP
		Alvocidib, HMR-1275, L-868275, MDL-107826A, NSC-649890, L-868276, Flavopiridol	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35
C21H20ClNO5	401.84		CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35
		Roscovitine, CYC202, Seliciclib	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35
C19H26N6O	354.449		CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35
C15H18N6O	298.343	Olomoucine	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35
			CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35
C20H25ClN6O3	432.904	Purvalanol B, NG 95	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35, GSK3
			CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35, GSK3
C16H11N3O3	293.277	Alsterpaullone	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35, GSK3
			CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35, GSK3
C12H17N5O	247.296	NU-2058	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35, GSK3
			CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35, GSK3
		Kenpaullone, NSC-664704	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35, GSK3
C16H11BrN2O	327.175		CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E, CDK5/p35, GSK3

			CDK5/p35, GSK3, LCK (p56lck)
			CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK3/Cyclin E, CDK4/Cyclin D1, CDK5/p35, CDK6/Cyclin D3
C16H18Cl3N5O2	418.705	AT-7519 hydrochloride, AT7519 hydrochloride	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK4/Cyclin D1
C13H11N3O2	241.245	SU-9516	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK2/Cyclin A, CDK9/Cyclin T1
C18H21N5O2S	371.457	AZD5438, AZD-5438	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK4/Cyclin D1
C21H21Cl2NO5	438.301	(+)-P276, P276-00	CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK5/p25, GSK3
C9H7N5	185.185	NSC 693868	cdc25
C15H16ClN3O3	321.759	NSC-663284	CDC7, CDK9/Cyclin T1, MAPKAPK2 (MK2)
C12H12ClN3O	249.696	PHA-767491 hydrochloride	CDK2/Cyclin A
C21H15N5O3S2	449.505	GW8510	
C17H21N3O2S	331.433	PHA690509, PHA-690509	CDK2/Cyclin A
			CDK2/Cyclin A, CDK5/p25, CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, CDK9/Cyclin T1
C21H28N6O2	396.486	Dinaciclub, MK-7965, NSC-727135, SCH-727965	CDK2/Cyclin A, CDK7/Cyclin H, CDK4/Cyclin D1, CDK5/p35, CDK2/Cyclin E, CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B
C25H32N8O	460.575	PHA-848125, PHA-848125AC	CDK2/Cyclin E, CDK7, CDK9/Cyclin T1
C17H25ClN4O2S2	416.989	SNS-032, BMS387032, BMS-387032	CDK4/Cyclin D1
C15H13NO2S	271.334	NSC 625987	CDK4/Cyclin D1
C15H12N2O2S	284.333	Ryuvidine	

C20H11N3O2	325.32	Arcyriaflavin A	CDK4/Cyclin D1, CaMK2b (CaMK2)
C24H29N7O2	447.533	PD-0332991, PD-332991	CDK4/Cyclin D1, CDK6/Cyclin D3
C27H32F2N8	506.593	LY2835219, LY-2835219	CDK4/Cyclin D1, CDK6/Cyclin D3, PIM1
C22H33ClN6	416.991	BS-181 hydrochloride	CDK7
C9H10N6O	218.215	CAN-508	CDK9/Cyclin T1
C25H26Cl2N4O2	485.406	TCS 2312	
C23H22ClN5O	419.907	dihydrochloride	CHK1
C25H24N4O2	412.484	CHIR-124	CHK1
		TCS 2312	CHK1
C24H15N3O3	393.394	SB-218078	CHK1, CDC2 (CDK1, p34cdc2, CDC28p)/Cyclin B, PKC
C15H18BrN7	376.254	SCH900776, SCH-900776, MK-8776	CHK1, CDK2/Cyclin A, CDK2/Cyclin E
C17H20ClFN4O2S	398.883	AZD 7762, AZD-7762	
		hydrochloride	CHK1, CHK2
C17H20ClFN4O2S	398.883	AZD 7762, AZD-7762	
		hydrochloride	CHK1, CHK2
C20H12N2O3	328.321	PD-407824	CHK1, WEE1
C20H14ClN3O2	363.797	Chk2 Inhibitor II, 339253	
		NSC 109555 ditosylate,	CHK2
C33H40N10O7S2	752.864	DDUG	CHK2
C18H17N7O3	379.373	PV-1019, NSC-744039	CHK2
		2-Dimethylamino- 4,5,6,7-tetrabromo-1H- benzimidazole, DMAT,	
C9H7Br4N3	476.788	Casein Kinase II	CK1a (Casein kinase 1, CK1)
C18H17NO4	311.332	IC-261, SU-5607	CK1a (Casein kinase 1, CK1)
C23H18N4O3	398.414	D4476	CK1a (Casein kinase 1, CK1)
C19H22Cl2FN5	410.316	PF-670462	CK1e, CK1d
C14H6O8	302.193	Ellagic acid	CK2a1 (CK2, CKII, Casein kinase2)
C6HBr4N3	434.708	TBB, NSC 231634	CK2a1 (CK2, CKII, Casein kinase2)
C19H12ClN3O2	349.77	CX-4945	CK2a1 (CK2, CKII, Casein kinase2)
C14H8O6	272.21	Quinalizarin	CK2a1 (CK2, CKII, Casein kinase2)

C16H14N2O2S2	330.425	TTP 22	CK2a1 (CK2, CKII,
C9H4Br4O2	463.743	Casein Kinase II	Casein kinase2)
C13H15NO2S	249.329	Inhibitor, TBCA	CK2a1 (CK2, CKII,
C21H14ClFN6	404.827	TG003, TG-003	Casein kinase2)
C18H34O4	314.46	TPL2	CLK1, CLK4
C27H26FN3OS	459.578	Dioctanoylglycol	COT (TPL2, EST,
C17H12N2O2	276.289	R 59-022	MAP3K8)
C17H15NO3	281.306	TC-DAPK 6	DAGK (DGK,
C12H15NO3	221.252	NU-7026, LY-293646	Diacylglycerol kinase)
C16H15N3O2	281.309	IC86621, IC-86621	DAGK (DGK,
C14H12O3	228.243	Compound 401	Diacylglycerol kinase)
C19H21N3O2S	355.454	TX-1918	DAPK1, DAPK3 (ZIPK,
C27H45IN2	524.564	Compound 34	ZIP)
C22H29Cl3FN7	516.87	NH125	DNAPK (DNA-PKcs)
C18H21Cl3FN7	460.764	BIBU 1361	DNAPK (DNA-PKcs)
C16H14BrN3O2	360.205	dihydrochloride	DNAPK (DNA-PKcs),
C24H25ClFN5O3	485.938	BIBX 1382	FRAP (MTOR, FRAP1)
C17H18ClN3O5S	411.86	dihydrochloride,	eEF2K
C16H12N2O3	280.278	Falnidamol	eEF2K
C15H10O5	270.237	PD-153035, AG-1517,	eEF2K activator
C11H9N5	211.223	Compound 32, SU-5271,	EGFR (ERBB1, HER1)
C25H27ClN6O3	494.973	ZM-252868, WHI-P79	EGFR (ERBB1, HER1)
C24H25ClN6OS	481.013	BIBW-2992, Tovok,	EGFR (ERBB1, HER1)
C24H23ClFN5O2	467.923	Afatinib	EGFR (ERBB1, HER1)
C24H25ClN6O2	464.947	Tyrphostin AG 1478	EGFR (ERBB1, HER1)
C13H8N4O3	268.228	mesylate	EGFR (ERBB1, HER1)
C18H13BrN4O	381.226	AG 494	EGFR (ERBB1, HER1)
		Genistein	EGFR (ERBB1, HER1)
		PP-3	EGFR (ERBB1, HER1)
		WZ4002, WZ-4002	EGFR (ERBB1, HER1)
		WZ8040, WZ-8040	EGFR (ERBB1, HER1)
		Pelitinib, EKB-569,	EGFR (ERBB1, HER1)
		WAY-EKB-569	EGFR (ERBB1, HER1)
		WZ3146, WZ-3146	EGFR (ERBB1, HER1)
		AG-183, Tyrphostin 51	EGFR (ERBB1, HER1)
		EKI-785, CL-387785	EGFR (ERBB1, HER1) -
			Irreversible

C22H24ClFN4O3	446.902	Gefitinib, Iressa, ZD1839	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C43H42ClFN4O10 S3	925.461	Lapatinib ditosylate, Tykerb, GW572016	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C22H24ClN3O4	429.897	Erlotinib HCl, CP- 358774, OSI-774, Tarceva, NSC-718781, RG-1415, Ro-50-8231	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C23H21N7	395.46	GW2974	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C28H27Cl3FN5O3 S2	671.033	GW583340 dihydrochloride	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C18H16N2O3	308.331	Tyrphostin B44, (-) enantiomer	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C27H28ClFN6O	507.002	AV-412, MP-412	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C27H27N5O3	469.535	CP-724714	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C30H29ClN6O3	557.043	Neratinib, HKI-272, CPD-820	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C22H19ClN6O2S	466.943	ARRY334543, ARRY- 334543, ARRY-543, ASLAN-001, AR- 00334543, Varlitinib	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)
C24H25ClFN5O3	485.938	CI-1033, Canertinib, PD-183805, SN-26606	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU) - Irreversible
C27H27FN8O3	530.553	BMS-599626, AC-480	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU), ErbB3 (HER3), ErbB4 (HER4)
C14H12BrN5	330.183	PD-158780	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU), ErbB4 (HER4)
C17H11ClFN5O	355.754	HDS 029	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU), ErbB4 (HER4)

C24H28Cl2N6O3	519.424	JNJ 28871063 hydrochloride	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU), ErbB4 (HER4)
C24H27ClFN5O3	487.954	Dacomitinib hydrate, PF-00299804, PF-00299804-03, PF-299, PF-299804	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU), ErbB4 (HER4)
C27H32N6	440.583	AEE 788, AEE-788, NVP-AEE-788	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU), KDR (VEGFR2, VEGFR, FLK1)
C17H14N2O3	294.305	AG 490, Tyrphostin AG 490	EGFR (ERBB1, HER1), JAK2, JAK3/STAT, JAK3/AP-1, JAK3/MAPK
C22H24BrFN4O2	475.354	Vandetanib, Zactima, ZD6474, AZD-6474	EGFR (ERBB1, HER1), KDR (VEGFR2, VEGFR, FLK1), RET, ABL1 (ABL), KIT (c-KIT), FLT1 (VEGFR1), FLT4 (VEGFR3), TRKA (TRK)
C10H6N2O2	186.167	AG 18, RG-50810, TyrphostinA23	EGFR (ERBB1, HER1), PDGFRb (PDGFR, PDGFR1)
C10H8N2O2S	220.248	AG 213, Tyrphostin AG 213	EGFR (ERBB1, HER1), PDGFRb (PDGFR, PDGFR1)
C21H19NO6	381.379	Lavendustin A, RG 14355	EGFR (ERBB1, HER1), SRC (c-SRC)
C18H15Cl4N3OS	463.208	Sal003	eIF2a
C25H23F3N4O2	468.471	Mubritinib, TAK-165, D04025	ErbB2 (TKR1, HER2, NEU)
C19H15N3O3S2	397.471	AG 825, Tyrphostin AG 825	ErbB2 (TKR1, HER2, NEU)
C24H20Cl2FN5O2	500.352	ERK2 inhibitor	Erk2 (ERK, p38)
C24H22FN5OS	447.528	Compound 10	Erk2 (ERK, p38)
C28H31F3N6O5	588.578	XMD8-92, XMD-8-92	Erk5 (BMK1, PRKM7)
C22H20F3N5O3S	491.486	PF-573228	FAK (FAK1)
C6H14Cl4N4	284.014	FAK Inhibitor 14	FAK (FAK1)
C21H20F3N7O3S	507.489	PF562271, PF-562271	FAK (FAK1), PYK2 (FAK2, PTK2B, CAKB)
C22H21F3N6O3S	506.501	PF431396, PF-431396	FAK (FAK1), PYK2 (FAK2, PTK2B, CAKB)
C27H22Cl2N4O	489.396	Tipifarnib, NSC-702818, R-115777, Zarnestra, IND 58359	Farnesyltransferase

C28H41N7O3	523.67	PD-173074	FGFR1 (FLT2), FGFR2, FGFR3
C26H33N5O3	463.572	AZD-4547	FGFR1 (FLT2), FGFR2, FGFR3, FGFR4
C26H31Cl2N7O3	560.475	BGJ-398, NVP-BGJ-398, NVP-BGJ398	FGFR1 (FLT2), FGFR2, FGFR3, FGFR4
C22H24FN5O4	441.455	BMS-582664, Brivanib alaninate	FGFR1 (FLT2), FGFR2, FGFR3, FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3)
C21H19CIN4O4	426.853	E7080, E-7080, ER-203492-00, Lenvatinib	FGFR1 (FLT2), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1), FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3)
C20H21CIN4O4	416.858	KRN 633, KRN-633	FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3)
C21H24CIN7O2S	473.979	Pazopanib hydrochloride, GW-786034, Armala, Votrient	FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3), KIT (c-KIT), PDGFRa (PDGFR2), PDGFRb (PDGFR, PDGFR1)
C25H27FN4O3	450.505	Cediranib, AZD-2171, Recentin	FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1)
C22H19CIN4O5	454.863	AV951, KRN951, Tivozanib	FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1), p38 MAPK
C35H30N4O4	570.637	PKC-412, CGP41251, Midostaurin	
C15H10N2O	234.253	GTP 14564	FLT3 (STK1, FLK2)
C18H20N2O4S	360.427	TCS 359	FLT3 (STK1, FLK2)
C20H20N4O	332.399	KW 2449, KW-2449	FLT3 (STK1, FLK2), ABL1 (ABL), FGFR1 (FLT2), AurA (AuroraA)
C29H32N6O4S	560.667	AC220, AC-220, Quizartinib, AC010220	FLT3 (STK1, FLK2), FMS (c-FMS, CSF-1R, CSF1R), KIT (c-KIT),

C27H36N6O3S	524.678	TG-101348	PDGFRb (PDGFR, PDGFR1), RET FLT3 (STK1, FLK2), JAK2
C31H42N6O4	562.703	MLN-518, CT 53518, Tandutinib	FLT3 (STK1, FLK2), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1) FLT3 (STK1, FLK2), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1), RET, RAF1 (c-Raf), BRAF (B-raf), FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3)
C27H22ClF3N4O6S	623	Sorafenib, p-Toluenesulfonate Salt, Bay 43-9006, Nexavar	FLT3 (STK1, FLK2), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1), RET, RAF1 (c-Raf), BRAF (B-raf), FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3)
C21H16ClF3N4O3	464.825	Sorafenib, Bay 43-9006, Nexavar	FLT3 (STK1, FLK2), MET (c-MET, HGFR), KDR (VEGFR2, VEGFR, FLK1)
C15H14N2O	238.284	SU 5416, SU-5416, TSU-16, NSC-696819, Semaxanib, Semaxnib, Semoxind	FLT3 (STK1, FLK2), MET (c-MET, HGFR), KIT (c-KIT), RET, TIE2 (TEK), KDR (VEGFR2, VEGFR, FLK1)
C32H30FN3O10	635.593	XL-184, XL184, BMS-907351, Cabozantinib malate	FLT3 (STK1, FLK2), SYK
C23H24FN6Na2O9P	624.423	R788, R-788, Fostamatinib disodium, R935788, R-935788, NSC-745942	FLT3 (STK1, FLK2), SYK, (IL-2, IL-6 and TNFa) production inhibitor
C22H23FN6O5	470.454	R406, R-406, NSC-742317	FLT3 (STK1, FLK2), TRKA (TRK), JAK2
C26H21N3O4	439.463	Lestaurtinib, CEP-701, KT-5555, SPM-924	FLT4 (VEGFR3)
C21H18N2O	314.38	MAZ51, MAZ-51	
C20H22N4O3	366.414	5-(3-Methoxy-4-((4-methoxybenzyl)oxy)benzyl)-pyrimidine-2,4-diamine	FMS (c-FMS, CSF-1R, CSF1R)

C26H32N6O2	460.571	GSK2636771, GSK-2636771	FMS (c-FMS, CSF-1R, CSF1R) FMS (c-FMS, CSF-1R, CSF1R), KDR (VEGFR2, VEGFR, FLK1), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1)
C24H24N4O5S	480.536	Ki20227 (+/-), Ki-20227	
C56H87NO16	1,030.29	Temsirolimus, CCI-779,	
C16H16N6O	308.338	Torisel	FRAP (MTOR, FRAP1)
C51H79NO13	914.172	Pp242, Torkinib	FRAP (MTOR, FRAP1)
		Rapamycin, Sirolimus,	FRAP (MTOR, FRAP1),
		Rapamune	CCR5 expression inhibitor
			FRAP (MTOR, FRAP1),
C53H83NO14	958.224	Everolimus, RAD-001,	FKBP12 (Rotamase),
		Certican	Angiogenesis inhibitor
C25H31N5O4	465.545	KU0063794, KU-	FRAP (MTOR, FRAP1),
		0063794, KU-63794	mTORC1, mTORC2
C25H31N5O4	465.545	AZD-8055, AZD 8055,	FRAP (MTOR, FRAP1),
		AZD8055	mTORC1, mTORC2
C21H23ClN6O3	442.899	OSI-027 hydrochloride	FRAP (MTOR, FRAP1),
			mTORC1, mTORC2
C24H29N7O5	495.531	WYE-354	FRAP (MTOR, FRAP1),
			mTORC1, mTORC2
C28H30N8O	494.591	WAY-600	FRAP (MTOR, FRAP1),
		CHIR-98014 isomer,	mTORC1, mTORC2
C20H17Cl2N9O2	486.314	CT-98014	
C22H18Cl2N8	465.338	CT-99021, CHIR-99021	GSK3
		6-bromoindirubin-3'-	
C16H10BrN3O2	356.173	oxime, BIO	GSK3
C12H12N4O4S	308.313	AR-A014418	GSK3
C19H12Cl2N2O2	371.217	SB 216763	GSK3
C16H10ClN3O5	359.721	SB-415286	GSK3
C18H14N4O2	318.329	TWS-119	GSK3
C17H13F3N4O2	362.306	A 1070722, A-1070722	GSK3
		2-Thio(3-iodobenzyl)-5-	
		(1-pyridyl)-[1,3,4]-	
C14H10IN3OS	395.218	oxadiazole	GSK3B
			Histone Deacetylase
C16H22N2O2S	306.423	OICR0000857A	SIRT1 inhibitor
C18H16ClN3O2	341.792	PQ401	IGF1R (JTK13, IGFIR)
C25H26ClN5O3	479.959	BMS-536924	IGF1R (JTK13, IGFIR)
		NVP-AEW-541, AEW-	
C27H29N5O	439.552	541	IGF1R (JTK13, IGFIR)

C44H47F2N9O5S	851.963	GSK-1904529A, GSK1904529A	IGF1R (JTK13, IGFIR)
C26H23N5O	421.494	OSI-906, Linsitinib	IGF1R (JTK13, IGFIR)
C23H24FN9O	461.495	BMS-754807	IGF1R (JTK13, IGFIR)
		NVP-ADW742, ADW-742, ADW, GSK-552602-A, NVP-ADW-742	
C28H31N5O	453.579		IGF1R (JTK13, IGFIR)
			IKKa (IKK1), IKKb (IKK2)
C28H30CIN5OS	520.089	IKK 16	
C14H18CIN5	291.779	BMS-345541	IKKb (IKK2)
C17H13Cl3N4O	395.67	PS1145 dihydrochloride	IKKb (IKK2)
C9H8N2OS2	224.303	SC-514	IKKb (IKK2)
C12H10FN3O2S	279.29	TPCA-1	IKKb (IKK2)
C15H8CIF6NO2	383.673	IMD 0354, IMD-0354	IKKb (IKK2)
C11H10FN5O2S2	327.358	ILK inhibitor	ILK
C20H21N5O4	395.412	IRAK-1/4 Inhibitor I	IRAK1 (IRAK)
C16H16CIN5	313.785	IRAK4 Inhibitor	IRAK4
C21H26N6O	378.471	ASC-033, APY 33	IRE1 (IRE1a, ERN1)
C19H19N7	345.401	ASC-069, APY 69	IRE1 (IRE1a, ERN1)
C22H25N7	387.481	ASC-081, APY 81	IRE1 (IRE1a, ERN1)
C23H21N7	395.46	ASC-082, APY 82	IRE1 (IRE1a, ERN1)
C24H19N7S	437.52	ASC-086, APY 86	IRE1 (IRE1a, ERN1)
C14H14CIFN8	348.766	AZD-1480, AZD1480	JAK1, JAK2, JAK3
		JAK compound I (VI-53)	
C20H18FN5O3S	427.452		JAK1, JAK2, JAK3
		Rigel JAK Compound II, Prodrug of VI-53, R-348, R348	
C23H22FN5O4S	483.515		JAK1, JAK2, JAK3
		Merck-5, Mk-5, JAK Inhibitor I	JAK1, JAK2, JAK3, TYK2
C18H16FN3O	309.338	INCB018424, INC-424, INCB-018424, INCB-424, INCB-18424, Ruxolitinib, Jakafi,	
		Jakavi	JAK1, JAK2, JAK3, TYK2
C17H18N6	306.365		
C18H16F2N6	354.357	AZ-960	JAK2
		CYT11387, CYT387,	
C23H22N6O2	414.46	CYT-387, Momelotinib	JAK2
		LY2784544, LY-2784544	
C23H25CIFN7O	469.942		JAK2
C27H30Cl2F2N6O	563.47	NVP-BSK805	JAK2
		ZM 39923	
C23H26ClNO	367.912	hydrochloride	JAK3

		CP-690550, CP-690,550, Tasocitinib,	
C16H20N6O	312.37	Tofacitinib	JAK3
C16H15N3O3	297.309	Janex-1, WHI-P131	JAK3
			JAK3, EGFR (ERBB1, HER1), SRC (c-SRC), ABL1 (ABL), KDR (VEGFR2, VEGFR, FLK1), p38 MAPK, PI3K
C16H14BrN3O3	376.205	WHI-P 154, MASTPROM	JNK1 (JNK)
C20H16N6S	372.446	AS-601245	JNK1 (JNK)
C10H8N4O2S2	280.326	AEG 3482	JNK1 (JNK)
C14H8N2O	220.226	SP-600125	JNK1 (JNK)
C13H9N5O5S2	379.371	BI 78D3	JNK1 (JNK)
C22H24N6O	388.466	CC-401, JNK-401	JNK1 (JNK), JNK2
			JNK1 (JNK), JNK2, JNK3, p38a
C20H16N2OS	332.419	TCS JNK 5a	KDR (VEGFR2, VEGFR, FLK1)
C17H16N2O	264.322	SU 4312, DMBI	KDR (VEGFR2, VEGFR, FLK1)
		Tyrphostin SU 1498, SU 1498	KDR (VEGFR2, VEGFR, FLK1)
C25H30N2O2	390.518	ZM 306416	KDR (VEGFR2, VEGFR, FLK1)
C16H14Cl2FN3O2	370.206	hydrochloride	KDR (VEGFR2, VEGFR, FLK1)
		ZM 323881	KDR (VEGFR2, VEGFR, FLK1)
C22H19ClFN3O2	411.857	hydrochloride	KDR (VEGFR2, VEGFR, FLK1), FGFR1 (FLT2), FGFR3, FLT4 (VEGFR3), FGFR2
C19H19FN4O3	370.378	Brivanib, BMS-540215	KDR (VEGFR2, VEGFR, FLK1), FGFR1 (FLT2), PDGFRb (PDGFR, PDGFR1)
			KDR (VEGFR2, VEGFR, FLK1), FLT3 (STK1, FLK2), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1)
C17H16N2O3	296.321	SU-5402	KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1)
			KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1)
C21H21FN6O	392.429	Dovitinib, CHIR-258, TKI-258, GFKI-258	KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3), KIT (c-KIT), PDGFRb (PDGFR, PDGFR1)
			KDR (VEGFR2, VEGFR, FLK1), KIT (c-KIT), PDGFRa (PDGFR2), FGFR2, FLT3 (STK1, FLK2)
C20H16ClN5O3	409.826	BAY 57-9352, Telatinib	
C24H18F3N3O4	469.413	Ki-8751	

C20H17Cl3N4	419.735	Vatalanib dihydrochloride, PTK- 787, ZK-222584, CGP79787D	KDR (VEGFR2, VEGFR, FLK1), PDGFRb (PDGFR, PDGFR1) KDR (VEGFR2, VEGFR, FLK1), PDGFRb (PDGFR, PDGFR1), FMS (c-FMS, CSF-1R, CSF1R), FLT3 (STK1, FLK2)
C21H18FN5O	375.399	ABT-869, Linifanib, AL-39324, RG-3635	KDR (VEGFR2, VEGFR, FLK1), PDGFRb (PDGFR, PDGFR1), MET (c-MET, HGFR)
C31H33N5O4	539.625	BIBF-1120, Intedanib, Vargatef, Nintedanib	KDR (VEGFR2, VEGFR, FLK1), RET, KIT (c-KIT)
C15H17ClN6O3	364.787	JNJ-38158471	KIT (c-KIT), FLT3 (STK1, FLK2), FMS (c- FMS, CSF-1R, CSF1R), KDR (VEGFR2, VEGFR, FLK1), PDGFRb (PDGFR, PDGFR1)
C26H33FN4O7	532.561	Sunitinib Malate, Sutent, SU-11248, PHA- 290940AD	KIT (c-KIT), KDR (VEGFR2, VEGFR, FLK1), FLT1 (VEGFR1), FMS (c-FMS, CSF-1R, CSF1R), RAF1 (c-Raf), LCK (p56lck), PDGFRb (PDGFR, PDGFR1)
C22H16F3N3O2S	443.442	OSI-930	KIT (c-KIT), PDGFRb (PDGFR, PDGFR1), BRAF (B-raf), RAF1 (c- Raf), KDR (VEGFR2, VEGFR, FLK1)
C24H16F6N6O	518.414	RAF265, RAF-265, CHIR-265	KIT (c-KIT), PDGFRb (PDGFR, PDGFR1), FGFR3
C29H34N6O4S2	594.748	Masitinib mesylate, AB1010, AB-1010	KIT (c-KIT), PDGFRb (PDGFR, PDGFR1), FGFR3
C28H30N6OS	498.642	Masitinib, AB-1010, AB1010	KIT (c-KIT), PDGFRb (PDGFR, PDGFR1), KDR (VEGFR2, VEGFR, FLK1)
C22H29N5O9P2	569.441	Motesanib diphosphate salt, AMG-706	KIT (c-KIT), PDGFRb (PDGFR, PDGFR1), RET,
C21H15ClF4N4O3	482.815	BAY 73-4506, Regorafenib	

			RAF1 (c-Raf), BRAF (B- raf), TIE2 (TEK), KDR (VEGFR2, VEGFR, FLK1), p38 MAPK LCK (p56lck), FYN (p59fyn), HCK (p56hck), SRC (c-SRC)
C15H16ClN5	301.774	PP-2, PP2, AG-1879 7-Cyclopentyl-5-(4- phenoxyphenyl)-7H- pyrrolo[2,3-d]pyrimidin- 4-ylamine	
C23H22N4O	370.447		LCK (p56lck), SRC (c- SRC) LCK (p56lck), SRC (c- SRC), KDR (VEGFR2, VEGFR, FLK1), p38a
C29H28F3N5O2	535.56	AMG-47a	
C17H12Cl2F2N4O S	429.271	BMS-3	LIMK1 (LIMK)
C17H14Cl2F2N4O S	431.287	BMS-5	LIMK1 (LIMK)
C20H21BrN8	453.338	Lim2 Kinase Inhibitor	LIMK2 LRRK2 (Leucine-Rich Repeat Kinase 2, Dardarin)
C17H20ClN5O3	377.825	HG-10-102-01	LRRK2, ROCK1, ROCK2 (ROCKa), Calcium sensitizer
C14H21N3O	247.336	Y-27632	LRRK2, ROCK1, ROCK2 (ROCKa), Calcium sensitizer
C14H21N3O	247.336	Y-27632	MAP2K1 (MEK1), BRAF (B-raf), RAF1 (c-Raf)
C21H18FN5O5S	471.462	RO-5126766	MAP2K1 (MEK1), DNA Topoisomerase II, HIV Integrase, NFkB, AP-1
C21H24O6	372.412	Arctigenin, (-)- Arctigenin	MAP2K1 (MEK1), MAP2K2 (MEK2)
C16H12F3N3S	335.347	SL327 RDEA-119, AR-119, RDEA119, BAY- 869766, BAY-86-9766,	
C19H20F3IN2O5S	572.337	Refametinib PD-0325901, PD 0325901	MAP2K1 (MEK1), MAP2K2 (MEK2) MAP2K1 (MEK1), MAP2K2 (MEK2)
C16H14F3IN2O4 C17H13BrClF2IN 2O2	482.193 557.556	PD-184161	MAP2K1 (MEK1), MAP2K2 (MEK2)
C18H16F3IN2O2	476.232	PD-198306	MAP2K1 (MEK1), MAP2K2 (MEK2)

C15H15FIN3O3	431.201	AS-703026, MSC1936369B	MAP2K1 (MEK1), MAP2K2 (MEK2)
C26H23FIN5O4	615.395	GSK-1120212, Trametinib	MAP2K1 (MEK1), MAP2K2 (MEK2)
C16H17FIN3O4	461.227	AZD8330, AZD-8330, ARRY-424704, ARRY- 704	MAP2K1 (MEK1), MAP2K2 (MEK2)
C16H13BrF3IN2O 4	561.089	PD318088	MAP2K1 (MEK1), MAP2K2 (MEK2)
C16H13NO3	267.279	PD-98059	MAP2K1 (MEK1), MAP2K2 (MEK2), Erk1
C17H15BrCIFN4O 3	457.681	AZD6244, ARRY- 142886, AZD-6244, Selumetinib, ARRY-886	MAP2K1 (MEK1), MAP2K2 (MEK2), Erk2 (ERK, p38)
C17H14CIF2IN2O 2	478.66	PD-184352, CI-1040	MAP2K1 (MEK1), MAP2K2 (MEK2), Erk2 (ERK, p38), RAF1 (c-Raf)
C18H16N6S2	380.49	U0126, U-0126, U 0126	MAP2K1 (MEK1), MAP2K2 (MEK2), JAK2, Erk2 (ERK, p38)
C27H28N4O2	440.537	BIX 02189, BIX-02189	MAP2K5 (MEK5), Erk5 (BMK1, PRKM7)
C16H15F3N6O	364.325	S-99	MAP3K5 (ASK1)
C19H13NO4	319.311	NQDI-1	MAP3K5 (ASK1)
C21H18N4OS	374.459	PF 3644022, PF- 3644022, MK2 inhibitor, compound 35	MAPKAPK2 (MK2)
C21H18N4OS	374.459	PF 3644022, PF- 3644022	MAPKAPK2 (MK2)
C24H26N8O2	458.516	GLPG-0259, Compound A	MAPKAPK5 (PRAK)
C28H30CIN5O4S	568.087	SU-11274, PKI- SU11274	MET (c-MET, HGFR)
C19H16N8O	372.383	PD04217903, PF- 04217903, PF-4217903	MET (c-MET, HGFR)
C18H13N7S	359.408	SGX-523	MET (c-MET, HGFR)
C19H13F2N7	377.35	JNJ-38877605	MET (c-MET, HGFR)
C25H16F2N4O3	458.416	BMS-2	MET (c-MET, HGFR)
C19H16N8O	372.383	PD04217903, PF- 04217903, PF-4217903	MET (c-MET, HGFR)
C29H28N6O2	492.572	EMD-1214063	MET (c-MET, HGFR)
C25H21N5O3S	471.531	MK-8033	MET (c-MET, HGFR)
C23H19N3O2	369.416	ARQ-197, Tivantinib	MET (c-MET, HGFR)
C30H29N5O5	539.582	AMG458	MET (c-MET, HGFR)

C25H19CIF2N4O4	512.893	BMS 777607, BMS-777607	MET (c-MET, HGFR), AXL (Ark), RON (MST1R), TYRO3 (RSE, SKY, TIF, BYK)
C23H15CIF2N4O3	468.84	BMS 794833, BMS-794833	MET (c-MET, HGFR), KDR (VEGFR2, VEGFR, FLK1)
C34H34F2N4O6	632.654	XL-880, GSK-1363089, EXEL-2880, GSK-089, foretinib	MET (c-MET, HGFR), KDR (VEGFR2, VEGFR, FLK1), FLT3 (STK1, FLK2), TIE2 (TEK)
C32H34Cl2N4O4S	641.608	PHA-665752	MET (c-MET, HGFR), RON (MST1R), KDR (VEGFR2, VEGFR, FLK1)
C26H20FN5O2S2	517.598	MGCD-265	MET (c-MET, HGFR), RON (MST1R), TIE2 (TEK), FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3)
C11H9FN6	244.228	CGP-57380, CGP 57380	MNK1
C11H9FN6	244.228	CGP 57380, CGP-57380	MNK1
C19H17CIN6O2	396.83	NIK Kinase Inhibitor	NIK
C20H13FN4O2	360.341	PD-169316	p38 MAPK
C16H12FN3S	297.35	SKF-86002, SKF 86002	p38 MAPK
C21H16FN3OS	377.435	SB 203580, SB-203580	p38 MAPK
C20H21FN4O2	368.405	SB 239063	p38 MAPK
C9H8N2O	160.173	SD 169	p38 MAPK
C16H14Cl2FN3S	370.272	SKF 86002 dihydrochloride, SKF-86002 dihydrochloride	p38 MAPK
C19H12F4N4O2	404.318	VX-702, VX-850, VX-954, KVK-702	p38 MAPK
C20H14FN3O	331.343	SB-202190, FHPI	p38 MAPK
C19H19CIF2N4O3	424.829	R1487, R-1487	p38 MAPK
C19H20FN5O	353.393	SB242235, SB-242235	p38 MAPK
C20H20CIN5O2	397.858	SD-06, SD-0006	p38 MAPK
C24H21N3OS	399.508	TAK-715	p38 MAPK
C31H37N5O3	527.657	BIRB 796,	
C19H9Cl2F2N3OS	436.262	Doramapimod	p38 MAPK
C21H25NO2S	355.494	VX-745	p38 MAPK
C20H16BrCIN2O	415.711	JX-401	p38a
C27H24FN3O	425.497	EO-1428	p38a, p38b
		RWJ-67657	p38a, p38b

C26H37FN6O6S2	612.737	LY2228820, LY-2228820	p38a, p38b
C19H21F3N6	390.405	PF 4708671, PF-4708671	p70S6K (S6K, S6K1)
C17H18N4O3S	358.415	S6K-18	p70S6K (S6K, S6K1)
C20H14O2S2	350.454	IPA 3	PAK1 (PAKa)
C25H30N8OS	490.624	PF-3758309, PF-03758309	PAK4
C28H26N4O3	466.531	Staurosporine	Pan kinase
C17H16FN5	309.341	Dianilinopyrimidine_01	Pan kinase
C26H29N5O2	443.541	Crenolanib, ARO-002, CP-868596	PDGFRa (PDGFR2), PDGFRb (PDGFR, PDGFR1)
C16H14N2O2	266.295	Tyrphostin AG-1296	PDGFRb (PDGFR, PDGFR1)
C18H16FN3O2	325.337	JNJ-10198409, RWJ-540973	PDGFRb (PDGFR, PDGFR1)
C16H16Cl2N2O2	339.216	DMPQ dihydrochloride	PDGFRb (PDGFR, PDGFR1)
C22H18N4OS	386.47	Axitinib, AG-013736, Inlyta	PDGFRb (PDGFR, PDGFR1), FLT1 (VEGFR1), KDR (VEGFR2, VEGFR, FLK1), FLT4 (VEGFR3), FMS (c-FMS, CSF-1R, CSF1R)
C24H22N2O3	386.443	SU 16f	PDGFRb (PDGFR, PDGFR1), KDR (VEGFR2, VEGFR, FLK1)
C26H22FN7O	467.498	AG13958, AG-013958	PDGFRb (PDGFR, PDGFR1), KDR (VEGFR2, VEGFR, FLK1)
C18H18N2O3	310.347	SU-6668, TSU-68, Orantinib	PDGFRb (PDGFR, PDGFR1), KDR (VEGFR2, VEGFR, FLK1), FGFR1 (FLT2), AurA (AuroraA), AurB (AuroraB), TBK1
C29H31N7O	493.603	Imatinib (free base), Gleevec, Glivec, CGP-57148B, STI-571	PDGFRb (PDGFR, PDGFR1), KIT (c-KIT), ABL1 (ABL)
C26H20ClF3N4O	496.911	OSU-03012 hydrochloride, AR-12, NSC-728209	PDK1 (PDPK1)

C2HCl2NaO2-	150.924	DCA, Dichloroacetate	PDK1 (PDPK1)
C20H23BrN8O	471.354	BX912, BX-912	PDK1 (PDPK1)
C25H34N8O	462.59	GSK 2334470, GSK-2334470	PDK1 (PDPK1)
C23H26IN7O2S	591.468	BX-795	PDK1 (PDPK1), TBK1, IKKe
C22H20BrN7	462.345	PERK inhibitor	PEK (PERK)
C24H19F4N5O	469.434	GSK PERK Inhibitor	PEK (PERK)
C25H35N7O9S4	705.847	GDC-0941 bismesylate, RG-7321, Pictilisib	PI3K
C30H23N5O	469.537	NVP-BEZ235, BEZ235	PI3K
C16H15BrClN5O4S	488.743	PIK-75 hydrochloride	PI3K
C11H5F2NO4S	285.224	AS604850, AS-604850, AS 604850	PI3K
C19H16N4O3	348.355	PI-103	PI3K
C19H17NO3	307.343	LY-294002	PI3K
C19H18N2O3	322.358	PI-828	PI3K
C15H10O7	302.236	Quercetin	PI3K
C19H21F2N7O2	417.413	ZSTK474	PI3K
C14H8FNO4S	305.281	AS-252424	PI3K
C21H24N4O2	364.441	TGX-221	PI3K
C21H16N6O2S2	448.521	XL-147, XL147, SAR-245408	PI3K
C18H21F3N6O2	410.394	BKM120, BKM-120, NVP-BKM-120	PI3K
C16H15N3O2S	313.374	PI3-Kinase a Inhibitor 2	PI3K
C18H11N3O2S	333.364	GSK-1059615, GSK-615	PI3K
C12H7N3O2S	257.268	AS605240, AS-605240	PI3K
C22H24N4O4	408.45	AZD6482, AZD-6482	PI3K
C18H14N6O2	346.343	TG100-115, TG-100-115, TG-100115	PI3K
C23H27N7O3S2	513.636	GDC-0941	PI3K
C23H24O8	428.432	Wortmannin, KY 12420	PI3K - Irreversible
C24H29N3O4	423.505	PF-04691502, PF-502	PI3K, FRAP (MTOR, FRAP1)
C32H29F3N6O6	650.604	BGT-226, NVP-BGT-226, NVP-BGT226	PI3K, FRAP (MTOR, FRAP1)
C27H33N7O4	519.595	WYE-125132, WYE-132	PI3K, FRAP (MTOR, FRAP1), mTORC1, mTORC2
C31H29N5O6S	599.657	XL765, XL-765, SAR245409	PI3K, mTORC1, mTORC2

		PX-866, DJM-166, DJM-2-166, Sonolisib, NSC-722134	PI3K, PTEN inhibitor
C29H35NO8	525.59		
C22H22F3N3O3	433.424	GSK-2636771	PI3K/p110b
C18H17N5O3	351.359	PIK-90	PI3Ka
C14H16ClN3O4S2	389.878	PI-93, PIK-93	PI3Ka
C28H23N7O2	489.528	PIK-294	PI3Ka
C28H23N7O2	489.528	PIK-294	PI3Ka
C19H22F3N5O2S	441.47	BYL-719, NVP-BYL- 719	PI3Ka
C22H19N7O	397.433	IC87114, D-030, IC- 87114	PI3Kd
C22H18FN7O	415.423	CAL-101, GS-1101	PI3Kd
C25H21N7O3	467.479	YM201636, YM-201636	PIKfyve (PIP5K)
C19H18F4N4O2	410.365	OICR0000666A01	PIM1
C13H13NO3S	263.312	PIM1/2 Kinase Inhibitor VI	PIM1, PIM2
C18H11BrN2O2	367.196	TCS PIM-1 1	PIM1, PIM2
C17H11ClN4O	322.748	PIM 1 Inhibitor 2	PIM1, PIM2
C20H23ClF3N5O	441.878	SGI-1776	PIM1, PIM2, FLT3 (STK1, FLK2)
C20H22F3N5O	405.417	SGI-1776	PIM1, PIM2, FLT3 (STK1, FLK2)
C20H22BrCl2N3O 2S	519.283	H 89 dihydrochloride, H-89 dihydrochloride	PKACa (PKA)
C26H27N5O5S2	553.653	Ro-31-8220 mesylate, Bisindolylmaleimide IX Bisindolylmaleimide I hydrochloride, GF	PKC
C25H25ClN4O2	448.945	109203X HCl, Go 6850 HCl	PKC
C21H18ClNO4	383.825	Chelerythrine chloride K-252c, Staurosporine	PKC
C20H13N3O	311.337	Aglycone	PKC
C13H15N3O2S	277.342	C-1	PKC
C26H25ClN4O2	460.955	Bisindolylmaleimide X, HCl salt	PKC
C44H38O14	790.764	Calphostin C, UCN 1028C	PKC
C24H18N4O	378.426	Go 6976, PD-406976	PKC, JAK2
C29H32N4O6S	564.653	LY 333531 mesylate, Ruboxistaurin	PKCb
C30H28O8	516.539	Rottlerin	PKCd, TGM2 (Tissue Transglutaminase), signal

			transduction pathway inhibitor
C28H29N7	463.577	PKC theta inhibitor	PKCt
C12H11NO3	217.221	CID 755673	PKD1 (PKD)
C13H8N4OS	268.294	Imidazolo-oxindole	PKR (EIF2AK1, EIF2AK2)
C28H39N7O3	521.654	PKR inhibitor C16	PLK1 (PLK, STPK13)
		BI 2536, BI-2536	
		Cyclapolin 9,	
C9H4F3N3O4S	307.206	Cyclapolin9	PLK1 (PLK, STPK13)
		GSK-461364,	
C27H28F3N5O2S	543.604	GSK461364	PLK1 (PLK, STPK13)
C34H50N8O3	618.813	BI-6727, volasertib	PLK1 (PLK, STPK13)
		Cyclapolin 9,	
C9H4F3N3O4S	307.206	Cyclapolin9	PLK1 (PLK, STPK13)
C22H20N2O5S	424.47	HMN-214, IVX-214	PLK1 (PLK, STPK13)
		ON-01910, Estybon,	
C21H24NNaO8S	473.472	Rigosertib	PLK1 (PLK, STPK13)
			PLK1 (PLK, STPK13),
C22H18F3N3O4S	477.456	GW-843682X	PLK3 (PRK, FNK, CNK)
		AB12134C3, PNK	
C30H38N4O5	534.647	inhibitor	PNK (PNKP)
C15H8Br2INO2	520.942	GW-5074	RAF1 (c-Raf)
C23H23N3O3	389.447	ZM 336372	RAF1 (c-Raf)
		L-779450, Raf Kinase	
C20H14ClN3O	347.798	Inhibitor IV	RAF1 (c-Raf)
		GSK-2118436B,	
C24H24F3N5O5S		GSK2118436B,	RAF1 (c-Raf), BRAF (B-raf)
3	615.668	Dabrafenib mesylate	RAF1 (c-Raf), SRC (c-SRC), ABL1 (ABL), EphB4
			Ras-NET (Elk3), Tubulin polimerization
C26H20F3N7O	503.479	NVP-BHG712	
		XRP44X, XRP-44X,	
C21H21ClN4O	380.871	WCH-44	
		RET Inhibitor Example	
C25H26N4O4S2	510.628	8 (Free base)	RET
			RET, KDR (VEGFR2, VEGFR, FLK1), FLT1 (VEGFR1), SRC (c-SRC), KIT (c-KIT)
C25H27N5O4S	493.578	Apatinib mesylate, YN-968D1	
C13H13N3OS	259.327	Necrostatin-1	RIPK1 (RIP)
			ROCK1, ROCK2 (ROCKa)
C29H30N8O5	570.599	GSK-269962A	
		N-(4-Pyridyl)-N'-(2,4,6-trichlorophenyl)urea,	
C12H8Cl3N3O	316.57	Rho Kinase Inhibitor II	ROCK1, ROCK2 (ROCKa)

C18H15N3O3	321.33	Rho Kinase Inhibitor V	ROCK1, ROCK2 (ROCKa)
C18H26Cl2N4O3S	449.395	H 1152, Glycyl dihydrochloride	ROCK1, ROCK2 (ROCKa)
C14H18ClN3O3S	343.829	HA 1100 hydrochloride, Hydroxyfasudil	ROCK1, ROCK2 (ROCKa)
C21H16F4N4O2	432.371	RHO-15	ROCK1, ROCK2 (ROCKa)
C16H17ClN4O	316.785	Y-39983, RKI-983, SNJ-1656	ROCK1, ROCK2 (ROCKa)
C14H18ClN3O2S	327.83	Fasudil HCl, AT-877, HA-1077 (diHCl), Eril	ROCK1, ROCK2 (ROCKa), Calcium sensitizer
C22H24N4O4	408.45	SR3677	ROCK2 (ROCKa)
C19H23F2N5O2	391.415	BI-D1870	RSK1 (RSK, S6Ka, MAPKAPK1C)
C22H20N6O2	400.433	BIX-RSK2 Inhibitor	RSK1 (RSK, S6Ka, MAPKAPK1C), RSK2 (S6K-a3, p90-RSK2)
C25H22N2O2	382.454	GSK-650394	SGK1 (SGK), SGK2
C27H27N9O3	525.562	SMG-1 Compound 467	SMG1 (ATX, LIP)
C15H18Cl2N2O2S	361.287	ML 9 hydrochloride	smMLCK (MLCK, myosin light chain kinase, MYLK)
C16H22Cl2N2O2S	377.329	W-7 hydrochloride	smMLCK (MLCK, myosin light chain kinase, MYLK)
C14H18Cl2N2O2S	349.276	W-13 hydrochloride	smMLCK (MLCK, myosin light chain kinase, MYLK)
C15H12Cl2N2OS	339.24	2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole, HCl, SKI-II, ABC-294640	SPK1 (SPHK, SK1, Sphingosine Kinase)
C18H37NO2	299.492	Sphingosine kinase Inhibitor 2	SPK1 (SPHK, SK1, Sphingosine Kinase)
C15H11ClN2OS	302.779	2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole, SKI-II, ABC-294640	SPK1 (SPHK, SK1, Sphingosine Kinase)
C16H19N5	281.356	PP-1, PP1, AGL-1872, Tyrphostin-PP1	SRC (c-SRC)
C19H19N5	317.388	1-Naphthyl PP1, 1-NA-PP 1	SRC (c-SRC), FYN (p59fyn), ABL1 (ABL)

C22H19N3O3	373.405	Src I1	SRC (c-SRC), LCK (p56lck), KDR (VEGFR2, VEGFR, FLK1)
C19H21N3O3S	371.453	SU 6656	SRC (c-SRC), YES, LYN, FYN (p59fyn)
		BAY 61-3606	
		hydrochloride, sc-	
C20H19CIN6O3	426.856	202351	SYK
C22H24N2O5	396.436	ER 27319 maleate	SYK
		(5Z)-7-Oxozeaenol, C-292, FR-148083, L-783278, LL-Z1640-2, f-	
C19H22O7	362.374	152A1	TAK1 (TGF1a, MAP3K7)
C24H23N5O2S	445.537	OSI-TAK1 inhibitor	TAK1 (TGF1a, MAP3K7)
C17H20N4O3S	360.431	AZ TAK1 Inhibitor	TAK1 (TGF1a, MAP3K7)
C28H35N7O4	533.622	TBK1 Inhibitor	TBK1
		SB-505124	
C20H24CIN3O3	389.876	hydrochloride hydrate	TGFbR1 (ALK5)
C17H10ClFN6	352.753	SD 208	TGFbR1 (ALK5)
C28H28CIN3O3	489.993	SIS3	TGFbR1 (ALK5)
		LY-364947, HTS	
C17H12N4	272.304	466284	TGFbR1 (ALK5)
C22H16N4O3	384.387	SB 431542	TGFbR1 (ALK5)
C25H19N5S	421.517	A 83-01	TGFbR1 (ALK5)
C17H13N5	287.319	SJN 2511	TGFbR1 (ALK5)
C21H21N5	343.425	SB-525334	TGFbR1 (ALK5)
		GW 788388, GW-	
C25H23N5O2	425.482	788388	TGFbR1 (ALK5)
			TGFbR1 (ALK5),
C22H19N5O	369.419	LY-2157299	TGFbR2
C25H20F3N5O2	479.454	AMG-Tie2-1	TIE2 (TEK)
C27H21N3O5	467.473	K-252a, K-2151	TRKA (TRK)
		GW441756	
C17H14CIN3O	311.766	hydrochloride	TRKA (TRK)
		Trk Inhibitor III, GNF-	
C28H21F4N5O2	535.492	5837	TRKA (TRK), TRKB
			TTK (ESK, MPS1L1,
			Monopolar spindle 1
C24H32N6O3	452.549	AZ 3146, AZ-3146	(Mps1) kinase)
C19H18N2O3S	354.423	ST638, ST-638	Tyrosine kinase inhibitor
		AT-7519 derivative,	
C23H19Cl4N5O3	555.241	AT-7519M derivative	Unknown
C27H32N8O2	500.595	MK-1775, MK1775	WEE1

6.2 Articles published

Contribution of co-authors. Review by William Chen and David Datzkiw was conceptualized and written by both authors. William Chen contributed significantly to sections 1-4, and David Datzkiw contributed significantly to sections 5 and 6. Figures were conceptualized by William Chen and created by David Datzkiw.

Review



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Satellite cells in ageing: use it or lose it

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Individuals that maintain healthy skeletal tissue tend to live healthier, happier lives as proper muscle function enables maintenance of independence and actuation of autonomy. The onset of skeletal muscle decline begins around the age of 30, and muscle atrophy is associated with a number of serious morbidities and mortalities. Satellite cells are responsible for regeneration of skeletal muscle and enter a reversible non-dividing state of quiescence under homeostatic conditions. In response to injury, satellite cells are able to activate and re-enter the cell cycle, creating new cells to repair and create nascent muscle fibres while preserving a small population that can return to quiescence for future regenerative demands. However, in aged muscle, satellite cells that experience prolonged quiescence will undergo programmed cellular senescence, an irreversible non-dividing state that handicaps the regenerative capabilities of muscle. This review examines how periodic activation and cycling of satellite cells through exercise can mitigate senescence acquisition and myogenic decline.

1. Introduction

Skeletal muscle accounts for approximately 40% of the human body weight and its functions include maintaining posture, mobility, regulating body temperature, energy storage and soft tissue support. Given its many important functions, it is unsurprising that muscle strength is positively correlated with better quality of life and negatively correlated with all-cause mortality [1–4].

The relationship between muscle strength and morbidity is more apparent in geriatric populations where sarcopaenia, age-related muscle atrophy, can lead to falls, disability and mortality. For example, the majority of hip fractures occur within individuals over 65 years of age [5]. Atrophy of stabilizer muscles can lead to loss of balance, important in preventing falls. Diminishing grip strength reduces one's ability to catch oneself in the event of a fall, and in the event of an accident, there is less muscle mass to cushion upon impact. Together, the greater risk of falls combined with sarcopaenia and osteoporosis are why the elderly are at a heightened risk of hip fractures, one of the highest predictors of morbidity and mortality in geriatric populations, with 1 year mortalities ranging from 14% to 58% [5,6]. Overall muscle decline begins around the age of 30, with an average annual muscle mass loss of 3–5%, with a total loss of up to 30% within an individual's lifetime [7–9].

Fortunately, skeletal muscle has remarkable regenerative capabilities, and age-related muscle loss can be drastically attenuated with exercise. On a physiological level, regular exercise has been shown to increase vascularization and perfusion, important for waste removal; muscle efficiency and greater recruitment of myofibres; flexibility and muscle tension; and bone strength and density, required to support muscles [10–12].

On the cellular level, exercise has been shown to increase mitochondrial numbers and quality, enhance innervation by neuromuscular junctions, stimulate supporting cells in the interstitial area of muscle (i.e. fibroapodogenic progenitors,

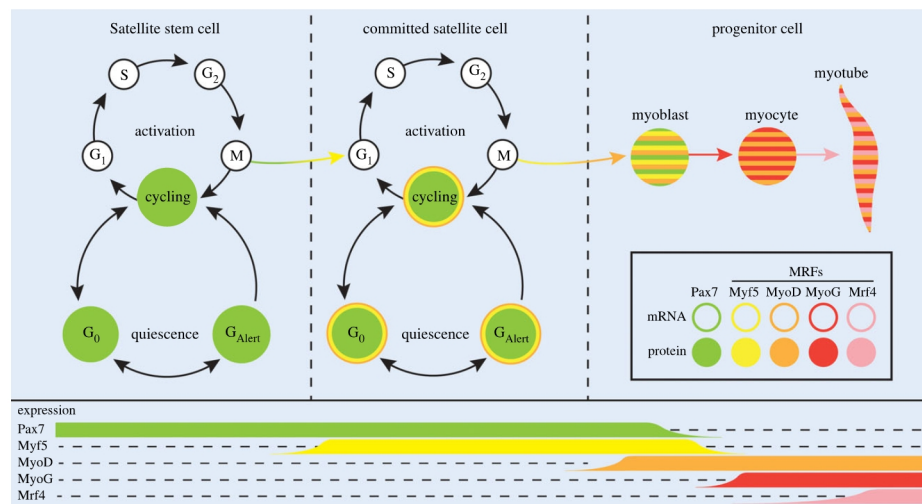


Figure 1. Satellite cell quiescence versus activation. Satellite stem (Pax7⁺;Myf5⁻) and committed cells (Pax7⁺;Myf5⁺) are able to exit the cell cycle and arrest in a reversible quiescent G₀ state. Satellite stem and committed cells are primed for rapid cell cycle reentry by transitioning to a second reversible quiescent state called G_{Alert}. Committed satellite cells in G_{Alert} begin transcribing downstream MRFs *Myf5* and *MyoD*. Satellite cells in G_{Alert} are primed to quickly respond to regenerative demands by entering the cell cycle faster than G₀ quiescent satellite cells. Active progenitor cells initiate the terminal differentiation programme to upregulate *MyoG* and *Mrf4* in myoblasts and myocytes before fusion into myotubes.

FAPs), and stimulate satellite cells to activate for muscle repair and growth [13–16].

In this review, we address the role of quiescence in muscle regeneration, the mechanisms that prevent senescence in quiescent muscle stem cells, and how exercise contributes to long-term satellite cell viability at a cellular and molecular level.

2. Muscle regeneration and the satellite cell niche

Molecular and genetic studies in mice have greatly elucidated our understanding of the mechanisms that govern satellite cell function during the growth and regeneration of skeletal muscle [17–20]. Satellite cells, named after their satellite position on the myofibre, are a heterogeneous population of stem cells and committed cells that are required for muscle repair. Satellite cells highly express the transcription factor *Pax7* and sequentially express myogenic regulatory factors (MRFs) (*Myf5*, *MyoD*, *MyoG*, *Mrf4*) as they proliferate and then differentiate [21–24]. Pax7⁺ satellite stem cells mark their commitment to the myogenic lineage as committed myogenic cells by upregulating *Myf5*. The onset of *Myf5* and *MyoD* protein expression marks the transition into myoblasts. Myogenin induction marks the entry of myoblasts into the terminal differentiation programme and subsequent fusion with other differentiating cells or with existing myofibres (figure 1) [25–27].

Approximately 10% of the satellite cell population appear to be a long-term self-renewing stem cell population expressing *Pax7* but not *Myf5*. The remaining 90% of the satellite cell population are committed cells that have expressed *Myf5* and appear to form a short-term self-renewing population. Transplantation and serial injury experiments have demonstrated the satellite stem cell population to be capable of replenishing

the stem cell population in addition to re-establishing the pool of committed satellite cells [22].

Central to a satellite cell's behaviour is the immediate microenvironment that envelops the cell with structural proteins, and the various signalling factors from other nearby cell types. The nearest structures in proximity to a quiescent satellite cell include matrices of the fibrous sheath of basal lamina closest to the apical side of the satellite cell, and the plasma membrane of the myofibre at the basal side of the satellite cell. Satellite cells are often closely associated with capillary endothelial cells, as muscle tends to be a highly vascularized tissue [28]. Other cell types of the local milieu including fibrocyte/adipocyte progenitors (FAPS), pericytes and motor neurons can be found in the muscle interstitium [17]. Under homeostatic conditions, the satellite cell niche provides cues to actively maintain quiescence signalling pathways while suppressing activation pathways [29,30]. Upon injury, these cues activate satellite cells to prepare for the anticipated demands of regeneration [31]. Inflammatory cytokines are also released to provide chemotactic cues for leucocytes, such as the macrophages required to clear debris and remove toxic waste [32–36]. Preservation of the stem cell pool is critical for maintaining regeneration potential, and thus the niche also provides cues for satellite cells to return to quiescence when regeneration is complete.

While the young niche favours stem cell quiescence, the aged niche provides cues that promote activation and differentiation [17,29,37–47]. Importantly, aged satellite cells demonstrate intrinsic defects that exposure to young niche environments cannot overcome, favouring differentiation and senescence rather than quiescence [38,48–52]. The aged niche and age-related stem cell defects lead to the typical fibro-adipogenic phenotype seen in aged and dystrophic muscle. These findings suggest that prolonged, chronic activation leads

to stem cell exhaustion, and that maintenance of stem cell quiescence is paramount to maintaining a healthy stem cell population throughout the ageing process.

3. Satellite cells prefer quiescence

Homeostatic quiescence and activation is critical for establishing short- and long-term regenerative potential of the muscle by activating satellite cells and creating new progenitors for muscle repair, while preserving a small population of stem and committed satellite cells that can enter quiescence for the future regenerative demands.

In response to injury or inflammation, satellite cells will activate and enter the cell cycle to begin dividing and creating new cells for regeneration. Depending on the degree of regeneration required, satellite stem cells ($\text{Pax7}^+/\text{Myf5}^-$) will change their preference for asymmetric self-renewal or symmetric expansion of the stem cell pool. Under normal homeostatic turnover or minor damage, satellite cells preferentially undergo asymmetric division that will produce one stem cell ($\text{Pax7}^+/\text{Myf5}^-$) and one committed cell ($\text{Pax7}^+/\text{Myf5}^+$), which can then become a progenitor and undergo successive rounds of division [17,22,53]. Conversely, under more severe circumstances where damage is greater and demand for regenerative cells higher, satellite stem cells initially undergo symmetric division in order to rapidly expand the stem cell pool to facilitate large-scale generation of progenitors through asymmetric divisions [18,22,53,54].

Under homeostatic conditions, or following injury when active muscle repair is no longer required, satellite cells must be able to exit the cell cycle and enter a reversible state of quiescence to preserve and maintain long-term regeneration potential of its resident muscle [55,56]. Multiple studies have demonstrated that the loss of quiescence and the ability to exit and re-enter quiescence leads to a loss of 'stemness', homeostatic decline of the satellite cell population, and eventual inability to regenerate damaged muscle [57–59].

Transcriptomics and proteomic analysis of quiescent satellite cells have revealed a number of transmembrane receptors that are highly expressed within this population, including M-/N-cadherin junctions, Sprouty1, integrin β 1, frizzled receptors, OSM β , CalcR and Notch receptors [60,61]. These receptors are part of a number of pleiotropic signalling cascades involved in actively maintaining quiescence by preserving cell/niche polarity, inhibition of cell motility and migration, regulating cytoskeletal organization, inhibiting gene of transcription/translation, inhibition of cell proliferation, and repression of activation-driving signalling axes such as ERK and hippo pathways [57,62–71]. Quiescent cells also maintain their G_0 state by regulating chromatin compaction and slowing protein synthesis [72,73].

In response to inflammation and injury, satellite cells can enter a second reversible quiescent state before activation called G_{Alert} [74]. Committed satellite cells in G_{Alert} are primed to express downstream targets of the myogenic lineage and withhold protein expression through post-transcriptional and post-translational modifications [75,76]. Satellite cells in G_{Alert} enter the cell cycle at least twice as fast as satellite cells in G_0 , facilitating a rapid response to injury. These findings suggest a hierarchical cascade model of quiescence and satellite cell recruitment during regeneration wherein quiescent G_0 and G_{Alert} populations are present at each stage of the myogenic lineage (figure 1).

Unlike the serious injury models and techniques (i.e. cardiotoxin, BaCl_2 , freeze and crush injuries) routinely performed on animal models to investigate muscle regeneration [77], humans do not typically experience such traumatic injuries during their lifetime. Thus, the real-world demand on quiescent satellite cells to activate and regenerate is comparatively lower, involving mainly homeostatic turnover and the response to exercise.

Given the impressive regenerative properties of skeletal muscle and lower demands of the modern day lifestyle, the reasons behind early adult onset of muscle decline remain elusive [7,8]. Recent findings on satellite cell quiescence and exercise in ageing populations provide a new paradigm on how to prevent age-related muscle regenerative decline.

4. Quiescence is a double-edged sword

Reversible quiescence is necessary in maintaining long-term regenerative potential, as a subset of regenerative stem cells and progenitors need to be preserved to adequately respond to future demands. However, periods of prolonged satellite cell quiescence may negatively impact long-term viability, leading to impaired regenerative potential (figure 2a). Despite lower protein synthesis, metabolism and general cellular activity, quiescent satellite cells still require cellular processes to actively maintain quiescence. These processes generate cellular waste, organelle damage and mitochondrial ROS from energy production (figure 2b) [72–74,78]. Accumulation of these waste products can harm the long-term viability of quiescent satellite cells. Fortunately, quiescent satellite cells have a number of protective mechanisms to prevent a build-up of cell waste products, DNA damage and transition to senescence [51,79–83].

Quiescent satellite cells under prolonged periods of quiescence are at risk of proteotoxicity (figure 2b). During deep quiescence, accumulation of dysfunctional proteins and organelles that would otherwise be diluted through rounds of cell division can increase cytotoxicity leading to senescence and apoptosis [84–86]. To combat proteotoxicity, systems for cell product turnover are important for maintaining cell viability.

The ubiquitin–proteasome system is one of the primary systems involved in pruning the proteomic landscape. This system is critical for maintaining cell identity. For example, Pax7 expression is regulated by Nedd4, a ubiquitin-ligase that is expressed during both quiescence and activation [87]. Satellite cell-specific knockout studies of the proteasomal subunit *Rpt3* demonstrate the importance of the ubiquitin–proteasome system in maintaining quiescence [83]. Disruption of the proteasome through deletion of *Rpt3* significantly depletes the quiescent satellite cell population in resting muscle. Moreover, post-injury muscle regeneration is disrupted under *Rpt3* knockout conditions, with a significant decline in Pax7-expressing quiescent satellite cell numbers at both 5 and 15 days post injury.

Autophagy also plays a pivotal role in the cell's capacity to maintain cellular integrity through recycling of intracellular components, such as defective or surplus proteins and organelles [85,88]. For the purpose of this review, autophagy will be used in reference to macroautophagy, one of the main forms of autophagy that occur [89]. Autophagy is a critical cellular process in maintaining cell viability, and its disruption in satellite cells promotes muscle atrophy and mitochondrial

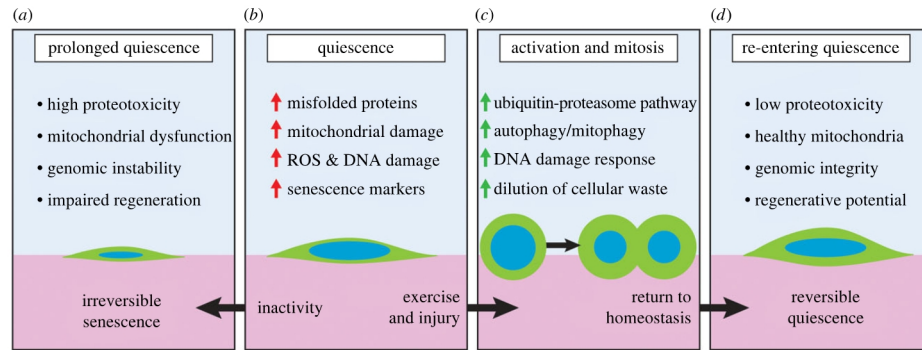


Figure 2. Healthy quiescence requires periodic activation. (a) Prolonged quiescence is met with detrimental effects, including high proteotoxicity, mitochondrial dysfunction, genomic instability and impaired regeneration. As satellite cells age, they are prone to prolonged states of quiescence during times of inactivity. (b) Quiescent satellite cells accumulate defects in the form of misfolded proteins, mitochondrial damage, reactive oxygen species (ROS) and DNA damage, and begin to express senescence markers. (c) Activation and mitosis of satellite cells induced through exercise or injury upregulates several processes that actively clear cellular waste, including the ubiquitin/proteasome pathway (UPP), autophagy/mitophagy and the DNA damage response (DDR). Passive dilution of cellular waste is also achieved through increased cytoplasm during mitosis. (d) During the return to homeostasis, satellite cells re-enter quiescence, exhibiting lower levels of proteotoxicity, healthy mitochondria, intact genomic integrity and a high regenerative potential.

dysfunction [90–93]. Proper autophagy function is required throughout the cell cycle, including active cycling and quiescence [51,94].

Pharmacological inhibition of autophagy and satellite cell-specific ablation of *Atg7*, an essential component of macroautophagy, demonstrate the requirement for a basal level of homeostatic autophagy in preventing quiescent satellite cells from transitioning into senescence [51]. Indeed, disrupted autophagy results in increased p62 and ubiquitin-positive aggregates, which mark damaged organelles and substrates for degradation respectively. Conversely, increased autophagy by *Atg7* overexpression is able to rescue the proliferative defect and reduce numbers of senescent satellite cells in geriatric mice [51].

All cells accumulate DNA damage from several intrinsic and extrinsic sources during ageing [95]. Satellite cells actively employ DNA damage responses (DDRs) as they activate and progress through the cell cycle towards differentiation [81,96]. Relatively little is known specifically concerning DDRs and satellite cell function. However, quiescent haematopoietic stem cells activate DDRs upon entry into the cell cycle to combat age-related DNA damage [97]. Presumably satellite cells upregulate DDRs in a similar fashion upon exiting quiescence and entering the cell cycle. The importance of a robust DDR in satellite cells can easily be expected, as this stem cell population must maintain in-tact genetic integrity to seed a lifetime of regeneration.

Quiescent satellite cells in G_0 display a detectable low level of energy metabolism and predominantly derive their energy needs from glycolysis, unlike most G_1 -arrested cells that rely on oxidative phosphorylation [98–100]. Despite a preference for glycolysis in quiescent satellite cells, which does not rely on mitochondrial function [101], the ability to remove damaged mitochondria remains integral to maintaining cell viability. Mitophagy impairment results in increased reactive oxygen species (ROS), DNA damage and senescence markers that can be attenuated using pharmacological ROS inhibition [51]. Recent reports have shed light on this paradox and have demonstrated that fatty acid metabolism is also required to maintain an in-tact quiescent state [102,103], and

peroxisome-targeted inhibition of fatty acid oxidation lead to premature differentiation of myoblasts *in vitro* [103]. As satellite cells exit quiescence and enter the cell cycle, a metabolic switch to oxidative phosphorylation occurs, with an expected increase in mitochondrial density and recycling [99].

Unfortunately, these protective mechanisms in place are not enough to maintain long-term satellite cell viability and regenerative capabilities of muscle over the lifetime of an individual. Quiescent geriatric satellite cells eventually enter a pre-senescent state by de-repression of $p16^{INK4a}$ (*Cdkn2a*), which inhibits multiple quiescence-inducing pathways and increases DNA damage through a ROS-positive feedback loop [50,104]. Geriatric muscle satellite cells are not as capable and efficient in transitioning from G_0 quiescence to activation required for creating new progenitors and consequently are unable to keep up with muscle degradation. Indeed, muscle cross-sections of adult and geriatric mice compared to young mice exhibit signs of muscle decline: fibre atrophy, loss of innervation, and re-expression of embryonic myosin heavy chain and central nucleation [50,51,80,105–107].

It appears the autophagy and mitophagy pathways active during prolonged quiescence are not sufficient to prevent satellite cells from transitioning into senescence [82]. What, then, can we do to prevent this seemingly inevitable decline in homeostatic satellite cell regenerative function? We postulate that periodic activation and cycling of satellite cells is required to remove proteotoxic waste through cytoplasmic dilution and upregulation of autophagy to maintain long-term cell viability (figure 2c,d).

5. Exercise activates satellite cells

Exercise bestows several benefits to overall health, including improved bone strength, offset obesity [12], and improved cardiovascular [108], cognitive [109], and immune system functions [110]. One of the most apparent outcomes of exercise is improved function and health of skeletal muscle [11,111,112].

Here we will discuss the effect of exercise on the satellite cell population within skeletal muscle.

The muscle regeneration field has been focused on muscle formation during embryogenesis and regeneration following severe injury and disease progression. Less clear are the mechanisms regulating satellite cell activity during homeostasis, response to eccentric exercise and ageing. The satellite cell population maintains a level of functional heterogeneity, with subpopulations of satellite cells mounting differential responses depending on environmental stimuli [113,114]. Satellite cell lineage tracing experiments using multi-colour *Pax7Cre^{ERTM};R26RBrainbow2.1* mice reveal that clonal complexity is maintained during homeostatic ageing and undergoes clonal selection during severe muscle injury [54]. This suggests that the satellite cell response differs depending on different thresholds of stress. However, it is not clear as to what type and to what degree of response is potentiated following stress caused by various types of exercise.

Eccentric exercise enhances myofibre hypertrophy, and stimulates satellite cell activation and proliferation [115]. Aerobic endurance training stimulates satellite cell activation, and human strength training studies with both single and long-term training sessions result in a marked induction of satellite cell proliferation [116–126]. Proper satellite cell function is indeed responsible for exercise-induced muscle mass gain, as satellite cell depletion by irradiation results in the loss of expected hypertrophy following weight bearing exercises [127,128]. Indeed, satellite cell activation and fusion into existing myofibres are an integral part of myofibre growth and accumulation of myonuclei following exercise [129–131]. It is possible that impairment of satellite cell activation partly contributes to muscle atrophy observed in sedentary lifestyles [132–134] and during space flight [135] due to a loss of force-induced activation.

Paradoxically, activation of satellite cells through exercise enhances muscle function, yet the age-related dysfunction of satellite cells arises due to their heightened propensity to activate and differentiate. As discussed earlier, this is in part due to age-related changes intrinsic to satellite cells [38,48–52], and to the aged stem cell niche [17,29,37–47]. However, it appears that in contrast to the exhaustive chronic satellite cell activation associated with age, acute activation of satellite cells following exercise leads to a regenerative response that is met with a subsequent return to quiescence. Supporting this notion are studies demonstrating the benefits to overall muscle health in elderly people who perform resistance training, which stimulates satellite cell activation [115,136–142]. As such, exercise may drive significantly different satellite cell activation signalling compared to the response observed due to age-related defects. Besides signalling differences within the satellite cells, systemic factors may also be at play. For example, factors associated with exercise such as increased vascularization [143] may reduce chronic inflammation, allow for more efficient removal of cellular debris and waste build-up in the niche, and promote satellite cell function.

Current understanding of the factors that activate satellite cells during eccentric exercise is limited as most molecular studies describe satellite cells either in the non-stressed or severely injured state. It appears that satellite cell activation during exercise is mediated by elements released from the myofibre and interstitial cell populations. Signalling cascades that appear to be responsible for driving satellite cell activation in response to exercise induced muscle stress include IGF1, IL-6/JAK/STAT3, hippo and SIRT1 pathways [79,144–147].

IGF-1 is released from muscle fibres following mechanical strain and exercise, driving muscle hypertrophy [144,148]. Barton-Davis *et al.* observed that the hypertrophic effect of IGF1 was approximately halved in the absence of satellite cells in irradiation experiments [149]. Supporting these findings, other transient IGF1 overexpression studies in muscle report the accumulation of myonuclei, an event mediated by satellite cell fusion to existing myofibres [150]. Interestingly, a splice variant of IGF1 (IGF-1Eb), termed mechano-growth factor, is produced and released from muscle following weight-bearing exercise, and is thought to drive satellite cell proliferation [151]. However, this effect is contested with *in vitro* studies on C2C12 and human primary myoblast cultures failing to demonstrate any significant increase in proliferation following treatment with this IGF1 splice variant [152,153].

Interleukin 6 (IL-6) is a pro-inflammatory cytokine that is released from growing muscle [154]. IL-6 has been shown to be released following resistance training exercise, with concomitant satellite cell proliferation and STAT3 signalling activation [145]. STAT3 signalling is implicated in satellite cell progression through the myogenic lineage [145,155–157]. However, it should be noted that age-related chronic STAT3 activity inhibits satellite cell expansion and has been implicated in age-related satellite cell exhaustion [48,158].

Non-myogenic cells that reside within the interstitial spaces of muscle appear to also play a role in the IL-6 signalling axis post-exercise, namely FAPs and infiltrating eosinophils. FAPs provide functional support for satellite cells and influence their activation [155,159–163]. Following muscle injury, eosinophils infiltrate the interstitial space in close proximity to FAPs and satellite cells, and release interleukin 4 (IL-4) [160]. IL-4 release from eosinophils stimulates FAPs, which secrete IL-6 when activated, providing an additional source of this cytokine within a regenerating muscle [160,162,164]. Interestingly, eosinophils have been shown to secrete IL-4 when activated by Meteorin-like (Metln), a myokine that is secreted following exercise [165]. These findings demonstrate that satellite cell activation post-exercise is a multi-faceted phenomenon, with several cell types and factors working in concert to mediate a regenerative environment, similar to the response following severe injury.

Hippo signalling through YAP and Taz has been demonstrated to play a role in satellite cell activation and proliferation [166–168]. Interestingly, it is becoming more evident that mechanotransduction is a primary activator of the hippo pathway [169,170]. This is in line with a recent findings by Eliazar *et al.* that demonstrate Rho-dependent mechanotransduction to repress YAP during quiescence to prevent satellite cell activation [146]. These findings give rise to the idea that exercise-induced mechanotransduction may activate satellite cells through involvement of the hippo pathway. Additionally, physical activation by massage has been reported to activate and increase satellite cell numbers, suggesting that a sensitive mechano-sensing mechanism is involved in satellite cell activation under healthy conditions, which could potentially involve hippo signalling [171].

Lastly, SIRT1 is another signalling effector that has been implicated in satellite cell activation and is upregulated in muscle following exercise [147,172]. SIRT1 is among the family of sirtuin deacetylase proteins, which exert NAD⁺-dependent activities and act as metabolic sensors [173]. SIRT1 is thought to upregulate autophagy as the cell exits quiescence to provide additional energy for activation and proliferation [79]. Deletion

of SIRT1 leads to a delay in satellite cell activation [79]. Indeed, satellite cell-specific SIRT1 knockout prevents muscle regeneration, and its overexpression improves muscle regeneration in older mice [174]. Since SIRT1 influences satellite cells through regulation of autophagy, it would be interesting to determine whether an age-related loss of SIRT1 expression is involved in the decline of satellite cell function linked to age-related autophagy dysfunction. Perhaps exercised-induced expression/activity of SIRT1 is capable of ameliorating dysfunctional autophagy in satellite cells.

Despite the well-established beneficial effect of resistance training, exercise alone does not prevent all age-related defects in skeletal muscle. Concomitant with sarcopaenia is a change in muscle composition within geriatric muscle: a shift of type 2 towards type 1 myofibres [175,176]. Even in cases of lifelong exercise, the shift from type 2 to type 1 myofibres persists [177] and the satellite cell population still diminishes to some degree over age [39,178–181]. Exercise-induced satellite cell activation also experiences some degree of impairment during ageing, as demonstrated by expression of myostatin within satellite cells, an inhibitor of satellite cell activation and self-renewal [178,181,182]. Regardless, lifelong resistance exercise should be pursued as the effects of sarcopaenia are greatly diminished when compared with sedentary lifestyles. As demonstrated in this review, the beneficial effects of exercise to muscle are derived from not only direct stimulation of muscle fibres, but also simultaneous activation of the muscle stem cell pool.

6. Concluding remarks

Higher quality of life and happiness is strongly associated with an individual's ability to exercise self-agency, and maintaining locomotion is a key aspect of this pursuit. Skeletal muscle health and exercise deserve greater attention in today's world, where a sedentary lifestyle is the norm, while obesity and cardiovascular disease are the leading causes of death. Although age-related muscle atrophy is the focus of many geriatric studies, it is clear that the decline in satellite cell number and function coincides with the gradual muscle loss that begins much earlier, at approximately 30 years of age.

Many additional mechanisms behind satellite cell function decline in ageing remain to be discovered. However, it appears to be multi-factorial and involve defects and misregulation of

cell maintenance, quiescence and activation pathways that prevent cell damage. It is evident that satellite cells are activated through various signalling pathways following exercise. Of particular relevance, cellular processes involved in the upkeep of cellular integrity (proteasome-mediated protein degradation, autophagy and DDRs) are upregulated during activation and proliferation. Moreover, satellite cells increase their cytoplasm during rounds of proliferation, which dilutes cellular waste and dysfunctional organelles between daughter cells. It is evident that waste and DNA damage accrued within periods of satellite cell quiescence are most effectively managed during periods of activation. Frequent activation of muscle satellite cells through exercise is a key process required to offset age-related waste/damage accumulation that leads to senescence. Physical inactivity becomes more detrimental with age as satellite cells accumulate higher levels of cellular waste and DNA damage during longer periods of deep quiescence.

Currently, our understanding of satellite cell behaviour in response to exercise is relatively limited compared with more severe models of injury. Future work in this field is needed to decipher the clonal dynamics of satellite cells following exercise. For example, is satellite cell replacement through asymmetric division sufficient to respond to exercise-induced stress, or is pre-expansion of the satellite cell pool required to mount a more robust response? Moreover, what is the threshold and range of exercise-induced mechanical stress that drives satellite cell activation, and perhaps different clonal dynamics? Are certain subpopulations of satellite cells more sensitive to mechanical stress within the normal range of exercise? Elucidating these mechanisms will reveal important information relevant to combating age-related muscle decline, as well as designing therapeutics to offset muscle atrophy that arises from sedentary lifestyle, space flight and disease.

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Chapter 7. Footnotes/Endnotes

Chapter 8. References

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